Transport and Metabolism of Folates by Bacteria*

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SUMMARY

Transport of labeled folic acid (PteGlu), pteroylpolyglutamates (PteGlu<sub>2</sub>...<sub>n</sub>), 5-methyl-tetrahydrofolate (5-methyl-H<sub>4</sub>PteGlu<sub>n</sub>), and methotrexate in late-log phase cells of Lactobacillus casei was active, and subject to inhibition by unlabeled pteroylmonoglutamates, pteroylpolyglutamates, and iodoacetate, but not glutamate or glutamate dipeptides. Pteroylpolyglutamates were transported without prior hydrolysis and shared a common uptake system with pteroylmonoglutamates. The affinity and maximum velocity of pteroylpolyglutamates were transported without prior hydrolysis and shared a common uptake system with pteroylmonoglutamates. The affinity and maximum velocity of pteroylpolyglutamates decreased with increasing glutamic acid chain length (K<sub>m</sub>: PteGlu<sub>1</sub>, 0.03 μM; PteGlu<sub>2</sub>, 0.32 μM; PteGlu<sub>3</sub>, 1.9 μM; PteGlu<sub>4</sub>, 3.7 μM) and comparisons with growth response curves suggested that polyglutamates were more effectively utilized by L. casei, once transported, than monoglutamate. No concentration of 5-methyl-H<sub>4</sub>PteGlu<sub>4</sub> inside the cells was observed.

The major folate metabolites found in L. casei preloaded with high levels of [3H]PteGlu (0.5 μM) were 10-formyl-H<sub>4</sub>PteGlu<sub>n</sub> and 10-formyl PteGlu<sub>n</sub>. Both compounds were released, the monoglutamate more rapidly. Pteroylglutamate formation appeared to be a rate-limiting step in intracellular metabolism. No 10-formyl-PteGlu was found in iodoacetate-treated cells and efflux was inhibited. Cells preloaded with low levels of [3H]PteGlu (7 nM) metabolized the vitamin to polyglutamate forms, the major derivatives being H<sub>4</sub>PteGlu<sub>n</sub>. First order exit rates of labeled folate from preloaded L. casei indicated an inhibition of PteGlu uptake with time. Exit rates dropped from 0.05 min<sup>-1</sup> to <0.002 min<sup>-1</sup> as intracellular folate was metabolized from monoglutamate to polyglutamate derivatives (n ≥ 3). In the latter case, materials lost by efflux were breakdown products and no folate of glutamate chain length greater than two was released.

Pediococcus cerevisiae actively transported 5-methyl-H<sub>4</sub>PteGlu but did not take up 5-methyl-H<sub>4</sub>PteGlu<sub>2</sub>. No active accumulation of 5-methyl-H<sub>4</sub>PteGlu was observed in Streptococcus faecalis.

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5-Methyl-H₄PteGlu was prepared by reduction of 5,10-methenyl-H₂PteGlu with sodium borohydride (28). H₄PteGlu was prepared by reduction of PteGlu in glacial acetic acid with sodium dithionite (29), and 10-formyl-PteGlu was prepared by formylation of PteGlu (30).

Folic acid polyglutamates PteGlu₂ to PteGlu₈-[U-¹³C]-Glu-Glu (specific activity, 310 μCi per mmol), PteGlu₂-[U-¹⁴C]-Glu-Glu (specific activity, 100 μCi per mmol), and PteGlu₂-[U-¹⁴C]-Glu-Glu (specific activity, 410 μCi per mmol) were synthesized by the method of Baugh et al. (12). (+)-5-Methyl-[³H]H₄PteGlu-8-Glu-Glu (specific activity, 100 βCi per mmol), and PteGlu₈-[U-¹⁴C]-Glu-Glu (specific activity, 410 βCi per mmol) were synthesized by the method of Baugh et al. (12). Polyglutamates were purified by chromatography on QAE-Sephadex and DEAE-cellulose. The identity of each compound was unambiguously confirmed by its chromatographic behavior on Sephadex G-25 and by differential microbiological response after hog kidney conjugase treatment (13, 15, 31).

γ-L-Glutamyl-L-glutamic acid was obtained from Sigma Chemical Co., and α-L-glutamyl-L-glutamic acid was obtained from Schwartz-Mann.

Organisms and Growth Conditions—Lactobacillus casei (ATCC 7409), Streptococcus faecalis (ATCC 8043), and Pediococcus cerevisiae (ATCC 8051) were cultured by the procedure of Bird et al. (31) with slight modifications employed in this laboratory (15).

Folates added to the media were PteGlu (1 ng per ml for L. casei, 2 ng per ml for S. faecalis), or 5-formyl-H₄PteGlu (1 ng per ml for P. cerevisiae). Folates added to the media were PteGlu (1 ng per ml for L. casei, 2 ng per ml for S. faecalis), or 5-formyl-H₄PteGlu (1 ng per ml for P. cerevisiae). Centrifugation from growth media in late log phase (20 to 24 hours) was used for growth experiments. S. faecalis, L. casei but not S. faecalis, had removed all of the 5-methyl-H₄PteGlu as determined by paper chromatography at pH 6.0. S. faecalis, L. casei but not S. faecalis, had removed all of the 5-methyl-H₄PteGlu as determined by paper chromatography at pH 6.0.

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Measurement of Folate Uptake—Bacteria were harvested by centrifugation from growth media in late log phase (20 to 24 hours at 37°C), washed twice with double-strength buffer, resuspended in buffer, and stored at 0-4°C during the course of an experiment until used. Unless otherwise indicated, the buffer was used (single strength) with a pH of 6.0. All buffers contained 0.2 M potassium phosphate, pH 6.0. Cells (0.02 to 1.0 mg per ml) were preincubated in a shaking water bath at 37°C for 5 min before addition of labeled vitamin. Aliquots (0.5 or 1.0 ml) were removed at various intervals and filtered on HA filters (Millipore Corp., 25-mm diameter, 0.45-μm pore size). After washing with cold buffer (twice with 1 ml), cells plus filter were added directly to counting vials. Aquasol (New England Nuclear) or Triton X-100-toluene (1:2) scintillation mix plus filter were added directly to counting vials. Aquasol (New England Nuclear) or Triton X-100-toluene (1:2) scintillation mix was added to each vial.

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Identification of Labeled Folates—Folates were identified by their chromatographic behavior on Sephadex G-25 and DEAE-cellulose before and after hog kidney conjugase treatment (13).

Identification of Labeled Folates—Folates were identified by their chromatographic behavior on Sephadex G-25 and DEAE-cellulose before and after hog kidney conjugase treatment (13). The chromatographic behavior of folates on Sephardex G-25 has been described in detail by Coleman et al. (30). However, fornyl derivatives were retarded less than PteGlu, H₂PteGlu, and 5-methyl-H₂PteGlu. This was also true for polyglutamates of folic acid and for 10-formyl-H₂PteGlu, which eluted approximately at the position of PteGlu₂₅₃. [³H]Folate samples were chromatographed on Sephadex G-25 columns that had been previously standardized with authentic standards and individual peaks were rerun on Sephadex together with appropriate ¹⁴C-labeled or unlabeled folate standards.

Further identification of the various folate derivatives was achieved by chromatography of [³H]folate samples, before and after hog kidney conjugase treatment, on DEAE-cellulose as described by Buehring et al. (13). Columns (25 X 0.9 cm) were eluted with an exponential phosphate gradient formed with 0.01 M potassium phosphate, pH 6.0 (0.6 μl buffers contained 0.2 m mercaptoethanol. A standard solution containing unlabeled PteGlu and 10-formyl-PteGlu and 5-[¹⁴C]methyl-H₂PteGlu was added to each [³H]folate sample before application to the column. The 5-[¹⁴C]methyl-H₂PteGlu standard had been stored for several months at 4°C and, under these conditions, about 50% had been converted to a degradative product of unknown composition, indicated by X in Figs. 6, 7, and 9. The elution position of this compound, which had no growth-promoting activity for L. casei, was useful in the interpretation of data from DEAE-cellulose chromatography. Calculation of Kₐ, values for folate transport were calculated by the method of Wilkinson (32).

As loss of intracellular vitamin followed first order kinetics, rate constants were determined by a least squares log-linear regression method.

RESULTS

5-Methyl-H₂PteGlu Uptake by Streptococcus faecalis—Although intracellular levels of 5-[¹⁴C]methyl-H₂PteGlu slowly equilibrated with extracellular vitamin at 37°C, the only concentration effects noted could be explained by intracellular breakdown of labeled vitamin to the degradative product X. These effects were noted in a variety of glucose-containing buffers (20 mM potassium phosphate, pH 6; acetate-phosphate buffer, pH 6), and in buffer containing acetate-phosphate buffer, pH 6, and 100 μM azide (50 μM) or azide (50 μM) plus iodoacetate (10 mM), and culturing in thymidine instead of PteGlu did not significantly change this uptake pattern. Uptake rates were approximately proportional to substrate concentration over the range tested (0.01 to 50 μM) and were reduced slightly at 0°C. A slight pH effect on uptake with a maximum at pH 6.0 was noted, but at low levels of relatively low specific activity vitamin was being measured, it was difficult to distinguish whether this was a transport phenomenon or a nonspecific binding effect.
levels of the Glus to Glus forms were taken up (Table I) and it is possible that these results reflect binding to the cells and filter PteGlu. mono- and polyglutamyl folates by P. cerevisiae. As shown by folates, with the exception of 5-methyl-H4PteGlu, for growth. of a growth inhibitor, as has been suggested for Pediococcus removal of a non-folate growth factor by S. javécalis or excretion studies carried out with P. cerevisiae, an organism that requires reduced low folate concentrations were used, and in the first 5 min at typical effects of time on the uptake of [3H]H4PteGlu and 5-[14C]-methyl-H4PteGlu by L. casei. Intracellular concentrations of rather than transport as such. least, most of this transported vitamin had not been metabolized to cell concentration up to 0.05 mg per ml (dry weight). Above this concentration, a falling off from linearity was observed. A rapid initial uptake or binding occurred within 60 s and was unable to deplete the medium of [3H]H4PteGlu, and an overshoot effect was often noted in intracellular tritium levels. Uptake of 5-methyl-H4PteGlu and PteGlu was proportional to cell concentration up to 0.05 mg per ml (dry weight). Above this concentration, a falling off from linearity was observed. [14C]Methotrexate was also concentrated by L. casei (Fig. 2A). A rapid initial uptake or binding occurred within 60 s and was followed by a gradual accumulation of tritium label. Iodoacetate (10 mM) inhibited initial uptake rates by 40 to 80%. At higher levels (100 mM), intracellular levels of 5-[14C]-methyl-H4PteGlu equilibrated with extracellular levels and only a slight concentration of [14C]PteGlu was observed. Azide (50 mM), arsenite (20 mM), and arsenate (20 mM) had no apparent effect on monoglutamate uptake in L. casei. Uptake at 0°C was about 10% of the 37°C rates.

**Table I**

| Folate                      | Extracellular concentration (nm) | Intracellular concentration (nm after min) |
|-----------------------------|---------------------------------|-------------------------------------------|
|                             |                                 | 1  | 2  | 5  | 10 | 30 | 60 | 120 |
| (±)-5-CH2-H4PteGlu          | 30                              | 72 | 155 337 | 635 | 2007 |
| (+)-5-CH2-H4PteGlu          | 2.81                            | 0.02 | 0.43 | 0.21 | 0.71 | 0.10 | 0.24 |
| (-)-5-CH2-H4PteGlu          | 23.0                            | 2.78 | 6.19 | 3.64 | 3.12 | 3.51 | 1.28 |
| (+)-5-CH3-H4PteGlu          | 0.21                            | --- | --- | --- | --- | --- | --- |
| (+)-5-CH3-H4PteGlu          | 0.30                            | --- | --- | --- | --- | --- | --- |
| (-)-5-CH3-H4PteGlu          | 0.49                            | --- | --- | --- | --- | --- | --- |
| (+)-5-CH3-H4PteGlu          | 0.61                            | --- | --- | --- | --- | --- | --- |
| PteGlu                      | 14.0                            | 2  | 2  | 14  | 5  | 15  | 14  |
|                             | 1340                            | 111 | 32 | 53  | 20 | 186 | 254 |

* Dashes indicate no uptake detected.
chain length polyglutamates were found, and it was apparent that these compounds were transported without prior hydrolysis. In fact, if a similar concentration of PteGlu had been taken up under similar conditions (see “Metabolism of [3H]PteGlu by L. casei”), practically all of the intracellular vitamin would have been in the monoglutamate form.

Kinetics of Folate Uptake by L. casei—Kinetic data for folate uptake in L. casei are shown in Table III. In each case, uptake followed typical Michaelis-Menten-type kinetics. Initially, PteGlu uptake was measured at pH 6, the pH of the growth medium. The values obtained at the optimum pH, 5.5, were practically identical. It can be seen that increasing the glutamate chain length leads to a decrease in affinity for the transport system and a lowering of maximal uptake rates. A comparison of these transport parameters with growth data for PteGlu obtained previously by Tamura et al. (15), and making the assumption that growth of the bacteria was purely a function of intracellular vitamin at suboptimal folate concentrations, suggests that, once transported, the longer chain polyglutamates are better utilized by L. casei than is PteGlu.

(+)-5-Methyl-[3H]PteGlu demonstrated a similar affinity for the uptake system as PteGlu (Table III), but showed a higher \( V_{max} \) value. However, the compound was a mixture of two diastereoisomers and the true affinity of the active (+)-stereoisomer would be about 14 nM, assuming no competition from the biologically inactive (−)-form. Of course, if the (−)-stereoisomer is a competitive inhibitor of ( +)-5-methyl-[3H]PteGlu uptake, and has a similar affinity for the uptake system but is not transported, then the true \( K_m \) for (+)-5-methyl-[3H]PteGlu uptake would be 27 nM and its \( V_{max} \) value would be about 47 \( \mu \)M·min\(^{-1}\).

It can be seen that ionic (or buffer) strength, as noted earlier with 5-methyl-[3H]PteGlu and PteGlu transport, affects the affinities for the transport system(s) but not maximal uptake rates (Table III).

An apparent \( K_m \) value of approximately 1 \( \mu \)M was obtained statistically for [3H]methotrexate uptake (Fig. 2A) at the 60-s level but uptake only approximated Michaelis-Menten-type kinetics.

The effects of various unlabeled folate analogs on 5-[^14C]-methyl-[3H]PteGlu and [3H]PteGlu uptake by L. casei are shown in Table IV. Each compound tested inhibited uptake competitively with the exception of methotrexate. Although an apparent \( K_i \) value is given for methotrexate, \( V_{max} \) values obtained in the presence of this compound were considerably lower than in its absence.

![Fig. 1. Uptake of PteGlu and 5-methyl-H<sub>2</sub>PteGlu by Lactobacillus casei.](image1)

![Fig. 2. Uptake of methotrexate (MTX) and PteGlu<sub>1-5</sub> by Lactobacillus casei.](image2)

**Table II**

| Folate                        | Intracellular concentration nm | Intracellular concentration nm after min |
|-------------------------------|--------------------------------|----------------------------------------|
|                               | 1     | 2     | 5     | 10    | 20    | 60    | 120   |
| (±)-5-CH<sub>3</sub>H<sub>2</sub>PteGlu | 35.5  | 3755  | 4312  | 4350  | 4498  | 3843  | 3.20  | 3.57  |
| (+)-5-CH<sub>3</sub>H<sub>2</sub>PteGlu   | 2.61  | 0.48  | 0.69  | 1.31  | 2.60  | 3.20  | 5.97  | 0.31  |
| (+)-5-CH<sub>2</sub>H<sub>3</sub>PteGlu    | 23.2  | 4.80  | 6.76  | 6.17  | 5.26  | 6.64  | 0.47  | 0.31  |
| (+)-5-CH<sub>3</sub>H<sub>2</sub>PteGlu    | 0.28  | 0.31  | 0.30  | 0.28  | 0.29  | 0.51  | 0.47  | 0.31  |
| (+)-5-CH<sub>3</sub>H<sub>2</sub>PteGlu    | 0.28  | 0.01  | 0.02  | 0.06  | 0.03  | 0.12  | 0.22  | 0.30  |
| (+)-5-CH<sub>3</sub>H<sub>2</sub>PteGlu    | 0.45  | 0.02  | 0.05  | 0.09  | 0.13  | 0.02  | 0.45  | 0.15  |
| (+)-5-CH<sub>3</sub>H<sub>2</sub>PteGlu    | 0.56  | 0.08  | 0.05  | 0.03  | 0.26  | 0.09  | 0.15  | 0.15  |
absence. It appeared to be a mixed inhibitor of monoglutamate uptake.

As all compounds tested had similar affinities for both the 5-

\[ ^{14} \text{C}\]methyl-H$_2$PteGlu and [HI]PteGlu uptake systems, it seems unlikely that these compounds are transported by separate systems. Also, $K_I$ values for PteGlu are similar to their uptake $K_m$ values (Table III), which suggests that all the compounds tested are transported by the same system. The slight difference between $K_m$ and $K_I$ values for PteGlu, are easily explained by the high cell concentrations needed to measure uptake of these compounds with the consequent removal of much of the substrate from the medium.

L-Glutamic acid, $\alpha$-L-glutamyl-L-glutamic acid, and $\gamma$-L-glutamyl-L-glutamic acid (100 $\mu$M) had no effect on 5-$^{14}$C]methyl-H$_2$PteGlu or [HI]PteGlu transport.

**Efflux of Intracellular Vitamin**—The efflux of labeled vitamin was studied with *L. casei* that had been preloaded for various times with [HI]PteGlu. Exit followed first order kinetics, and rate constants are shown in Table V. With cells that had been preloaded for 5 min with relatively large amounts of [HI]PteGlu (0.5 $\mu$M), considerable reuptake of vitamin lost by efflux took place, as evidenced by the increased exit rate when uptake was

![Fig. 3. Sephadex G-25 column chromatography of intracellular and extracellular labeled folates after incubating Lactobacillus casei with [14C]PteGlu (A) or [14C]PteGlu (B). Cells (2 mg per ml) were preincubated at 37°C for 5 min in 50 mM K$_2$HPO$_4$-100 mM sodium acetate-H$_3$PO$_4$ buffer, pH 6, containing 1% glucose before the addition of [14C]PteGlu (7 $\mu$M, A) or [14C]PteGlu (5 $\mu$M, B). After 60 min, cells were filtered, and intracellular vitamin was extracted with boiling buffer. The elution positions of reference compounds under comparable conditions are indicated beneath the abscissa.](image)

**TABLE IV**

| Analog | $K_I$ | $A$ | $B$ |
|--------|------|-----|-----|
| Methotrexate* | 0.031 | 0.033 |
| 10-CHO-PteGlu | 0.035 | 0.040 |
| (+)-5-CHO-H$_2$PteGlu | 0.038 | 0.031 |
| (+)-5-CH$_3$-H$_2$PteGlu | 0.033 | 0.037 |
| PteGlu | 0.016 | 0.020 |
| PteGlu$_3$ | 0.005 | 0.063 |
| PteGlu$_2$ | 0.15 | 0.14 |
| PteGlu$_4$ | 1.2 | 1.4 |
| PteGlu$_5$ | 1.2 | 1.8 |
| PteGlu$_6$ | 7.0 | 7.2 |
| PteGlu$_7$ | 10.2 | 9.7 |
| Glu | >500 | >500 |
| Glu-\(\alpha\)-Glu | >500 | >500 |
| Glu-\(\gamma\)-Glu | >500 | >500 |

* Mixed inhibitor of folate uptake.

**TABLE III**

| Folate | pH | $K_m$ ± S.E. | $V_{\text{max}}$ ± S.E. | Concentration at 50% maximum growth | Velocity at 50% maximum growth |
|--------|----|-------------|----------------|-------------------------------|-------------------------------|
| PteGlu | 6.0 | $0.28 \pm 0.005$ (7) | $14.2 \pm 1.1$ (7) | 0.93 | 456 |
| PteGlu$_2$ | 6.0 | $0.32 \pm 0.13$ (5) | $15.6 \pm 2.0$ (5) | 0.93 | 45.2 |
| PteGlu$_3$ | 6.0 | $1.9 \pm 0.3$ (5) | $13.5 \pm 2.4$ (5) | 1.52 | 10.8 |
| PteGlu$_4$ | 0.0 | $3.7 \pm 1.2$ (5) | $0.0 \pm 0.7$ (5) | 4.49 | 7.3 |
| PteGlu$_5$ | 5.5 | $0.110 \pm 0.010$ (7) | $15.1 \pm 0.6$ (7) | 14.0 | 0.9 |
| PteGlu$_6$ | 5.5 | $0.030 \pm 0.001$ (7) | $23.1 \pm 0.9$ (7) | 23.7 | 0.7 (7) |
| (+)-5-CH$_3$-H$_2$PteGlu* | 5.5 | $0.071 \pm 0.004$ (7) | $23.7 \pm 0.7$ (7) | 23.7 | 0.7 (7) |

* Uptake in 10 mM K$_2$HPO$_4$-H$_2$PO$_4$ buffer containing 1% glucose.

* From data of Tamura et al. (15).
TABLE V
First order exit rates (k) of labeled folate from Lactobacillus casei

| Preloading time | Efflux media | Preloaded with 7 nm PteGlu | Preloaded with 0.5 μM PteGlu |
|-----------------|--------------|--------------------------|-----------------------------|
| min             |              | S₀ μM min⁻¹ | k μM min⁻¹ | S₀ μM min⁻¹ | k μM min⁻¹ |
| 5               | Buffer       | 7.10       | 0.020     | 64.3       | 0.015     |
|                 | + azide (50 mM) + iodoacetate (20 mM) + PteGlu (50 μM) | 6.05 | 0.0017 | 90.3 | 0.035 |
| 60              | Buffer       | 7.00       | 0.0016    | 94.6       | 0.054     |
|                 | + azide (50 mM) + iodoacetate (20 mM) + PteGlu (50 μM) | 6.85 | 0.0008 | 92.6 | 0.0079 |
| 120             | Buffer       | 7.12       | 0.0024    |           |           |
|                 | + azide (50 mM) + iodoacetate (20 mM) + PteGlu (50 μM) | 6.01 | 0.0015 |           |           |
| 240             | Buffer       | 6.24       | 0.0006    |           |           |

inhibited by excess unlabeled PteGlu. Exit rates were very low in the presence of metabolic inhibitors. Preloading cells for 60 min with [3H]PteGlu (0.5 μM) did not significantly affect the exit rate of labeled vitamin when reuptake was inhibited by excess unlabeled PteGlu, but the net exit rate in buffer alone was increased. It appeared that influx of vitamin was decreased compared to cells that had been preloaded for 5 min and suggests some metabolic control over the transport of PteGlu. This would also explain the overshoot effect noted earlier with PteGlu uptake.

Cells preloaded with low levels of [3H]PteGlu (7 nm) for 5 min exhibited a similar exit rate to that found with 0.5 μM PteGlu-loaded cells; so it was apparent that the exit system was not saturated under the conditions of these experiments. With longer preloading times with 7 μM [3H]PteGlu, and consequent intracellular metabolism to polyglutamate forms, the exit rate dropped to very low levels. This was not a result of increased reuptake, as excess unlabeled PteGlu had little effect on these rates. Metabolic inhibitors increased the exit rates under these conditions but the rates were still very low. It should be noted that the intracellular vitamin concentration was similar irrespective of preloading time, so the change in exit rate must have been solely a function of folate form.

Metabolism of [3H]PteGlu by L. casei—Duchning et al. (13) have shown that L. casei grown in the presence of [3H]PteGlu (25 nm) for 24 hours contain mainly pteroylglutamates while growth medium apparently contained shorter length polyglutamates. In our studies, preloading times and [3H]PteGlu concentrations were varied to control the degree of metabolism of PteGlu. Addition of glutamic acid (1 mM) to the preloading and efflux media did not discernibly affect metabolism. Metabolism of intracellular vitamin was investigated in preloaded cells at 0, 5, 20, and 60 min after resuspension in fresh media. Exit rates were checked to ensure that they corresponded to the rates shown in Table V.

The Sephadex G-25 elution patterns of cell extract and medium after a 5-min preloading with [3H]PteGlu (0.5 μM) are shown in Fig. 4A. Practically all of the tritium label was associated with the monoglutamate fraction. The Sephadex G-25 elution patterns of intracellular labeled vitamin after resuspension of these cells in fresh buffer for 60 min are shown in Fig. 4B. By this time,
FIG. 5. Metabolism of [H]PteGlu by Lactobacillus casei. Experimental conditions are as described in the legend to Fig. 4, except cells were preloaded for 60 min with [3H]PteGlu (0.5 μM; specific activity: 0.5 Ci per mmol). Cells incubated in buffer alone had metabolized PteGlu to several compounds, including some polyglutamates eluting before standard PteGlu. One metabolite (Peak A in Fig. 4B) eluted just after standard PteGlu at the approximate position of a 10-formyl-PteGlu derivative, while another (Peak B in Fig. 4B) eluted at the approximate position of a 10-formyl-PteGlu derivative. A similar elution pattern was seen in cells suspended in buffer containing excess unlabeled PteGlu although less labeled vitamin was retained by these cells as reuptake was inhibited (Fig. 4B). Conversely, cells resuspended in buffer plus iodoacetate contained higher levels of labeled vitamin as efflux was inhibited (Fig. 4B). In this case, a large part of the intracellular vitamin was metabolized to the compound resembling a 10-formyl-PteGlu derivative (Peak A in Fig. 4B) and a smaller polyglutamate fraction was observed. The Sephadex G-25 elution patterns of tritium in the media after resuspending the cells for 60 min are shown in Fig. 4C. Extracellular vitamin released from cells incubated in buffer alone consisted mainly of the 10-formyl-PteGlu derivative (Peak A) plus some monoglutamate (Peak D) while vitamin from cells incubated in buffer containing excess unlabeled PteGlu was, as expected, mainly in the monogluta- mate form (Peak D) plus a compound with the chromatographic properties of a 10-formyl-PteGlu derivative (Peak B in Fig. 4C). Comparison of this elution pattern with the pattern obtained after a 5-min resuspension of cells in buffer containing excess unlabeled PteGlu (not shown) indicated that practically all of Peak B was released in the first 5 min. Most of the intracellular folate released from the cells after a 60-min resuspension in buffer containing metabolic inhibitors eluted at the position of the 10-formyl-PteGlu derivative (Peak A in Fig. 4C).

Fig. 5, A to C, shows comparable results for cells that had been preloaded for 60 min with [H]PteGlu (0.5 μM) before resuspension in fresh buffer for 60 min. After preloading, the intracellular vitamin was mainly in the monoglutamate form (Fig. 5A) as was found after a 5-min preloading (Fig. 4A). However, resuspension of the cells led to further metabolism of intracellular vitamin (Fig. 5B). The 10-formyl-PteGlu and 10-formyl-PteGlu derivatives built up (Peaks A and B, Fig. 5B) as well as a compound with the elution position of a PteGlu derivative (Peak C). A similar pattern was found with cells resuspended in buffer containing excess unlabeled PteGlu while most of the intracellular vitamin in iodoacetate-treated cells eluted at the position of a 10-formyl-PteGlu derivative (Peak A, Fig. 5B). The Sephadex G-25 elution patterns of label in the media of cells incubated in buffer alone or buffer plus excess unlabeled PteGlu (Fig. 5C) showed that the main released forms after 60 min were 10-formyl-PteGlu and PteGlu derivatives (Peaks B and C) with some 10-formyl-PteGlu derivative (Peak A) and monoglutamate (Peak D). Again, the net release of the 10-formyl-PteGlu derivative

Fig. 6. DEAE-cellulose chromatography of 10-formyl-PteGlu derivatives. [H]Folate peaks eluted from Sephadex G-25 columns at the approximate position of a 10-formyl-PteGlu derivative (Peak A, Figs. 4 and 5) were treated with hog kidney conjugase and chromatographed on DEAE-cellulose as described in the legend to Fig. 7. A, the elution pattern obtained with folate derivative from the medium of cells suspended in buffer alone (Peak A, Fig. 5C); B, the pattern with intracellular derivative from iodoacetate-treated cells (Peak A, Fig. 5B).
FIG. 7. DEAE-cellulose chromatography of the 10-formyl-PteGlu derivative. [3H]folic acid eluted from Sephadex G-25 columns at the approximate position of a 10-formyl-PteGlu derivative (Peak B, Fig. 5C) was applied to a DEAE-cellulose column (25 X 0.9 cm). The column was eluted by an exponential phosphate gradient formed with 0.01 M potassium phosphate buffer, pH 6, (100 ml) in a closed mixing chamber attached to a reservoir of 0.6 M potassium phosphate buffer, pH 6. The elution positions of reference compounds, applied with the [3H]folic acid sample, are indicated beneath the abscissae.

(Peak B) appeared to be complete by 5 min. No 10-formyl-PteGlu derivative was found in the medium of iodoacetate-treated cells (Fig. 5C), only a buildup of the 10-formyl-PteGlu derivative (Peak A) plus some monoglutamate.

The identity of the 10-formyl-PteGlu derivative (Peak A, Fig. 4, B and C; Fig. 5, B and C) was confirmed by DEAE-cellulose chromatography. The folic acid eluted as a single peak after conjugase treatment at the position of 10-formyl-H$_4$PteGlu (Fig. 6) indicating it to be 10-formyl-H$_4$PteGlu. Similarly, the 10-formyl-PteGlu derivative (Peak B, Figs. 4C and 5, B and C) was identified to be 10-formyl-PteGlu (Fig. 7).

It was apparent that only a small amount of polyglutamate of chain length greater than two was formed under the described conditions and it appeared that triglutamate synthesis was a rate-limiting step in metabolism of PteGlu. It also appeared that iodoacetate inhibited the synthesis of polyglutamates. In order to study the further metabolism of PteGlu and to study efflux of higher polyglutamates, cells were preloaded for 2 or 4 hours with low levels of [3H]PteGlu (7 nM). Although some variation in polyglutamate composition was found under these conditions, the results shown in Fig. 8, A to C, are typical of the effects noted.

The Sephadex G-25 elution pattern of intracellular labeled vitamin after preloading with [3H]PteGlu (7 nM) for 120 min (Fig. 8A) showed that most of the vitamin consisted of polyglutamates with chain length ≥3. Folate in the medium was of shorter glutamate chain length (Fig. 8A) with considerable amounts of mono- and diglutamate forms. Recycling of these cells for 60 min in buffer alone led to further chain elongation of intracellular folate (Fig. 8B) and practically no net release of labeled vitamin (Fig. 8C). With cells incubated in buffer containing iodoacetate, a small release of mono- and diglutamates was observed as well as a folate which eluted just before standard PteGlu from Sephadex G-25 (Fig. 8C). There was less apparent intracellular chain elongation with iodoacetate-treated cells (Fig. 8B) and in some experiments no chain elongation was observed. Intracellular labeled folate from cells suspended in buffer alone for 60 min was treated with conjugase and chromatographed on DEAE-cellulose (Fig. 9A). Most of the tritium label eluted at the position of H$_4$PteGlu (Peak D, Fig. 9A, 72%) with some 10-formyl-H$_4$PteGlu (Peak B, 13%), 5-methyl-H$_4$PteGlu (Peak C, 10%), and a trace of PteGlu (5%). Material eluted straight through the column (Peak A, Fig. 9) represented breakdown products of unknown composition. These breakdown products were always observed when high specific activity (37 Ci per mmol) PteGlu was used (13). Iodoacetate-treated cells, after conjugase treatment, also contained 10-formyl-H$_4$PteGlu (Peak B, Fig. 9B, 19%), 5-methyl-H$_4$PteGlu (Peak C, Fig. 9B, 6%), H$_4$PteGlu (Peak D, Fig. 9B, 19%), as well as breakdown products (Peak A, Fig. 9B). However, most of the radioactive label eluted from DEAE-cellulose at the position of X (54%), the 5-methyl-H$_4$PteGlu breakdown product (Fig. 9B). The material eluted at Peak X was rechromatographed on Sephadex G-25 (Fig. 10) and, although it had been treated with conjugase, still eluted just prior to standard PteGlu. It was apparent from this that the chain lengths of polyglutamates found in iodoacetate-treated cells (Fig. 8B) were lower than their elution positions from Sephadex G-25 suggested.

The small amounts of labeled vitamin released from cells sus-
and aliquots (equivalent to 0.4-mg cells) were chromatographed on DEAE-cellulose as described in the legend to Fig. 7. Intracellular vitamin was extracted with boiling buffer, treated with hog kidney conjugase, and reteined against a concentration gradient and the transport process was dependent on energy, pH, temperature, ionic effects, and displayed structural specificity and saturation kinetics. PteGlulA uptake also demonstrated many of the properties of an active process. As suggested by Buehring et al. (13), PteGlu polyglutamates were transported without prior hydrolysis.

Although Cooper (38) suggested that PteGlu and 5-methyl-H4PteGlu were transported by separate uptake systems in L. casei, the similarities we found between K, values for uptake and K, values measured against both [3H]PteGlu and 5-[3H]-methyl-H4PteGlu uptake suggests that all folates tested were transported by the same system. The K, value of 0.03 μmol for PteGlu uptake was similar to that reported by Cooper (38) (0.045 μmol). The only qualitative difference between uptake of PteGlu compared to other folates was an inhibition of uptake with time. This apparent metabolic control was probably a consequence of culturing the cells with PteGlu as folate source.

A decrease in transport system affinities and maximal transport rates was observed with increasing pteroylglutamate chain length and glutamic acid dipeptides did not affect uptake. It appears that the specificity of the uptake system was for the pteroyl moiety of folate compounds.

Comparisons of transport parameters with growth data obtained previously by Tamura et al. (15) suggest that, although pteroylglutamates are transported at a slower rate, transport is more effectively utilized by L. casei for growth than is PteGlu.

[3H]Methotrexate uptake did not follow Michaelis-Menten-type kinetics, probably because the compound is rapidly broken down and released by L. casei (39).

The study of the exit mechanism was complicated by metabolism and reuptake of vitamin. Exit was inhibited by iodoacetate, suggesting a carrier-mediated system. When cells were loaded with relatively large amounts of [3H]PteGlu, very little folate containing more than 2 glutamate residues was formed and it appeared that triglutamate formation was rate-limiting. Sakami et al. (40), in studies with Neurospora crassa, reported the presence of two polyglutamate synthetases, one specific for the formation of pteroyldiglutamate and the other for synthesis of longer chain polyglutamates. Our results could also be explained by two synthetases with similar properties to, but was distinct from, 10-formyl-H4PteGlu, and a pteroyldiglutamate built up. 10-Formyl-PteGlu was rapidly released by L. casei and was not detected in iodoacetate-poisoned cells, which metabolized PteGlu mainly to 10-formyl-H4PteGlu. This latter compound was probably the "early eluting folate" described by Cooper (38) which had similar properties to, but was distinct from, 10-formyl-PteGlu, and whose formation was inhibited by methotrexate. These results are also in agreement with the observation of Ohara and Silber (41) that the specific activity of L. casei 10-formyl-H4PteGlu synthetase was highest in late-log phase cells. Although some differences in exit rates with iodoacetate-treated cells might be

![Fig. 9. DEAE-cellulose chromatography of labeled folates in Lactobacillus casei. Cells (0.2 mg per ml) suspended in 50 mM K2HPO4-100 mM sodium acetate-H2PO4 buffer, pH 6, containing 1% glucose were preloaded at 37°C for 120 min with [3H]PteGlu (7 nCi per mmol), filtered, and reincubated in A, fresh buffer or B, buffer containing azide (50 mM) and iodoacetate (20 mM) (0.5 vol) for 60 min. Intracellular vitamin was extracted with boiling buffer, treated with hog kidney conjugase, and aliquots (equivalent to 0.4-mg cells) were chromatographed on DEAE-cellulose as described in the legend to Fig. 7.](http://www.jbc.org/Downloaded from http://www.jbc.org)

![Fig. 10. Sephadex G-25 chromatography of Peak X. The conjugase-treated [3H]folate derivative from iodoacetate-treated Lactobacillus casei, which eluted at the position of X, the 5-CH3-H4PteGlu breakdown product (Fig. 9B), was chromatographed on Sephadex G-25. The elution positions of reference compounds are indicated beneath the abscissa.](http://www.jbc.org/Downloaded from http://www.jbc.org)
expected due to differences in intracellular metabolism, it was apparent that exit per se was inhibited by this compound. Iodoacetate also appeared to inhibit polyglutamate formation in these cells, which again suggests two polyglutamate synthetases in *L. casei*.

Buehring et al. (13) were unable to detect conjugase activity in *L. casei*. Our results confirm this observation as the metabolism of H4PteGlu in cells resuspended in buffer plus excess unlabeled PteGlu was similar to that found in cells resuspended in buffer alone. If conjugase had been present, an equilibration of tritium between mono- and polyglutamyl folates would have occurred with a much larger proportion of the label in the monoglutamate fraction in cells resuspended with excess PteGlu.

Sakami et al. (40) reported that only H4PteGlu compounds were substrates for the polyglutamate synthetase of *N. c. mossae*, while Gawthorne and Smith (42) have reported that 5-formyl-H4PteGlu and 5-methyl-H4PteGlu would also serve as substrates for the sheep liver enzyme. In our studies, with cells metabolizing low levels of [3H]PteGlu, the major polyglutamates present were H4PteGlu or,H4PteGlu or. Although this would suggest a specificity of glutamate fraction in cells resuspended with excess PteGlu.

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by higher concentrations, although we consider this to be unlikely. It may well be that the same carrier is used by *L. casei* for folate uptake and exit and that this carrier will not transport reduced pteroylglutamate compounds, thus explaining their retention by the cells.
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