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Carrier-Mediated Transport of Folate in a Mutant of *Pediococcus cerevisiae*

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A mutant strain of *Pediococcus cerevisiae* (*P. cerevisiae*/PteGlu) was isolated which grows on low-folate (PteGlu) concentrations (200 pg/ml). The growth response of the parent and mutant strains to folinate (5-CHO-H_4PteGlu) was the same. The transport of ^{14}C-PteGlu by *P. cerevisiae*/PteGlu was temperature-dependent (Q_{10} between 27 C and 37 C was about 2), energy-dependent, and pH-dependent and was inhibited by iodoacetate, 2,4-dinitrophenol, potassium fluoride, and sodium azide. The uptake obeyed saturation kinetics with an apparent K_m of 6.6×10^{-6} M and v_{max} of 4.0×10^{-10} mol per min per mg (dry weight). At the steady state the intracellular concentration of PteGlu was 120-fold higher from that of the medium. Reduced folates like 5-CHO-H_4PteGlu and methyl-tetrahydrofolate (5-CH_3-H_4PteGlu) as well as 2,4-diamino-analogues (amethopterin and aminopterin) were shown to compete for the PteGlue-carrier.

We previously reported that *Pediococcus cerevisiae*, a folinic acid (5-CHO-H_4PteGlu) auxotroph, possesses an active transport system for this compound and that neither folate (PteGlu) nor its analogues (amethopterin or aminopterin) compete with this system (13).

Nichol (15, 16), searching for bacterial mutants sensitive to aminopterin, isolated a strain of *P. cerevisiae* which could grow on relatively low concentrations of PteGlu and accumulated the labeled compound. Zakrzewski reported later that the abovementioned mutant, when grown in a PteGlu-containing medium, accumulated the vitamin as its tetrahydroderivatives (S. F. Zakrzewski, Fed. Proc., p. 943, 1970). In a further study, Zakrzewski et al. showed that the uptake of PteGlu by cells grown without folate in a medium supplemented with thymidine and purine was inhibited by amethopterin (S. F. Zakrzewski and B. Grzelakowska-Sztabert, Fed. Proc., p. 1142, 1971). The same authors assumed, therefore, that uptake of PteGlu by the mutant strain was markedly influenced by metabolic alterations.

Recently, a mutant strain of *P. cerevisiae* was obtained in our laboratory which is about 10-fold more responsive to PteGlu than the one isolated by Nichol (15, 16). It was therefore of interest to investigate the properties of the folate transport system in the mutant strain in comparison with those of the parent (13).

The present study provides data on the transport mechanism of folates in the mutant strain of *P. cerevisiae*.

MATERIALS AND METHODS

Chemicals. PteGlu and DL-5-CHO-H_4PteGlu were obtained from Sigma Chemical Co., St. Louis, Mo. Folic acid-2-^{14}C (potassium salt), specific activity 55.3 mCi/mmol, and tetrahydrofolic acid-*5-methyl-*^{14}C, specific activity 61 mCi/mmol, were purchased from the Radiochemical Centre, Amersham, England. 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. 5-CH_3-H_4PteGlu was synthesized from 5-CHO-H_4PteGlu by the method of Chanarin and Perry (2).

PteGlu and the analogues were purified by ascending chromatography on Whatman no. 1 paper with 0.1 M phosphate buffer (pH 7.0) saturated with isoamyl alcohol as solvent and folinate, as described previously (13). Quantitation of aminopterin was based on a molar extinction coefficient of 26.4×10^3/cm at 284 nm in 0.1 M NaOH and, of amethopterin, 22.0×10^3/cm at 302 nm in 0.1 M NaOH (17). The concentrations of PteGlu and 5-CHO-H_4PteGlu were determined by bioassay with the *P. cerevisiae* mutant (see Results).

Isolation of a PteGlu auxotroph from the P. cerevisiae parent strain. The parent strain of *P. cerevisiae* was grown for 24 h in a medium supplemented with folinate (8) and then transferred to the same medium containing PteGlu (2 μg/ml) instead of folinate. The cells were successively transferred every

24 h (incubation at 37 C) to liquid media with decreasing concentrations of PteGlu. When growth was obtained in a medium with low PteGlu (5 ng/ml), the cells were washed and transferred to petri dishes containing solid medium with a gradient concentration of PteGlu. Colonies which grew on the lowest concentrations of the vitamin were picked out and screened for the sensitivity of their response to PteGlu. A strain was obtained which grew on PteGlu as well as on 5-CHO-H_4PteGlu (20–200 pg/ml) and was labeled as *P. cerevisiae*/PteGlu. The growth was measured in a Klett-Summerson photoelectric colorimeter, with a no. 66 filter.

Both the mutant and the parent strains grew poorly on 5-CH_3-H_4PteGlu (2–5%) in comparison with folinate and are methionine-dependent. No differences in morphology and in the colony form between them were noticed.

Uptake of ^{14}C-PteGlu by the mutant strain. The cells were grown in a liquid medium supplemented with PteGlu (150 pg/ml) and prepared for uptake experiments as reported previously (12, 13). Cells (about 1.0×10^9, equivalent to 1 mg, dry weight) were incubated in a water bath at 37 C with gentle shaking in a reaction mixture consisting of potassium citrate buffer (pH 5.7, 20 μmol), glucose (1%), and the labeled compound as indicated. The total volume was 1 ml. The uptake was terminated by rapid filtration through membrane filters and washing three times with 2 ml of ice-cold saline. The filters were dried and counted in vials containing 10 ml of toluene (12) in a Tri-Carb scintillation spectrometer, model 3310 (Packard Instrument Co. Inc., Downers Grove, Ill.).

Extraction and identification of the incorporated material. Cells containing radiofolate were washed, resuspended in 2 ml of water, and heated in a boiling water bath for 20 min. The extracted compound was identified by ascending chromatography on Whatman no. 1 paper with 0.1 M phosphate buffer (pH 7.0) as solvent, and the radioactivity peak was quantitated in a Packard radiochromatogram scanner (model 7201) supplemented with an integrator.

RESULTS

Growth of the P. cerevisiae mutant on PteGlu and 5-CHO-H_4PteGlu. The mutant strain shows a considerable lag when grown on PteGlu in comparison with its growth on folinate. After 24 h of incubation, the growth started at PteGlu concentrations above 50 pg/ml and at 200 pg/ml was only about 25% of that in the presence of 100 pg/ml 5-CHO-H_4PteGlu (considering that only the L-isomer was utilized) (Fig. 1). After 48 h, the growth on PteGlu increased to 50% of that on CHO-H_4PteGlu. The growth response of the parent and mutant strains to 5-CHO-H_4PteGlu was the same, and no growth of the parent strain in the PteGlu concentrations used was obtained.

Time course of ^{14}C-PteGlu uptake and effects of temperature and energy source. Uptake of PteGlu was linear for up to 4 min of incubation at 37 C (Fig. 2), and the vitamin accumulated in the cells against the concentration gradient. On the assumption that the intracellular water volume was 4 μliters/mg of dry cells (9), the concentration of folate in the cellular pool at the steady state of uptake was about 120-fold higher than in the medium. For up to 20 min of incubation the accumulated PteGlu was found to be unmetabolized and about 95% of the radioactivity was identified as PteGlu (see Materials and Methods). Preincubation of the cells with a low concentration of aminopterin (0.05 nmol), to prevent metabolic transformation, did not affect the results (Table 1). Fig. 2 also shows that the uptake of PteGlu is energy (glucose)-dependent and temperature-dependent with a Q_{10} of approximately 2, between 27 and 37 C, and no uptake at 0 C. No radioactivity was found in cells incubated without glucose up to 10 min; on longer incubation (15–20 min), the amount of radioactivity incorporated was about 2% of that in the glucose-containing system.

Effect of pH on ^{14}C-PteGlu transport. The uptake of PteGlu shows a peak at pH of 5.5 to 5.7 with a sharp decline at more acidic and alkaline pH values (Fig. 3). The uptake in citrate buffer was considerably higher than in phosphate buffer.

Effect of metabolic inhibitors on ^{14}C-PteGlu uptake. Since the uptake of PteGlu was energy (glucose)-dependent, it was of interest to test the effect of metabolic inhibitors. Table 2 shows that PteGlu transport is very sensitive to iodoacetate and to 2,4-dinitrophenol. Potassium fluoride and sodium azide were effective only at 100-fold higher concentrations.

Effect of ^{14}C-PteGlu concentration on the rate of uptake. The rate of PteGlu uptake increased with increasing of the external concentration, showing saturation kinetics. The uptake was almost linear up to 1.0×10^{-6} M, and saturation was nearly complete at an external concentration of 5.0×10^{-6} M (Fig. 4). The apparent K_m for ^{14}C-PteGlu uptake, calculated from the Lineweaver-Burk plot (4), was 6.6×10^{-6} M, and the v_{max} was about 4.0×10^{-10} mol per min per mg of dry cells (Fig. 5).

Specificity of PteGlu transport. The above given data clearly indicate that the uptake of PteGlu by the *P. cerevisiae* mutant involves an active transport mechanism. We previously showed that the transport system for 5-CHO-H_4PteGlu or 5-CH_3-H_4PteGlu of the parent strain of *P. cerevisiae* was not interfered by PteGlu or by folate analogues (13). It

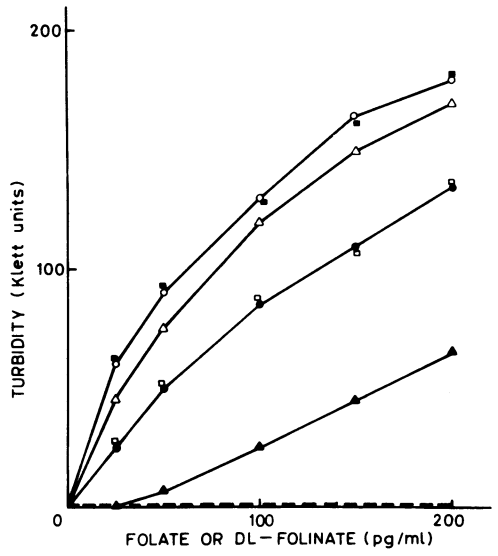

FIG. 1. *Growth response of P. cerevisiae parent and mutant strains to PteGlu and 5-CHO-H_4PteGlu. The cells were incubated at 37 C and the growth was determined turbidimetrically. Symbols: ●, growth of the mutant strain on 5-CHO-H_4PteGlu for 24 h; ○, the same grown for 48 h; ▲, growth of the mutant on PteGlu for 24 h; △, the same grown for 48 h; □, growth of the parent strain on CHO-H_4PteGlu for 24 h; ■, for 48 h; – – –, growth of the parent strain on PteGlu for 48 h.*

seemed of interest, therefore, to determine whether the ability of the mutant strain to accumulate PteGlu is due to a modification in the specificity of the folinate transport carrier or whether a new PteGlu-specific carrier appeared. Table 3 shows that the reduced folates (5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu) exhibit higher affinity for the uptake system than PteGlu itself. The analogues aminopterin and amethopterin also compete with PteGlu uptake, showing almost the same affinity as the reduced derivatives.

To rule out a possibility that the high affinity of the folate analogues for the uptake system is due to a competition with the vitamin for the dihydrofolate reductase (18), the effect of the analogues on 5-CH_3-H_4PteGlu uptake (which is neither a substrate nor a product of the enzyme) was determined. Table 4 shows that aminopterin and amethopterin compete effectively with CH_3-H_4PteGlu uptake, which provides further support for the data given in Table 3.

DISCUSSION

A mutant strain of *P. cerevisiae* was isolated which could grow on about 10^5-fold lower folate concentration than required by the parent strain (1, 7). The growth response of the mutant to PteGlu (200 pg/ml) was only about 25% in comparison with L-5-CHO-H_4PteGlu, after short incubation (24 h), and increased thereafter (48 h). Two factors may be responsible for the lower growth rate on PteGlu: (i) its

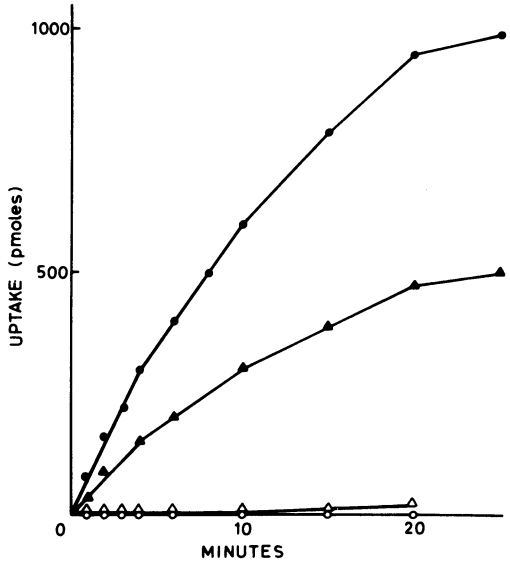

FIG. 2. *Time course of ^{14}C-PteGlu uptake by Pediococcus cerevisiae/PteGlu. 1.0 × 10^9 cells (equivalent to 1.0 mg dry weight) were incubated with ^{14}C-PteGlu (2 nmol) in citrate buffer (pH 5.7) and glucose as indicated. The uptake was terminated by rapid filtration through membrane filters and washing of the cells with ice-cold saline. Symbols: ●, uptake at 37 C; △, uptake at 37 C in the absence of glucose; ▲, uptake at 27 C; ○, uptake at 0 C.*

TABLE 1. *Effect of incubation time on PteGlu accumulation in cells of P. cerevisiae/PteGlu*[a]

Time (min)	^{14}C-PteGlu accumulated (pmol)		
	A[b]	B[c]	C[d]
4	300	295	300
10	580	560	560
20	900	855	860

[a] Experimental conditions were as in Fig. 2.

[b] A, The accumulated PteGlu was calculated from the total radioactivity taken up by the cells.

[c] B, The concentration of PteGlu was calculated after extraction and identification of the labeled compound.

[d] C, Cells were preincubated with aminopterin (0.05 nmol) for 10 min at 37 C prior to addition of the radiofolate. The PteGlu accumulated was calculated as in footnote c.

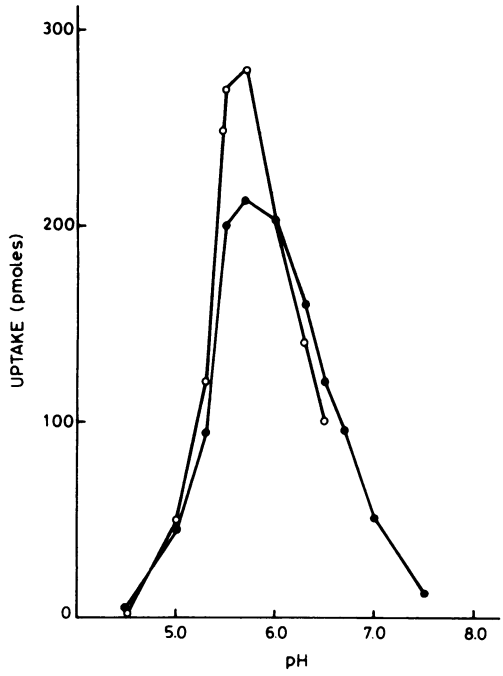

FIG. 3. *Effect of pH on the ^{14}C-PteGlu uptake. P. cerevisiae/PteGlu cells were incubated for 4 min in either phosphate or citrate buffers (20 μmol) at pH values as indicated, with glucose 1% and ^{14}C-PteGlu (2 nmol). Symbols: O, uptake in citrate buffer; ●, uptake in phosphate buffer.*

TABLE 2. *Effect of metabolic inhibitors on the uptake of ^{14}C-PteGlu*[a]

Inhibitor added (M)	^{14}C-PteGlu accumulated (pmol)	Inhibition (%)
None	280	
Sodium azide, 1.0×10^{-1}	100	64.0
Potassium fluoride, 1.0×10^{-1}	15	94.7
Iodoacetate, 1.0×10^{-3}	22	92.0
1,4-dinitrophenol, 5.0×10^{-3}	40	85.7

[a] The cells (1.0×10^{9}) were preincubated for 5 min at 37 C in citrate buffer (pH 5.7) with glucose and the inhibitor, as indicated. After addition of ^{14}C-PteGlu (2 nmol), the incubation was continued for 4 min.

uptake is less efficient than that of 5-CHO-H_4PteGlu; (ii) PteGlu has to be reduced first to its coenzymatic derivatives, whereas folinate could be utilized rapidly (12).

The accumulated ^{14}C-PteGlu (120-fold higher concentration than in the medium) was identified as unmetabolized PteGlu, a finding which is different from that of S. F. Zakrzewski and B. Grzelakowska-Sztabert (Fed. Proc., p. 1142, 1971), who concluded that the PteGlu uptake is mainly due to metabolic alterations. These authors also suggested that inhibition of PteGlu uptake by amethopterin was due to inhibition of folate metabolism. In the strain used by us, amethopterin was shown to compete efficiently with the folate carrier. The different results obtained may be due to metabolic differences between the strains.

In contrast to the absence of transport ability

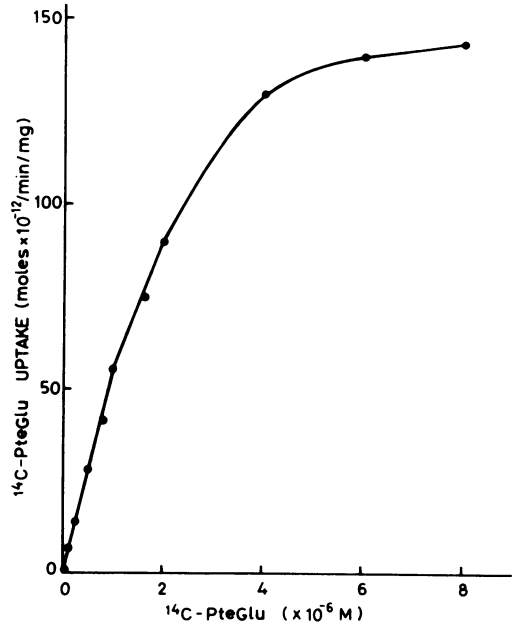

FIG. 4. *Effect of concentration on the rate of ^{14}C-PteGlu uptake. Cells (1 mg dry weight) were incubated for 1 min at 37 C in citrate buffer (pH 7.0), glucose, and varying concentrations of folate.*

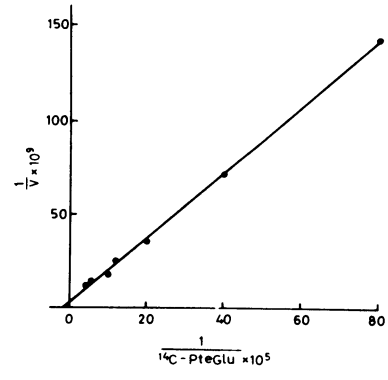

FIG. 5. *Lineweaver-Burk plot of ^{14}C-PteGlu uptake. The data are derived from Fig. 4. V = rate of ^{14}C-PteGlu uptake in moles per min per mg of cells (dry weight).*

TABLE 3. *Effect of structurally related compounds on ^{14}C-PteGlu uptake by P. cerevisiae/PteGlu*[a]

Additions (nmol)	^{14}C-PteGlu accumulated (pmol)	Inhibition (%)
None	141	0
PteGlu		
2.0	75	47
L-5-CHO-H$_4$PteGlu		
2.0	14	90
1.0	49	65
0.5	82	42
L-5-CH$_3$H$_4$PteGlu		
2.0	17	95
1.0	39	72
0.5	70	50
Amethopterin		
2.0	14	90
1.0	21	85
0.5	48	66
Aminopterin		
2.0	21	85
1.0	48	66
0.5	70	50

[a] Cells (1.0×10^9) were incubated for 2 min at 37 C with ^{14}C-PteGlu (2 nmol) in presence of the compound added, as indicated. The reaction was terminated by filtration through membrane filters.

TABLE 4. *Effect of folate analogues on the uptake of 5-^{14}CH$_3$-H$_4$PteGlu by P. cerevisiae/PteGlu*[a]

Additions (nmol)	5-^{14}CH$_3$-H$_4$PteGlu accumulation (pmol)	Inhibition (%)
None	260	0
Amethopterin		
1.0	135	50
0.33	160	39
Aminopterin		
1.0	175	33
0.33	220	16

[a] Cells (1.0×10^9) were incubated for 2 min with 5-^{14}CH$_3$-H$_4$PteGlu (1 nmol) in phosphate buffer (pH 6.0) and glucose (1%) in presence of the added analogue, as indicated. The reaction was terminated by filtration through membrane filters.

for unreduced folates in the parent strain (13), the mutant was shown to possess a very efficient system for PteGlu transport, which is shared by the unreduced analogues folinate and methyl-tetrahydrofolate (Table 3). The reduced derivatives and the analogues seem to have higher affinity for the carrier than PteGlu itself. To rule out the possibility of competition between the analogues and folate for binding to folate-reductase (16), affecting thereby the uptake process, we could show that the analogues compete very efficiently with the transport of 5-CH$_3$-H$_4$PteGlu (Table 4), which is neither a substrate nor a product of folate reductase.

The transport system for PteGlu derivatives in the *P. cerevisiae* mutant seems to be similar in its specificity to other biological systems (5, 6, 10, 11, 19). Wood and Hitchings found that, in *Streptococcus faecalis*, PteGlu and folinate interfered very efficiently with the uptake of aminopterin (19). PteGlu, folinate, and amethopterin were also shown to share the same carrier system in L1210 leukemia cells (6, 11). Goldman, in his studies on trans-stimulation of amethopterin influx in cells preloaded with 5-CH$_3$-H$_4$PteGlu, 5-CHO-H$_4$PteGlu or PteGlu, provided further data on a common transport system for folates in leukemia L1210 and in Ehrlich ascites tumor cells (5). Nahas et al. (14) reported recently that L1210 leukemia cells appear to use the same carrier system for the transport of amethopterin and 5-CH$_3$-H$_4$PteGlu, which is less efficient for PteGlu. In *Lactobacillus casei* a transport system was described which is apparently shared by PteGlu and amethopterin and not by folinate and methyl-tetrahydrofolate (3).

We conclude that the mutant strain of *P. cerevisiae* possesses different transport properties for unreduced PteGlu derivatives from those of the parent strain. A carrier system is present which is shared by the reduced and unreduced folates. Whether the new properties are due to conformational changes in the existing carrier, or to the presence of an additional carrier system cannot yet be answered.

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