**Folate Transport in *Lactobacillus salivarius***

CHARACTERIZATION OF THE TRANSPORT MECHANISM AND PURIFICATION AND PROPERTIES OF THE BINDING COMPONENT

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*Lactobacillus salivarius* cells contain an inducible transport system for folate. Influx via this system is time- and temperature-dependent, requires glucose and glutamine for optimum activity, and is half-maximal at folate concentrations in the nanomolar range. The folate internalized after 30 min at 30 °C is not released from the cells by excess extracellular folate and is recovered in cell extracts primarily in metabolized forms. A membrane-associated folate-binding protein is also present in cells that have been induced to transport folate. This binding protein constitutes 1% of total cellular protein, exhibits a high affinity for folate (*K_D* = 0.40 nM), and requires divalent cations for optimum binding activity. Folate binds rapidly to this protein, while the exchange of bound substrate with folate added subsequently is relatively slow and dependent on the metabolic state of the cell. The transport rate per binding site is 0.05/min at 30 °C. A comparison of substrate specificity showed that folate binding and transport are both inhibited to the same extent by several different folate compounds, and a parallel irreversible inhibition of both processes is observed by prior treatment of the cells with a carbodiimide-activated derivative of folic acid. Binding protein labeled covalently with [*H]folate and solubilized with Triton X-100 was purified by a fractionation procedure involving absorption and elution from microgranular silica and molecular sieve chromatography. The isolated protein appeared homogeneous by gel electrophoresis and had an apparent molecular weight of 21,000. Monoclonal antibodies to the folate transport protein of *Lactobacillus casei* showed a high degree of cross-reactivity to the isolated binding protein from *L. salivarius*, indicating that these proteins share common epitopes. These results suggest that folate uptake by *L. salivarius* proceeds via an abundant membrane-associated binding protein which facilitates the movement of folate across the membrane as an electroneutral complex with cations. The substrate then slowly dissociates from internalized binding sites and is metabolized sequentially to coenzyme forms and then to membrane-impermeable poly(poly)glutamates.

Folate is obtained by most bacterial cells from *de novo* biosynthesis and hence is not usually required as a growth factor (1). In addition, cells that synthesize folate *de novo* rarely express a transport system for folate, even when the vitamin is present in the extracellular environment. The few bacterial cells that must acquire folate from extracellular sources include *Lactobacillus casei* (1–4), *Streptococcus faecalis* (1, 5–7), and *Pediococcus cerevisiae* (1, 3, 8, 9). The folate transport system of *L. casei* has been studied in the most detail and has been shown to consist of a binding protein (10–12) and at least one additional component which is shared with transport systems for other vitamins (13). The binding protein, which acts both as an external folate receptor and a transmembrane folate carrier, is produced in moderately high amounts per cell (0.2% of total cellular protein), binds folate with a high affinity (*K_D* = 0.4 nM at pH 7.5), and has been purified to homogeneity (10, 11). The isolated protein is relatively small (*M*ₐ = 19,000) and consists of a high proportion of hydrophobic amino acids (10, 11). Optimum binding of folate requires divalent cations (12), suggesting that transfer of the folate diion across the cell membrane proceeds via a cation cotransport mechanism. The function of the second component is unresolved but has been suggested to involve the coupling of energy to active folate accumulation within the cell (14).

Evidence has been obtained in the present study that *Lactobacillus salivarius* also contains an abundant transport system for folate. These cells both require folate as a growth supplement and contain an inducible folate-binding protein which is remarkably similar to the corresponding folate binder from *L. casei*. The binding proteins from both sources have comparably high affinities for various folate compounds, are inactivated to a high extent by a reactive ester of folate, and have similar molecular weights. The distinguishing feature of these systems is that *L. salivarius* cells lack the energy-coupling system of *L. casei* which facilitates the concentrative uptake of free vitamin within the cell. This difference could explain the expression of higher amounts of binding protein by *L. salivarius*, and it suggests a mechanism in which transport is limited by a slow rate of dissociation of folate from internalized binding sites. Similar high-affinity binding proteins with a low capacity for releasing bound folate have been implicated in the transport of folate compounds in certain mammalian cells (15, 16).

**MATERIALS AND METHODS**

*Radioabeled Compounds*

[3',5',7,9-3H]Folate (40 Ci/mmole) and [3',5',9-3H]methotrexate (20 Ci/mmole) were obtained from Moravek Corp., diluted with unlabeled compound to a specific activity of 200,000 to 1,000,000 dpm/nmol, purified by thin-layer chromatography (17), and stored at

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Folate Transport in L. salivarius

For L. salivarius cells (10 g, wet weight) that had been washed with 200 ml of 50 mM potassium phosphate, pH 7.5, were suspended in 500 ml of 5 mM HEPES, 150 mM potassium chloride, 1 mM MgCl₂ and adjusted to pH 7.7 with potassium hydroxide. EDC-activated [³H]folate (specific activity, 10,000 cpm/nmol) was then added to the cells (at 0 °C) in an amount which exceeded the folate-binding activity by 3-fold and the mixture was stirred for 16 h at 0 °C. The resulting labeled cells were recovered by centrifugation for 5 min at 20,000 × g, suspended in 250 ml of 20 mM potassium phosphate, pH 6.5, containing 500 μM unlabeled folate, and incubated for 20 min at 23 °C to displace noncovalently bound [³H]folate. After centrifugation, the cells were suspended in 250 ml of 20 mM potassium phosphate, pH 6.5, containing 5% Triton X-100. Extraction of the labeled binding protein was achieved by five cycles of chilling to 0 °C and homogenization. The gradient was followed by an additional wash with 10 ml of the tetrabutylammonium phosphate/ammonium phosphate mixture containing 50% methanol.

Isolation of the Folate-binding Protein from Intact Cells

Step 1: Protein Labeling and Extraction from the Membrane From L. salivarius cells (10 g, wet weight) that had been washed with 200 ml of 50 mM potassium phosphate, pH 7.5, were suspended in 500 ml of 5 mM HEPES, 150 mM potassium chloride, 1 mM MgCl₂ and adjusted to pH 7.7 with potassium hydroxide. EDC-activated [³H]folate (specific activity, 10,000 cpm/nmol) was then added to the cells (at 0 °C) in an amount which exceeded the folate-binding activity by 3-fold and the mixture was stirred for 16 h at 0 °C. The resulting labeled cells were recovered by centrifugation for 5 min at 20,000 × g, suspended in 250 ml of 20 mM potassium phosphate, pH 6.5, containing 500 μM unlabeled folate, and incubated for 20 min at 23 °C to displace noncovalently bound [³H]folate. After centrifugation, the cells were suspended in 250 ml of 20 mM potassium phosphate, pH 6.5, containing 5% Triton X-100. Extraction of the labeled binding protein was achieved by five cycles of chilling to 0 °C and homogenization for 2 min in a Manton Gaulin homogenizer at 5000 p.s.i. A crude extract containing selectively labeled binding protein was obtained by centrifugation of the sample at 24,000 × g for 30 min at 4 °C. The extent of cell disruption under these conditions was 60–80%.

Step 2: Silica Fractionation—The crude cell extract containing the labeled binding protein was combined with 3 g of Q-Sep G-52 microgranular silica (Philaide Chemical, Inc.) and the mixture was stirred at 4 °C. After centrifugation at 16,000 × g (5 min, 4 °C), the absorbent was washed twice with 200 ml of 50 mM potassium phosphate, 0.1% Triton X-100, pH 7.5, and the binding protein was eluted by stirring the silica for 3 min at 0 °C with 100 ml of 0.2 M

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; MES, 2-(N-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography.

-80 °C in 10 mM K-HEPES, pH 7, containing 10% ethanol. (6R,6S)-5-methyl-¹⁴C[Tetrahydrofolate (61 mCi/mmol)] and [³⁵S]thiamine (189 mCi/mmol) were obtained from Amersham Corp., while [³H]biotin (20 Ci/mmol) was from New England Nuclear; each of the latter compounds was stored at -20 °C and employed without further purification. The specific activity of the [³H]biotin was reduced to 1,000,000 dpm/nmol by the addition of unlabeled biotin.

Chemicals

Folic acid, calcium (6R,6S-5-formyltetrahydrofolate, pteroic acid, p-aminobenzoic acid, 6-hydroxyethynylpterin, biotin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), HEPES, and MES were obtained from Sigma. (6R,6S)-5-Methyltetrahydrofolate was a gift of Dr. J. H. Whiteley, while 3,5-dichloromethotrexate and homofolate were provided by Dr. J. H. Hanscom. All other chemicals were obtained from the usual commercial sources and had the highest purity available.

Growth of Cells

L. salivarius cells (ATCC 11741) were grown for 18 h at 30 °C from a 2% inoculum of fresh or frozen cells in autoclaved medium consisting of the following components (per liter): glucose, 10 g; sodium acetate, 10 g; vitamin-free casein hydrolysate, 5 g; ammonium citrate, 0.5 g; Tween 80, 1 ml; monobasic potassium phosphate, 200 mg; sodium sulfate, 100 mg; magnesium sulfate, 10 mg; ferrous sulfate, 10 mg; ascorbic acid, 100 mg; adenine, 5 mg; guanine, 10 mg; thymidine, 10 mg; riboflavin, 2 mg; pyridoxine, 2 mg; nicotinic acid, 5 g; biotin, 1 mg; and folate, 2 nmol, pH 6.6, with NaOH. Cells employed for measurements of the binding and transport of thiamine and biotin were grown in the same medium except that the respective vitamins were reduced to 2 nmol/liter. In order to deplete vitamin reserves, the latter cells were transferred twice prior to use. Medium that was essentially free of folate was obtained by suspending "vitamin-free" casein hydrolysate (1 g) in 50 volumes of HEPES-Mg buffer (50 mM HEPES, 5 mM MgSO₄, pH 7.5, with KOH) and incubating the mixture for 1 h at 0 °C. After removing cells by centrifugation, the treated casein hydrolysate was combined with the other medium components and autoclaved for 5 min at 121 °C. Full-grown cultures (160 Klett units; A₅₅₀ = 1.2; 10⁶ cells/ml) were centrifuged at 16,000 × g, x (5 min, 4 °C), washed with 100 volumes of HEPES-Mg buffer or MES-Mg buffer (50 mM MES, 5 mM MgSO₄, pH 6.0, with KOH), and then resuspended in the same buffer to the desired cell density. The relationship between cell number and Klett unit was linear for 24 h at 23 °C to determine cell density. The energy reserves. ATP levels in control and preincubated cells were measured by the same procedure in cells preincubated for 2 min at 30 °C prior to the addition of [³H]folate (0.5 μM). After an additional 15 min at 30 °C, the cells were collected by filtration, washed with 2 ml of ice-cold buffer, and analyzed for radioactivity. Control samples were prepared similarly except that the temperature was reduced to 0 °C during the incubation with [³H]folate. After correction for the control, transport was expressed as the amount of folate accumulated by 10⁹ cells/min at 30 °C. Biotin transport was measured by the same procedure in cells preincubated for 2 min at 30 °C with 1 mM glucose and exposed subsequently to 0.5 μM [³H]biotin for 15 min.

Metabolism of Internalized Folate

L. salivarius cells (2 × 10⁹) that contained internalized [³H]folate were collected by centrifugation, washed twice at 4 °C with 100 volumes of saline, and resuspended in 1.2 ml of 1.0% ascorbic acid. The sample was then heated for 2 min at 80 °C. After centrifugation for 5 min at 15,000 × g, the supernatant fraction was split into two equal portions and either placed on ice or flushed with nitrogen and treated with hog conjugase (19) for 1 h at 37 °C. The latter sample was then processed for an additional 2 min at 80 °C and clarified by centrifugation at 15,000 × g (5 min, 4 °C). Samples (250 μl) that had been passed through a 0.22-μm filter were subjected to high-pressure liquid chromatography (HPLC) on a C18 Altex Ultrasphere-ODS column. Elution was achieved with a solvent consisting of 5 mM tetrabutylammonium phosphate and 10 mM ammonium phosphate in water. The elution gradient was in the buffer containing 0.1% methanol as described by Vitois et al. (19), except that the end of the gradient was followed by an additional wash with 10 ml of the tetrabutylammonium phosphate/ammonium phosphate mixture containing 50% methanol.

Binding and Transport Measurements

Folate-binding activity was determined as described previously (12) in 2 ml of HEPES-Mg buffer, pH 7.5. Duplicate assay mixtures contained 2 × 10⁸ energy-depleted cells and 0.5 μM [³H]folate. After incubation for 5 min at 0 °C, the cells were collected onto a 0.45 μm nitrocellulose filter, washed with 2 ml of ice-cold buffer, and analyzed for radioactivity in CytoScint (WestChem Products). Results were expressed in nanomoles of folate bound per 10⁹ cells. For measurements of dissociation constants (Kₐ), the assay volume was increased to 50 ml, lower and variable amounts of [³H]folate were added, and the incubation time at 0 °C was extended to 120 min. Cells were collected by filtration as above but were not washed prior to analysis for bound radioactivity. Kₐ was calculated from linear-regression analyses of double-reciprocal plots of the folate bound by cells versus the free folate concentration remaining in solution at equilibrium. Kₐ values were determined in 50-ml assay mixtures containing a fixed concentration (2.0 nM) of [³H]folate and variable amounts of the desired inhibitor; calculations employed linear-regression analyses of Dixon plots of the data. Binding of [³H]methotrexate, [³C]-methy-
sodium carbonate containing 0.1 Triton X-100. The eluant was recovered by centrifugation at 24,000 × g for 2 min at 4°C and immediately adjusted to pH 7.0 by the addition of a saturated solution of monobasic potassium phosphate. The supernatant fraction was then concentrated by vacuum dialysis to about 3 ml and dialyzed against 500 ml of 20 mM potassium phosphate, pH 7.5.

Step 3: Sepharose Chromatography—The dialyzed sample from the previous step was incubated for 10 min at 37°C, clarified by centrifugation, and then applied to a column (100 × 2.5 cm) of Sephacryl S-300 equilibrated (at 4°C) with 20 mM potassium phosphate, 100 mM KC1, and 0.1 mM Triton X-100, pH 7.5. Elution was achieved with the same buffer, and fractions containing the major portion of bound [3H]folate were pooled and concentrated by vacuum dialysis to about 3 ml. The sample was then re-applied to the same column, eluted as described above, concentrated to 3 ml, and stored at −20°C in the presence of 20% glycerol.

Concentrations of protein in crude cell extracts and after the silica fractionation step were determined using the biuret procedure (20), while purified protein was quantitated by the procedure of Lowry et al. (21) after precipitation with acetic acid (22). Bovine serum albumin was employed as a standard in both procedures, although Lowry determinations on purified samples were corrected using a ratio of 1.0 mg of binding protein/0.56 mg of serum albumin as determined previously for the folate binder from L. casei (22).

Gel Electrophoresis

SDS-polyacrylamide slab gel electrophoresis was performed by the general procedure of Laemmli (23). Prior to electrophoresis, samples of binding protein (50 μg) were precipitated by the addition of 3 volumes of aceton, recovered by centrifugation, suspended in 500 μl of sample buffer, and heated for 1 min at 80°C. Protein standards were ovalbumin, chymotrypsinogen, dihydrofolate reductase (L1210 cells), β-lactalbumin, and lysozyme.

Monoclonal Antibodies

Monoclonal antibodies were prepared against folate-binding protein from L. casei (11, 22) that had been dialyzed overnight against 100 volumes of 10 mM sodium phosphate, 150 mM NaCl, pH 7.0, precipitated by the addition of isopropl alcohol to 50%, washed with 100 volumes of water, and suspended to 0.5 mg/ml in Freund’s complete adjuvant. A BALB/c mouse was injected intraperitoneally with 20 μg of protein and then boosted twice with 100-μg amounts of protein 4 and 8 weeks after the initial injection. The spleen was removed aseptically 3 days after the final injection, and the splenocytes were recovered, fused with SP2/0 M-5 myeloma cells, selected on hypoxanthine/aminopterin/thymidine (24), and grown in 96-well plates by standard procedures (24). Supernatant solutions of growth-positive wells were screened for antibody to the folate-binding protein by standard techniques (25) using Falcon flexible 96-well plates treated first with glutaraldehyde and then exposed to volumes of acetone, recovered by centrifugation, suspended in 0.1% Triton X-100. Detection of antibody-binding protein complexes was achieved using anti-mouse goat antibodies conjugated with alkaline phosphatase. Clones producing the desired antibody were transferred to tissue culture flasks and then injected intraperitoneally (at 106 cells/mouse) into BALB/c mice that had been treated 10 days earlier with an intraperitoneal injection of pristane (26). Ascites fluid containing secreted antibody was collected from the peritoneum over a 7 to 14-day period subsequent to injection and stored at −20°C. The reactivity of binding proteins with monoclonal antibodies was determined in 96-well plates containing 100 ng/well of binding protein as described above.

RESULTS

Growth Characteristics of L. salivarius—An undefined medium had been developed previously for the growth of L. salivarius (27). This medium contained yeast extract and hence unknown amounts of folate and other vitamins that might interfere with the expression of corresponding vitamin transport systems. A modified medium was therefore developed (see “Materials and Methods”) that contained only vitamin-free components. The primary difference between the two mediums was the substitution of vitamin-free casein hydrolysate for the yeast extract. Uracil was also added since it was required in small amounts for optimum cell growth, although inhibition of growth was observed at concentrations above 2 mg/liter. The yield of L. salivarius cells in the revised medium was 3 g/liter, which was lower than had been obtained by L. casei (8 g/liter). L. salivarius cells failed to grow in the medium employed to propagate L. casei (2), although L. casei grew well in the above medium for L. salivarius.

L. salivarius cells have an absolute growth requirement for folate. When medium was prepared with casein hydrolysate that had been processed to remove trace amounts of residual folate (see “Materials and Methods”), growth after 16 h was less than 10% that of cells in the same medium with excess folate. The addition of varying amounts of folate revealed that growth was half-maximal at 0.2 nM folate and reached a maximum when the folate concentration exceeded 2 nM (Fig. 1). 5-Formyltetrahydrofolate also supported optimal cell growth at concentrations above 2 nM, while 5-methyltetrahydrofolate (10 nM) had no effect. Methotrexate (4-aminopterin) produced an inhibition of growth which was half-maximal at a concentration of 0.07 nM (Fig. 1).

Folate Uptake by L. salivarius—The uptake of folate by freshly harvested L. salivarius cells was biphasic at 30°C (Fig. 2). A rapid initial uptake phase was observed which reached a maximum immediately after the addition of [3H]folate, was not affected by the addition of glucose, and corresponded to a 1.9 ± 0.3 nmol/109 cells. This initial phase was followed by a much slower component whose activity was dependent upon glucose (filled triangles) and glutamine (filled circles) and exhibited a maximal rate of 0.1 nmol/min/109 cells. Buffer composition appeared to have little effect on uptake since similar uptake profiles were observed (in the presence of glucose) in HEPES-Mg buffer, pH 7.5, 50 mM potassium or sodium phosphate, pH 7.5, 50 mM potassium MES, pH 6.0, and 50 mM potassium MES, 5 mM MgSO4, pH 6.0 (data not shown).

The ability to displace [3H]folate from L. salivarius cells that had been exposed initially to [3H]folate, allowed to accumulate the label for 30 min at 30°C, and then exposed to an excess (100 μM) of unlabeled folate is also shown in Fig. 2.

![Fig. 1. Growth response of L. salivarius cells to varying concentrations of folate and methotrexate. Folate-free medium supplemented with either increasing concentrations of folate or a constant level of folate (2 nM) plus increasing concentrations of methotrexate were inoculated with cells (1%), incubated for 24 h at 30°C, and analyzed for cell growth by absorbance in a Klett colorimeter.](image-url)
of the total polyglutamate pool.

The rate was much slower than for folate. The profile after treatment with hog conjugase (Fig. 1A) was characterized by a rapid uptake of radioactive folate, followed by a slower uptake component (Fig. 1B) that was displaced within the elution profile after treatment with hog conjugase (Fig. 1C). Release of radioactive folate from cells that had accumulated the substrate in the presence of glucose and glutamine was initiated by the addition of 100 μM unlabeled folate. A time-dependent increase in the amount of folate in peak fractions of the control cells (see "Materials and Methods") was observed and the amount (1.6 nmol/10⁶ cells) was comparable to that of the initial uptake phase. Subsequent incubation, however, caused relatively little of the remaining [³H]folate to dissociate from the cells. When cells (point C, Fig. 2) that had been exposed to [³H]folate for 30 min and then to unlabeled folate for 20 min at 30 °C were analyzed for intracellular metabolites of folate (see "Materials and Methods"), a substantial amount of the folate (68%) was found to have been metabolized to other forms (Fig. 3A). Polyglutamates comprised 29% of the total cellular folate as determined from the amount of radioactivity in peak fractions of the control cells (see shaded areas, Fig. 3A) that was displaced within the elution profile after treatment with hog conjugase (Fig. 3B). Folate increased from 32% in untreated samples to 38% of the total radioactivity after treatment with conjugase, indicating that folate polyglutamates comprised a significant portion (21%) of the total polyglutamate pool.

Uptake and Release of Methotrexate—Methotrexate was also found to associate rapidly with L. salivarius cells (Fig. 4), and the amount of uptake (2.1 pmol/10⁶ cells) was comparable to the initial uptake component for folate (see Fig. 2). A second uptake component was also observed with methotrexate but the rate was much slower than for folate. The latter uptake was dependent upon glucose but was not enhanced by glutamine. The addition of excess unlabeled folate (100 μM) to cells that had been exposed to [³H]methotrexate for 30 min at 30 °C led to a rapid release of the labeled folate (68%) was found to have been metabolized to other forms (Fig. 3A). Polyglutamates comprised 29% of the total cellular folate as determined from the amount of radioactivity in peak fractions of the control cells (see shaded areas, Fig. 3A) that was displaced within the elution profile after treatment with hog conjugase (Fig. 3B). Folate increased from 32% in untreated samples to 38% of the total radioactivity after treatment with conjugase, indicating that folate polyglutamates comprised a significant portion (21%) of the total polyglutamate pool.

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FIG. 2. Effect of various conditions on the time dependence of [³H]folate uptake by L. salivarius cells and the release of accumulated [³H]folate by unlabeled substrate. Uptake of [³H]folate (0.5 μM) was determined at 30 °C in HEPES-Mg buffer, pH 7.5, with freshly harvested cells that had been preincubated for 30 min at 23 °C to exhaust energy reserves (D), preincubated for 2 min at 30 °C in the presence of 1 mM glucose (Glc) (A), or preincubated for 2 min at 30 °C in the presence of 1 mM glucose and 1 mM glutamine (Gln) (B). Release of [³H]folate from cells that had accumulated the substrate in the presence of glucose and glutamine was initiated by the addition of 100 μM unlabeled folate. A, time of [³H]folate addition; B, time of addition of excess unlabeled folate; C, time at which cells were analyzed for [³H]folate metabolism.

At the initiation of [³H]folate release (point B, Fig. 2), a rapid exchange of a portion of the total associated [³H]folate was observed and the amount (1.6 nmol/10⁶ cells) was comparable to that of the initial uptake phase. Subsequent incubation, however, caused relatively little of the remaining [³H]folate to dissociate from the cells. When cells (point C, Fig. 2) that had been exposed to [³H]folate for 30 min and then to unlabeled folate for 20 min at 30 °C were analyzed for metabolites of [³H]folate (see "Materials and Methods"), a substantial amount of the folate (68%) was found to have been metabolized to other forms (Fig. 3A). Polyglutamates comprised 29% of the total cellular folate as determined from the amount of radioactivity in peak fractions of the control cells (see shaded areas, Fig. 3A) that was displaced within the elution profile after treatment with hog conjugase (Fig. 3B). Folate increased from 32% in untreated samples to 38% of the total radioactivity after treatment with conjugase, indicating that folate polyglutamates comprised a significant portion (21%) of the total polyglutamate pool.

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FIG. 3. HPLC analysis of intracellular folates. L. salivarius cells that had been exposed progressively to 0.5 μM [³H]folate (1,000,000 cpm/nmol) for 30 min at 30 °C and then to 100 μM unlabeled folate for 20 min at 30 °C (conditions depicted by arrow C, Fig. 2) were collected by centrifugation, extracted with heat in the presence of ascorbic acid, and then evaluated for intracellular metabolites of folate as described under "Materials and Methods." Panel A, untreated sample; 105,000 cpm applied; recovery, 101%; panel B, sample treated with hog conjugase; 92,200 cpm applied; recovery, 100%. Arrows indicate elution positions of standard folate compounds: 1, 10-formyltetrahydrofolate; 2, tetrahydrofolate; 3, folate; 4, 5-methyltetrahydrofolate. Shaded areas in panel A correspond to folylpolyglutamates.

FIG. 4. Time dependence for the uptake of [³H]methotrexate and its subsequent release by excess unlabeled folate. Uptake of [³H]methotrexate (0.5 μM) was determined in HEPES-Mg buffer, pH 7.5, with freshly harvested cells that had been preincubated for 30 min at 23 °C to exhaust energy reserves (D), or preincubated for 2 min at 30 °C in the presence of either 1 mM glucose (E) or 1 mM glucose plus 1 mM glutamine (F). Glc, glucose; Gln, glutamine.
substrate (Fig. 4). The extent of this release was nearly quantitative in samples without added glucose, while a small portion of the label remaining associated with cells incubated with glucose and glutamine. The amount of label remaining with the latter cells (0.4–0.5 nmol/10^10 cells) was comparable to the uptake (0.4 nmol/10^10 cells) that had occurred via the second uptake component during the 30 min prior to the addition of excess folate.

**Characteristics of the Initial Uptake Component**—The initial uptake component exhibited the characteristics of a membrane-associated binding protein with a high affinity for folate; the association of folate with this component was rapid (see Fig. 2) and was unaffected by reducing the temperature to 0 °C (data not shown) or by incubating the cells at 23 °C for 30 min prior to the addition of [3H]folate (see Fig. 2). The latter procedure is effective in producing energy-depleted cells as judged by cellular ATP levels which were 80, 17, and <5 nmol/10^10 cells, respectively, in freshly harvested cells incubated (5 min, 30 °C) with 1 mM glucose, freshly harvested cells held at 0 °C, or cells incubated for 30 min at 23 °C with no addition. The amount of binding activity was maximal in cells grown with 2 nM folate but could be reduced by growth of the cells on higher folate concentration (Fig. 5). Binding decreased to 50% of maximum by growth in the presence of 25 nM folate, while a reduction in excess of 95% occurred when the folate in the growth medium was raised to 1 μM. In the latter measurements, binding sites were not masked by the presence of bound unlabeled folate since fully induced cells that had been exposed to unlabeled folate, washed with buffer, and then incubated for 5 min at 23 °C with [3H]folate exhibited the same binding activity as untreated cells. Induced cells grown with 2 nM folate and then transferred to medium containing excess folate (1 μM) were rapidly depleted of the binding protein (inset, Fig. 5). The binding activity per cell decreased by greater than 5-fold within the first doubling of the cell mass (see arrow, Fig. 5), indicating that a rapid turnover of the protein was occurring. Similarly, cells grown with a high concentration (200 nM) of folate and then placed in medium containing a limiting amount (2 nM) of the vitamin initiated a rapid synthesis of the binding protein after a brief lag period of about one cell division (inset, Fig. 5).

The concentration dependence for the binding of folate to *L. salivarius* cells is shown in Fig. 6. Saturation kinetics were observed both in HEPES-Mg buffer, pH 7.5 (line A), and in MES-Mg buffer, pH 6.0 (line B), and dissociation constants (K_d values) of 0.10 and 0.40 nM, respectively, were obtained. Measurements performed similarly (at pH 7.5) showed that methotrexate (K_d = 0.58 nM) and 5-methyltetrahydrofolate (K_d = 5.9 nM) were also bound by *L. salivarius* with a high affinity. The affinity for folate decreased substantially, however, when binding measurements were performed in a dilute HEPES buffer, pH 7.5, that lacked an added divalent cation (line C, Fig. 6). The observed K_d for folate under these latter conditions was 100 nM.

The concentration dependence for the inhibition of [3H]folate binding to cells by unlabeled substrate is shown in Fig. 7. The inhibition appeared monophasic and was complete at high concentrations of the unlabeled substrate, indicating that a single high-affinity binding component was present. Moreover, the calculated K_i, for unlabeled folate (0.39 nM) was comparable to the K_d for [3H]folate (0.40 nM) obtained by direct measurements (Fig. 6). Inhibition was also observed (inset, Fig. 7) upon treatment of the cells with increasing amounts of EDC-activated folate, an activated folate ester which irreversibly inhibits substrate binding to the folate transport protein of *L. casei* (18). Inhibition was half-maximal.

![Fig. 5](image-url)

**Fig. 5.** Dependence of folate concentration in the growth medium on the amount of folate-binding activity. Cells were grown in the presence of the indicated concentrations of folate, harvested by centrifugation, preincubated at 23 °C to exhaust energy reserves, and then analyzed for binding activity by incubation with 0.5 μM [3H]folate for 5 min at 23 °C. Inset, rate of repression and induction of folate-binding activity in cells transferred to medium containing high or low concentrations of folate, respectively. Cells that had been grown at 2 nM folate (line A) were transferred at zero time to fresh medium containing 200 nM folate, allowed to grow for the indicated times, and analyzed for binding activity (after depletion of energy reserves) by incubation for 5 min at 23 °C with 0.5 μM [3H]folate. Cells grown with 200 nM folate (line B) were transferred to medium containing 2 nM folate, incubated for the indicated times, and analyzed by the same procedure for folate-binding activity. Arrow indicates time at which the cells had undergone one cell division.

![Fig. 6](image-url)

**Fig. 6.** Effect of pH and buffer composition on the concentration dependence of [3H]folate binding by *L. salivarius* cells. Binding was determined after incubation of the cells for 2 h at 0 °C in the presence of the indicated concentrations of [3H]folate. Assay buffers: A, HEPES-Mg, pH 7.5; B, MES-Mg, pH 6.0; and C, 0.2 mM potassium-HEPES, pH 7.5.
Folate Transport in L. salivarius

FIG. 7. Inhibition profile for the binding of \(^{3}H\)folate by L. salivarius cells in the presence of increasing amounts of unlabeled folate. Inset, \(^{3}H\)folate bound by cells that had been pretreated with varying amounts of EDC-activated folate for 2 h at 0 °C. Assay buffer, HEPES-Mg, pH 7.5; buffer employed for EDC-folate pretreatment, 10 mM potassium HEPES, pH 7.5.

**TABLE I**

Affinity of the folate-binding proteins from L. salivarius and L. casei for various folate compounds

<table>
<thead>
<tr>
<th>Folate compound</th>
<th>L. salivarius</th>
<th>L. casei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_D) (nM)</td>
<td>(K_I) (nM)</td>
</tr>
<tr>
<td>Homofolate</td>
<td>0.40</td>
<td>0.4</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.58</td>
<td>0.5</td>
</tr>
<tr>
<td>3',5'-Dichloromethotrexate</td>
<td>7.30</td>
<td>7.3</td>
</tr>
<tr>
<td>5-Formyltetrahydrofolate</td>
<td>0.35</td>
<td>0.3</td>
</tr>
<tr>
<td>5-Methyltetrahydrofolate</td>
<td>5.90</td>
<td>5.9</td>
</tr>
<tr>
<td>Pterate</td>
<td>1.40</td>
<td>1.4</td>
</tr>
<tr>
<td>p-Aminobenzoylglutamate</td>
<td>58,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>6-Hydroxymethylpterin</td>
<td>19,000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

*From Ref. 1.

b, not determined.

**TABLE II**

Comparative inhibition of \(^{3}H\)folate binding and transport by L. salivarius by various folate compounds

<table>
<thead>
<tr>
<th>Folate compound</th>
<th>[^{3}H]Folate binding inhibition by 50%</th>
<th>[^{3}H]Folate transport inhibition by 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homofolate</td>
<td>0.28</td>
<td>0.25</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>5-Formyltetrahydrofolate</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>5-Methyltetrahydrofolate</td>
<td>3.0</td>
<td>3.8</td>
</tr>
</tbody>
</table>

At an EDC-activated folate concentration of 25 nM and exceeded 90% at high concentrations of this reagent. Competition experiments (see Fig. 7) performed at a fixed concentration of \(^{3}H\)folate and increasing amounts of other folate compounds revealed that homofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, methotrexate, and 3',5'-dichloromethotrexate interacted with the binding component with high affinity, while pterate, p-aminobenzoylglutamate, and 6-hydroxymethylpterin were ineffective (Table I). A similar binding specificity for folate compounds has been observed previously for the folate transport protein of L. casei (see Table I).

**Characteristics of the Second Uptake Component**—The second uptake component for folate was dependent upon time and an available source of energy (see Fig. 2) and thus had the properties of substrate internalization. This latter conclusion was also consistent with the observations that \(^{3}H\)folate taken up via this component was refractory to exchange with external unlabeled folate (Fig. 2) and was predominantly in metabolized forms (Fig. 3). In addition, uptake of \(^{3}H\)folate via the second component could be inhibited by various folate compounds, including folate, homofolate, methotrexate, 5-formyltetrahydrofolate, and 5-methyltetrahydrofolate, and the relative ability of each folate compound to inhibit the internalization component by 50% paralleled the ability of these same compounds to inhibit (by 50%) the binding component (Table II). Additional measurements revealed that the secondary uptake component did not vary in assay mixtures containing \(^{3}H\)folate at concentrations between 25 and 500 nM, but it could be reduced to 50% of control values by employing cells that had been grown in medium containing 25 nM folate. Complete inhibition was also observed upon pretreatment of the cells with 200 nM EDC-activated folate.

**Exchange of Bound Folate**—The binding of folate to L. salivarius cells can be measured without interference from the second uptake component by reducing the temperature to 0 °C. Under the latter conditions, the second time-dependent uptake component was not observed over a 20-min incubation period even in cells preincubated with glucose (data not shown). \(^{3}H\)Folate bound to cells at 0 °C could be displaced upon addition of excess unlabeled folate (Fig. 8). In energy-depleted cells, this exchange appeared monophasic, proceeded with a \(t_0\) of 13 min, and approached completion at the longer time intervals. In contrast, cells that had been preincubated for 5 min at 30 °C with 1 mM glucose bound the same amount of \(^{3}H\)folate (at 0 °C) but exhibited a substantially slower exchange rate. Excess unlabeled folate in the latter case displaced bound \(^{3}H\)folate with a \(t_0\) of 40 min. A similar exchange pattern has been observed previously in L. casei (28).

**Relationship between Folate Transport and the Transport of Other Vitamins**—Kinetic studies have established that a common cellular component is required for the transport of folate, thiamine, and biotin in L. casei (13). This common component was proposed to be an energy-coupling factor which can interact with the individual binding proteins for each vitamin. The basic observations supporting a second shared component were that the transport of one vitamin was inhibited by the transport of a second vitamin and the extent of inhibition depended upon the relative amounts of binding protein (13). When similar measurements were performed in L. salivarius, no evidence was obtained for a second component shared with other transport systems: (a) L. salivarius cells grown in the presence of limiting thiamine did not produce a thiamine-binding protein (Table III) and did not accumulate thiamine (at 30 °C) in the presence of glucose; and (b) Biotin-depleted cells expressed substantial amounts of a biotin-binding pro-
Folate Transport in *L. salivarius*

![Graph showing Folate Bound versus Time](image)

**FIG. 8. Effect of the energetic state of the cell on the exchange rate for bound [3H]folate.** Cells that had either been depleted of energy reserves by incubation for 30 min at 23°C or preincubated for 2 min at 30°C in the presence of 1 mM glucose were exposed to 0.5 μM [3H]folate for 3 min at 0°C and then examined for the rate of release of bound [3H]folate by the addition of 100 μM unlabeled folate. Binding and release buffer, HEPES-Mg, pH 7.5.

**TABLE III**

Comparison of the levels of binding proteins for folate, thiamine, and biotin in *L. salivarius* and *L. casei*

Levels of the indicated binding proteins were measured as described under "Materials and Methods" in 2.0 ml HEPES-Mg buffer, pH 7.5, containing 0.5 μM labeled substrate.

<table>
<thead>
<tr>
<th>Binding protein</th>
<th><em>L. salivarius</em></th>
<th><em>L. casei</em>&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate</td>
<td>1.90</td>
<td>0.35</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.00</td>
<td>0.72</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.24</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup>From Ref. 13.

**TABLE IV**

Cross-reactivity of monoclonal antibodies to the folate-binding protein of *L. casei* with the folate-binding protein from *L. salivarius*

Assays were performed with 100 ng of the indicated binding protein and monoclonal antibodies (HK-1, HK-2, and HK-3) that had been isolated against the folate-binding protein of *L. casei*. The response of each antibody with the *L. casei*-binding protein was normalized to 100% and compared with the response under the same conditions to the *L. salivarius*-binding protein.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Source of binding protein</th>
<th>Relative response %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK-1</td>
<td><em>L. casei</em></td>
<td>100</td>
</tr>
<tr>
<td>HK-2</td>
<td><em>L. casei</em></td>
<td>100</td>
</tr>
<tr>
<td>HK-3</td>
<td><em>L. salivarius</em></td>
<td>100</td>
</tr>
</tbody>
</table>

Disregard
high affinity for folate allows L. salivarius to acquire folate when extracellular vitamin levels are extremely low. Folate internalization, however, proceeds at the unusually slow maximum rate of 0.05 molecules transported per min per binding site (at 30 °C). The latter rate is considerably less than the 0.90 turnovers per min per binding site that is observed for folate transport in L. casei (12) and the 16 molecules of methotrexate transported per min per binding site in L1210 cells (30).

The folate-binding protein from L. salivarius has properties very similar to those of a binding protein characterized previously from L. casei (1, 11, 12, 18, 28). Both proteins have a comparable affinity for various folate compounds at both pH 7.5 and at pH 6.0 (see Table I), are irreversibly inactivated and covalently labeled by EDC-activated folate (Fig. 7), can exist in two forms that express different rates of self-exchange (Fig. 8), require cations for optimum binding of folate (Fig. 6), are membrane-associated proteins with similar molecular weights, and can be isolated by the same purification procedure. The L. salivarius and L. casei binding proteins also share common protein domains since two of three monoclonal antibodies to the L. casei-binding protein react nearly identically with both proteins (Table IV). The structural similarities between these proteins presumably reflect a close genetic relatedness between these cells or a convergence in evolutionarily distant organisms of structures required in facilitating the transport of folate compounds.

The folate transport system of L. salivarius is unable to facilitate the concentrative uptake of folate. The inability to accumulate free intracellular folate was supported by the observation that the [3H]folate accumulated after a brief time interval at 30 °C did not exchange with externally added unlabeled folate (Fig. 2). Metabolism studies (Fig. 3) confirmed that the accumulated folate had undergone a substantial conversion both to other coenzyme forms and to polyglutamylates. Intracellular accumulation was also enhanced by glutamine (Fig. 4), a monovalent cation co-transport mechanism in which cation binding (Fig. 11) suggests that substrate internalization may be occurring by a folate/cation co-transport mechanism. Co-transport of the folate dianion with a divalent cation (or two monovalent cations) would be desirable since an electroneutral substrate would be readily metabolized by cells (32-33). The energy required during folate transport thus appears to be consumed for substrate metabolism and not by energy-driven components of the transport system. This contrasts with the folate transport system of L. casei which appears to utilize energy directly for the active accumulation of free folate (14).

The requirement of cations for optimum binding of folate (Fig. 6) suggests that substrate internalization may be occurring by a folate/cation co-transport mechanism. Co-transport of the folate dianion with a divalent cation (or two monovalent cations) would be desirable since an electroneutral substrate complex could be formed with externally oriented binding sites and the resulting complex could move across the membrane without hindrance from the inward-negative membrane potential. A similar requirement for cations has been observed for substrate binding to the folate (12) and biotin (34) transport proteins of L. casei and has been interpreted in terms of a cation co-transport mechanism in which cation binding precedes the binding of the anionic vitamin. The substrate-binding site on the L. salivarius transport protein also appears to exist in two forms, one that exchanges with bound folate more rapidly and predominates in energy-depleted cells, and another form which mediates a slower exchange and is found in energy-replete cells (Fig. 8). Similar forms observed previously for the folate transport protein in L. casei (28) have been proposed to represent proteins with binding sites oriented toward the outer or inner membrane surface, respectively.

The present findings support a mechanism for the internalization of folate in L. salivarius which involves a sequence of at least five separate steps. The proposed initial step in this process is the binding of a cation or cations to an externally oriented binding site. Folate then binds to a second site, the folate/cation complex is transferred across the membrane, substrate dissociation occurs at the inner surface of the membrane, and the binding site on the unloaded carrier returns to the cell exterior. Energy is not coupled directly to the transport process but is assigned a role in the metabolism of folate once it enters the cell. Folate coenzymes would eventually accumulate within the cells as polyglutamate forms which, with increasing chain length, would be progressively less able to exit the cells via the transport system for folate monoglutamates (3). The unusually slow rate of transport (0.05 turnovers/min/binding site) could likewise be explained by the requirement that folate must dissociate from internalized binding sites which are presumed to remain in a high-affinity state. In L. casei, a similar sequence of steps has been proposed for the cation-dependent internalization of folate compounds (12), although in this case an active accumulation of free folate is observed and energy-coupling components are thought to facilitate the release of folate from inward-facing binding sites (13, 14). The presence of an energy-coupling mechanism would account for a rate of folate transport in L. casei that is 18-fold higher per binding site than in L. salivarius.

REFERENCES

Folate Transport in *L. salivarius*