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Transport of Folinate and Related Compounds in *Pediococcus cerevisiae*

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The properties of folinate and 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) transport mechanism of *Pediococcus cerevisiae* were studied. The uptake was dependent on temperature, *p*H (optimum for both compounds at *p*H 6.0), and glucose. Iodoacetate, potassium fluoride, and sodium azide inhibited the uptake. 5-CH_3-H_4-PteGlu was apparently not metabolized but folinate was metabolized. Metabolism of folinate was reduced by preincubation of cells with fluorodeoxyuridine. The transport system for folinate and 5-CH_3-H_4PteGlu were specific for the L-isomers. Pteroylglutamate, aminopterin, and amethopterin did not interfere with the uptake. Tetrahydrofolate competed with the uptake of folinate. The transport of folinate and 5-CH_3-H_4PteGlu at 37 C conformed to Michaelis-Menten kinetics; apparent K_m for both compounds was 4.0×10^{-7} M, and the V_{max} for folinate was 1.0×10^{-10} moles per min per mg (dry weight) and for 5-CH_3-H_4PteGlu it was 1.6×10^{-10} moles per min per mg (dry weight). Both compounds accumulated in the intracellular pool at a concentration about 80- to 140-fold higher than that in the external medium. Folinate inhibited competitively the uptake of 5-CH_3-H_4PteGlu with a K_i of 0.4×10^{-7} M. Unlike 5-CH_3-H_4PteGlu, which accumulated only at 37 C, folinate was also taken up at 0 C by a glucose- and temperature-independent process, which was not affected by the metabolic inhibitors mentioned above. Since at 0 C the intracellular concentration of folinate was also considerably higher than the external, binding of the substrate to some cellular component is assumed. The finding of an efficient transport system for L-5-CH_3-H_4PteGlu is of special interest, since this compound has no growth-promoting activity for *P. cerevisiae*.

In a previous study on the uptake of ^{3}H-thymidine by *Pediococcus cerevisiae* cells, we showed that the incorporation of this compound was inhibited by the addition of folinate (5-HCO-H_4-PteGlu) to the system. Thus, the uptake of folinate was indirectly evidenced from the inhibition of incorporation of ^{3}H-thymidine. Inhibition of thymidine uptake, caused by folinate, was shown to be due to de novo synthesis of thymidylate in the presence of the vitamin (18).

Wood and Hitchings (22) found that the uptake and degradation of folinate and pteroylglutamate (PteGlu) by *P. cerevisiae* were glucose-dependent and that PteGlu was very poorly incorporated. Although these investigators assumed that the uptake of folinate by *P. cerevisiae* was due to active transport, no direct support for this hypothesis was given.

In this report, a detailed study on the mechanism of transport of folinate and 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) in *P. cerevisiae* is presented.

MATERIALS AND METHODS

Chemicals. DL-Folinate and DL-tetrahydrofolate were obtained from Sigma Chemical Co., St. Louis, Mo. The ammonium salt of L-folinic acid-6-T was a gift from M. Friedkin and L. Pastore of Tufts University, Boston, Mass., and was purified by descending chromatography on Whatman no. 1 paper, with 0.1 M $(NH_4)_2CO_3$ used as solvent. The ultraviolet fluorescent spot, with R_F of about 1.0, was eluted with 0.01 M phosphate buffer (*p*H 7.0), and the vitamin concentration was determined by bioassay with *P. cerevisiae* (9).

The chemical 5(methyl-^{14}C)-5,6,7,8-tetrahydrofolic acid (specific activity 50.8 mCi/mmole) was purchased from The Radiochemical Centre, Amersham, England. Unlabeled 5-CH_3-H_4PteGlu was synthesized from 5-HCO-H_4PteGlu by the method of Chanarin and Perry (2). The spectra at *p*H 1.0 (0.1 M HCl) and at *p*H 7.0 (0.1 M phosphate buffer) were determined in a Perkin-Elmer spectrophotometer, model 137UV. Quantitation of the material was based on a molar coefficient of 31.7×10^3 per cm at 290 nm in phosphate buffer, *p*H 7.0 (10).

Aminopterin was obtained from Mann Research

Laboratories, Inc., New York, N.Y., and amethopterin from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y. Fluorodeoxyuridine was a product of Hoffman-LaRoche, Basel, Switzerland.

Uptake of ^{3}H-folinate and related compounds by P. cerevisiae. Growth conditions and the procedure for obtaining exponential-phase cells were as previously described (18). Washed cells (about 1.0×10^9, equivalent to 1 mg, dry weight) were incubated in potassium phosphate buffer (*p*H 6.0), 20 μmoles; glucose, 1%; and the labeled compound, as indicated, in a total volume of 1 ml. The reaction mixture was incubated in a water bath at 37 C (or at 0 C) with gentle shaking. The uptake was terminated (unless otherwise stated) by addition of 4 ml of ice-cold saline, chilling in an ice bath, and rapid removal of cells in a Sorvall centrifuge at 14,000 × *g* at 0 C. The cells were subsequently washed three times with 4 ml of ice-cold saline to remove the extracellular labeled compound.

Extraction and identification of the incorporated material. The washed cells were suspended either in 2 ml of water or 5% (w/v) trichloroacetic acid; they were then heated for 20 min in a boiling-water bath. Cell debris was removed by centrifugation, and the radioactivity of the supernatant fluid was measured in a Tri-Carb liquid scintillation spectrometer, model 3375 (Packard Instrument Co., Inc., Downers Grove, Ill.), as previously described (18). The biological activity of the accumulated folinate was determined in a hot-water extract by bioassay with *P. cerevisiae* (9). The 5-$^{14}CH_3$-H_4PteGlu was released from cells by boiling for 20 min in 1 ml of 0.01 M phosphate buffer (*p*H 7.0) containing 1 mg of ascorbate. The biological activity was assayed with *Lactobacillus casei*, which responds equally to L-5-CH_3-H_4PteGlu and to PteGlu (11, 14, 16). The compound was identified by descending chromatography on Whatman no. 1 paper, with 0.1 M phosphate buffer (*p*H 7.0), supplemented with 0.5% mercaptoethanol, as the solvent (10). The radioactivity of the chromatogram was determined in a Packard radiochromatogram, model 7200.

RESULTS

Kinetics of ^{3}H-folinate uptake. Folinate uptake in *P. cerevisiae* cells was found to be very rapid; in 2 min the cells (1.5×10^9) accumulated about half the amount of the substrate present in the medium at a concentration of 10^{-7} M (*see* Fig. 1). Assuming the intracellular water volume to be 4 μliters per mg of dry cells (13), the concentration of folinate in the cellular pool was about 80 times that present in the medium.

Since folinate participates in thymidylate synthesis (1, 19, 21), it was essential to establish whether all the accumulated radioactivity could be ascribed to folinate, or whether some of it was also incorporated into the nucleic acid fraction by transferring the hydrogen on carbon 6 of folinate to the methyl group of thymidylate. Therefore, the radioactivity accumulated in the

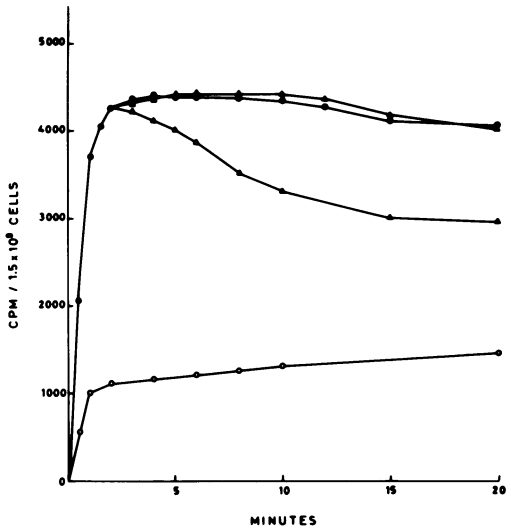

FIG. 1. *Time course of ^{3}H-folinate uptake by Pediococcus cerevisiae. The cells (1.5×10^9, equivalent to 1.5 mg, dry weight) were incubated with ^{3}H-folinate [0.1 nmole (9,500 counts/min)], phosphate buffer (pH 6.0), and glucose. Folinate uptake was simultaneously measured in cells that were preincubated with 10 μg of fluorodeoxyuridine (in buffer and glucose) for 10 min at 37 C. The uptake was terminated by dilution with ice-cold saline, rapid filtration through membrane filters, and washing of the cells with ice-cold saline. The radioactivity was released from the cells be extracting in hot water or in hot trichloroacetic acid. Symbols: ○, uptake at 0 C (hot-water extract); ●, uptake at 37 C (hot trichloroacetic acid extract); ▲, uptake at 37 C (hot-water extract); △, uptake at 37 C by cells preincubated with fluorodeoxyuridine (hot-water extract).*

pool (hot-water extract) was measured, and folinate was identified by bioassay. The total radioactivity (intracellular folinate plus labeled nucleic acids) was extracted with hot trichloroacetic acid. As shown in Fig. 1, the amount of folinate in the cellular pool (hot-water extract) was the highest at about 2 min of incubation and then started to decline, whereas the radioactivity of the hot trichloroacetic acid extract remained almost constant. A constant ratio of radioactivities in both fractions was achieved after 15 min of incubation.

To block the participation of folinate in the synthesis of thymidylic acid, cells were preincubated for 10 min at 37 C with 5-fluorodeoxyuridine, a potent inhibitor of thymidylate synthetase (4, 20). Indeed, 5-fluorodeoxyuridine prevented transfer of the label from folinate to thymidylate, as evidenced from the similar radioactivities of the hot trichloroacetic acid and hot-water extracts of cells preincubated with the

analogue (Fig. 1). For up to 5 min of incubation, folinate concentration in the hot water extract (assayed with *P. cerevisiae*) fully correlated with that calculated from the radioactivity values. However, on longer incubation, folinate was metabolized and derivatives were formed, which presumably decomposed during heating in the absence of a reducing agent. The biological activity decreased with time of incubation much faster than the radioactivity values (Table 1).

Effect of temperature on folinate uptake. Figure 1 shows that the uptake of folinate was temperature-dependent. The amount of folinate accumulated at 37 C within 2 min was about four times higher than at 0 C. At 0 C, no transfer of the label to the nucleic acid fraction was found.

Specificity of ^{3}H-folinate uptake in P. cerevisiae. PteGlu and its analogues aminopterin and amethopterin, at concentrations 100 to 500 times higher than folinate, did not affect the incorporation of folinate (Table 2). Similar results were obtained when *P. cerevisiae* cells were preincubated with these compounds for 10 min at 37 C prior to the addition of ^{3}H-folinate. In contrast to the ineffectiveness of the unreduced derivative (PteGlu), tetrahydrofolate (H_4PteGlu) and 5-CH_3-H_4PteGlu competed with the folinate uptake. Data on the competition between the labeled and unlabeled folinate indicate that only the L-isomer accumulated.

The finding that 5-CH_3-H_4PteGlu interferes with folinate uptake seems of special interest, since this compound is almost inactive as a growth factor for *P. cerevisiae* (11, 14, 16). According to our data, L-5-CH_3-H_4PteGlu had only 3 to 4% of the growth-promoting activity of L-folinate. The mechanism of 5-$^{14}CH_3$-H_4PteGlu uptake was further investigated.

Table 3 shows that unlabeled 5-CH_3-H_4PteGlu competes linearly with the labeled compound. On the other hand, PteGlu or aminopterin do not affect the uptake of 5-$^{14}CH_3$-H_4PteGlu.

TABLE 1. *Effect of incubation time on disappearance of intracellular folinate*[a]

Time	Counts per min accumulated	Folinate accumulated
min		*pmoles*
2	4,250	44
5	4,000	42
8	3,500	25
10	3,300	18
15	3,000	7
20	2,950	3

[a] Additional data on folinate uptake to that shown in Fig. 1. The accumulated folinate was assayed with *P. cerevisiae*.

TABLE 2. *Effect of structurally related compounds on ^{3}H-folinate uptake by Pediococcus cerevisiae*[a]

Additions	^{3}H-folinate accumulated	Inhibition
nmoles	*pmoles*	%
None	40	0
PteGlu		
10	40	0
20	40	0
50	40	0
Aminopterin		
10	40	0
20	38	5
Amethopterin		
10	40	0
20	40	0
DL-H_4PteGlu		
1	15	63
2	10	75
DL-5-CH_3-H_4PteGlu		
1	10	75
None[b]	80	0
DL-CHO-H_4PteGlu[b]		
0.4	40	50

[a] Cells (1.5×10^9) were incubated for 2 min with 0.1 nmole of L-^{3}H-folinate in presence of the added compound, as indicated. The radioactivity was extracted from the cells with hot water.

[b] Cells incubated with 0.2 nmole of L-^{3}H-folinate.

Time course of 5-$^{14}CH_3$-H_4PteGlu uptake and effect of temperature. Uptake of 5-CH_3-H_4PteGlu was linear for up to 2 min of incubation at 37 C (Fig. 2). The substrate accumulated against the concentration gradient and, at the steady state, the concentration in the pool was about 140-fold higher than in the medium. The accumulated radioactivity was identified as 5-CH_3-H_4PteGlu, by radiochromatography, and a peak with R_F 0.8, characteristic for this derivative (10), was obtained. The biological activity of the hot-water extract, measured with *L. casei*, indicated that only the L-isomer accumulated.

In contrast to folinate, the uptake of 5-CH_3-H_4-PteGlu at 0 C was negligible.

Effect of pH on ^{3}H-folinate and 5-$^{14}CH_3$-H_4-PteGlu uptake. The uptake of ^{3}H-folinate by *P. cerevisiae* was shown to be *p*H-dependent, with a peak at *p*H 6.0 (Fig. 3). Accumulation of 5-CH_3-H_4PteGlu was also maximal at *p*H 6.0, but de-

TABLE 3. *Effect of structurally related compounds on 5-$^{14}CH_3$-H_4PteGlu uptake by Pediococcus cerevisiae*[a]

Additions	5-$^{14}CH_3$-H_4PteGlu accumulated	Inhibition
nmoles	*pmoles*	%
None	137	0
DL-5-CH_3-H_4PteGlu		
0.98	70	49
0.65	95	30
0.33	114	17
0.16	125	9
PteGlu		
50	137	0
20	137	0
Aminopterin		
50	137	0

[a] Cells (1.5×10^9) were incubated for 5 min with DL-5-$^{14}CH_3$-H_4PteGlu (0.98 nmole) in presence of the compound added as indicated. Other conditions as in Table 2.

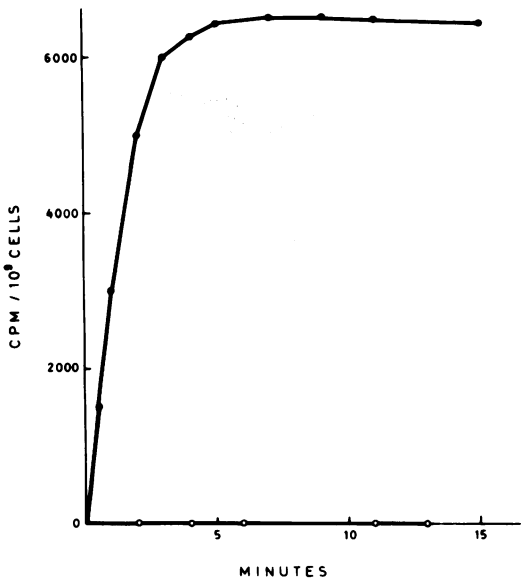

FIG. 2. *Time course of 5-$^{14}CH_3$-H_4PteGlu uptake by Pediococcus cerevisiae. The cells were incubated with 5-$^{14}CH_3$-H_4PteGlu (0.122 nmole), glucose, and buffer. The radioactivity was released by extraction with hot water. Symbols: ●, uptake at 37 C; ○, uptake at 0 C.*

clined much more rapidly at acidic and alkaline *p*H ranges.

Effect of glucose and metabolic inhibitors on ^{3}H-folinate and 5-$^{14}CH_3$-H_4PteGlu transport. The uptake of folinate and 5-CH_3-H_4PteGlu was energy (glucose)-dependent (Table 4). Cells incubated with folinate in the absence of glucose accumulated up to 25% of the radioactivity found in cells incubated in the presence of glucose. Metabolic inhibitors (iodoacetate, potassium fluoride, sodium azide) reduced the uptake considerably. The uptake of 5-CH_3-H_4PteGlu was much more energy-dependent and more sensitive to the above metabolic inhibitors.

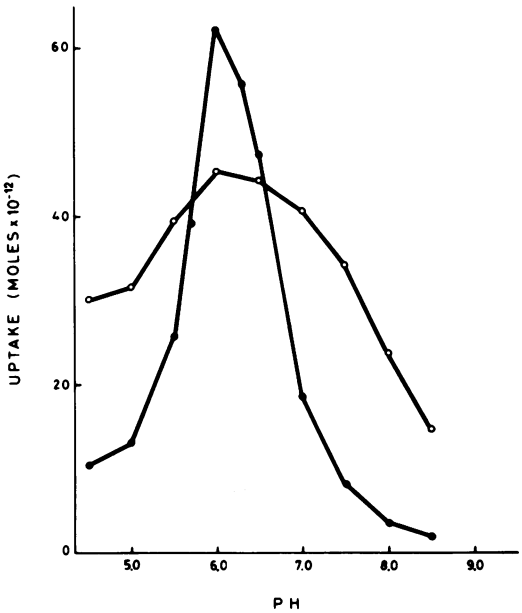

FIG. 3. *Effect of pH on the uptake of ^{3}H-folinate and 5-$^{14}CH_3$-H_4PteGlu. The reaction mixture consisted of P. cerevisiae cells, phosphate buffer (20 μmole), glucose (1%), and either folinate or CH_3-H_4PteGlu. Incubation was carried out at 37 C. Symbols: ○, Uptake of L-^{3}H-folinate (0.1 nmole) by 1.5 mg of cells (dry weight) incubated for 2 min; ●, uptake of 5-$^{14}CH_3H_4$PteGlu (0.122 nmole) by 1 mg of cells incubated for 3 min. The radioactivity was measured in hot-water extracts.*

Effect of concentration on the rate of uptake of ^{3}H-folinate and 5-$^{14}CH_3$-H_4PteGlu. Figure 4 shows rates of folinate uptake by cells incubated at 37 C in the presence and absence of iodoacetate, and by cells incubated at 0 C. The initial uptake velocities at 37 C (after subtraction of radioactivity incorporated at 0 C) appear to be a hyperbolic function of the external folinate concentration. The increase in rate of the temperature-dependent uptake with increase in concentration was almost linear up to 1.2×10^{-7} M. The uptake at 0 C, as well as that by cells poisoned with iodoacetate, increased almost linearly with increase in concentration. When the reciprocal of the initial uptake velocity at 37 C was plotted as a

TABLE 4. *Effect of glucose and metabolic inhibitors on the uptake of ^{3}H-folinate and 5-$^{14}CH_3$-H_4PteGlu*[a]

Glucose, 1%	Iodoacetate, 10^{-2} M	Potassium fluoride, 10^{-1} M	Sodium azide, 10^{-1} M	Compounds accumulated (pmole): ^{3}H-folinate[b]	Compounds accumulated (pmole): 5-$^{14}CH_3$-H_4PteGlu[c]
+	−	−	−	43	63
−	−	−	−	11	1
+	+	−	−	10	1
+	−	+	−	12	2
+	−	−	+	19	10

[a] *P. cerevisiae* cells were preincubated in phosphate buffer (*p*H 6.0) with glucose and the inhibitor for 5 min at 37 C, prior to addition of the labeled compound.

[b] Uptake of L-^{3}H-folinate (0.1 nmole) by 1.5×10^9 cells incubated for 2 min.

[c] Uptake of 5-$^{14}CH_3$-H_4PteGlu (0.122 nmole, calculated as the L-isomer) by 1.0×10^9 cells incubated for 3 min.

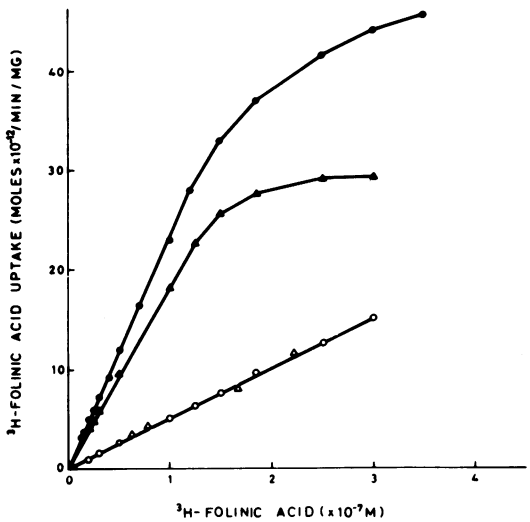

FIG. 4. *Effect of concentration on the rate of ^{3}H-folinate uptake. Cells (1 mg, dry weight) were incubated for 1 min at 37 C or at 0 C in buffer, glucose, and varying concentrations of ^{3}H-folinate. Symbols: ●, total uptake at 37 C; ○, uptake by cells preincubated for 5 min with iodoacetate (10^{-2} M); ▲, uptake at 37 C less the uptake by iodoacetate-treated cells; △, uptake at 0 C.*

function of the reciprocal of the external folinate concentration, a straight line was obtained (Fig. 5). The apparent K_m for folinate uptake, calculated from the Lineweaver-Burk plot (5), was 4.0×10^{-7} M, and the V_{max} was about 1.0×10^{-10} mole per min per mg of dry cells.

Essentially similar results were obtained with 5-CH_3-H_4PteGlu. At 37 C, the increase in the rate of uptake with increase of concentration was almost linear up to 1.0×10^{-7} M (Fig. 6). The uptake at 0 C was negligible. The apparent K_m for 5-CH_3-H_4PteGlu uptake, calculated from Fig. 7, was 4.0×10^{-7} M, and the V_{max} was 1.6×10^{-10} mole per min per mg of dry cells. When a constant concentration of folinate was added to increasing concentrations of 5-CH_3-H_4-PteGlu, the uptake of the latter was found to be competitively inhibited (Fig. 7). The K_i for folinate, as calculated from Fig. 7 (6), was 0.4×10^{-7} M.

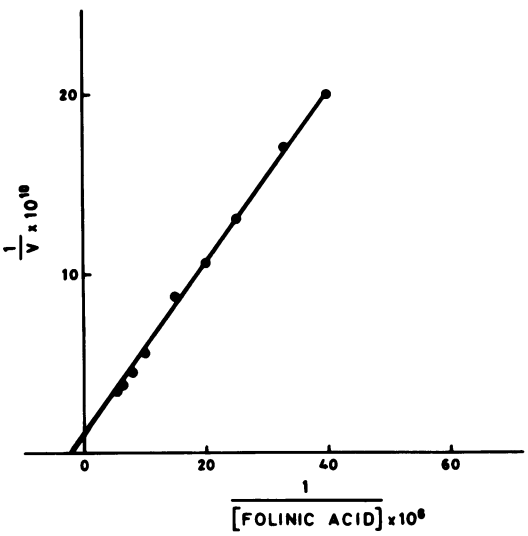

FIG. 5. *Lineweaver-Burk plot of ^{3}H-folinate uptake at 37 C. The data are derived from Fig. 4 (uptake at 37 C less the uptake by iodoacetate-treated cells). V = rate of uptake of ^{3}H-folinate in moles per minute per milligram of cells (dry weight).*

DISCUSSION

The present study showed that the uptake of ^{3}H-folinate and 5-$^{14}CH_3$-H_4PteGlu by *P. cerevisiae* depended on an energy source, temperature, and *p*H (optimum for both compounds at *p*H 6.0), and was inhibited by metabolic inhibitors. The uptake of both compounds at 37 C followed saturation kinetics, and its rate depended on the external substrate concentration, obeying Michaelis-Menten kinetics. To prevent the participation of folinate in thymidylic acid synthesis, the system was inhibited by preincubation with 5-fluorodeoxyuridine. The possibility of conversion of folinate to 10-formyltetrahydrofolate (12), or to

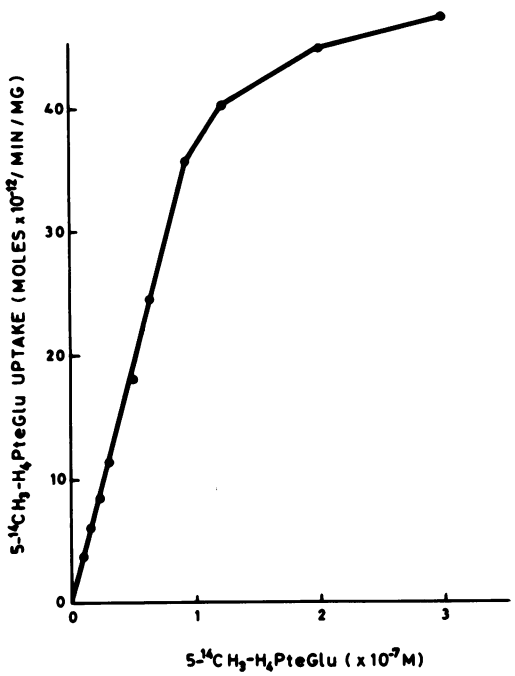

FIG. 6. *Effect of concentration on the rate of* L-5-$^{14}CH_3H_4$*PteGlu uptake. Cells (1 mg, dry weight) were incubated for 1 min at 37 C. Other conditions were as in Fig. 4.*

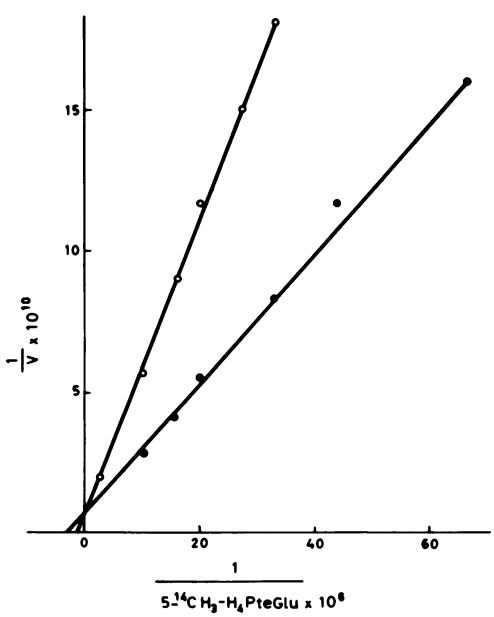

FIG. 7. *Lineweaver-Burk plot of the inhibition of 5-*CH_3-H_4*PteGlu uptake by folinate. V = rate of uptake of* L-5-$^{14}CH_3$-H_4*PteGlu in moles per minute per milligram (dry weight) of cells.* L-*Folinate (inhibitor) at a constant concentration of 0.05* $\times$ 10^{-6} M. *Symbols:* ●, *without folinate;* ○, *in the presence of folinate.*

5,10-methylene-tetrahydrofolate (8), was also eliminated, since good correlation was found between the accumulated radioactivity and the biological activity. Under the same conditions, 5-CH_3-H_4PteGlu was not metabolized.

At equilibrium, the intracellular concentration of folinate at 37 C was about 80-fold of the external concentration, and at 0 C it was 20-fold; the accumulation of 5-$^{14}CH_3$-H_4PteGlu at 37 C was almost 140-fold, and no uptake was found at 0 C.

The initial rate of uptake of 5-CH_3-H_4PteGlu was similar to that of folinate. The very low (and identical) K_m values for both compounds (4.0×10^{-7} M) indicate a high affinity of the transport systems for these substances. The high capacity of intracellular concentration of folinate in *P. cerevisiae* explains the growth-promoting activity of relatively low concentrations of the vitamin in the medium [folinate at 100 pg/ml supported half-maximal growth (9)].

Folinate competitively inhibited the uptake of 5-CH_3-H_4PteGlu with a K_i of 0.4×10^{-7} M; it is therefore suggested that both compounds compete for the same carrier. However, since folinate, unlike 5-CH_3-H_4PteGlu, is also taken up at 0 C, it is assumed that the former possesses an additional transport process which is not affected by metabolic inhibitors (Table 4, Fig. 4). As folinate accumulates against a concentration gradient also at 0 C, it is further assumed that it binds to some component inside the cell, or on its membrane, or both.

The specificity of folinate uptake is remarkable. Only the L-isomer was taken up. Tetrahydrofolate interfered efficiently with the uptake of folinate, whereas PteGlu, aminopterin, and amethopterin had no effect. The inability of unreduced folate derivatives to interfere with folinate uptake is contrary to the data of other investigators who studied different biological systems (15, 17, 22; M. C. Hakala, Fed. Proc., p. 183, 1963). Wood and Hitchings (22) demonstrated that, in *Streptococcus faecalis*, folinate interfered with the uptake of aminopterin. Folinate was shown to inhibit noncompetitively the uptake of amethopterin in sarcoma 180 cells (Hakala, Fed. Proc., p. 183, 1963). Amethopterin transport in Ehrlich ascites carcinoma and L 1210 cells was also inhibited by folinate (15). Goldman et al. (7) found competitive inhibition between these compounds in L 1210 leukemia cells. Recently, Lichtenstein et al. (17) suggested that, in L 1210 leukemia cell, folate, folinate, and amethopterin share, at least partly, the same carrier mechanism.

Considering the results, it is assumed that the uptake of folinate and of 5-CH_3-H_4PteGlu fulfills the criteria of active transport (3, 13). The finding that 5-CH_3-H_4PteGlu was accumulated by *P. cerevisiae* as efficiently as folinate, or even

more, is of special interest, since this compound is almost inactive as a growth factor for this microorganism (11, 14, 16). The 5-CH_3-H_4PteGlu being unmetabolized, presumably due to a lack or repression of transmethylation or other mechanisms, provides a choice substrate for further investigation of the mechanism of transport of folic acid derivatives in *P. cerevisiae*.

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