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Effect of Folinate on Thymidine Uptake by *Pediococcus cerevisiae*[1]

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Uptake of ^{3}H-thymidine by resting cells of *Pediococcus cerevisiae* was found to be energy- and temperature-dependent. The *p*H optimum was between 6.5 and 8.0, and after 2 min of incubation most of the radioactivity was found in the deoxyribonucleic acid (DNA) fraction. Iodoacetate at a concentration of 10^{-2} M caused a 50% inhibition of uptake. Preincubation of resting cells for 10 min with folinate ($10^{-3}\mu$ mole/ml) diminished the ^{3}H-thymidine uptake by 75%. In growing cells, the folinate-induced inhibition was still more striking. Deoxyuridine augmented the folinate effect, whereas fluorodeoxyuridine and aminopterin or amethopterin abolished it. Preincubation with folinate did not interfere with the uptake of ^{3}H-amethopterin, and thus the inhibitor did not compete for uptake sites within the cell. The role of these inhibitors in reversing the folinate effect is discussed. Cells preincubated with folinate showed an increased incorporation of ^{14}C-uracil into DNA, presumably after prior conversion to thymidylate. We concluded that the folinate effect was due to stimulation of de novo thymidylate synthesis with concomitant inhibition of the uptake of external thymidine.

Pediococcus cerevisiae ATCC 8081 (*Leuconostoc citrovorum*) responds quantitatively to 5 - formyltetrahydropteroylglutamate (5 - HCO - H_4PteGlu), also known as leucovorin or folinic acid, and to other derivatives of tetrahydropteroylglutamate (H_4PteGlu), but not to physiological amounts of dihydrofolate or to the unreduced pteroylglutamate (4, 12, 21).

Thymidine was found to spare, but not to replace, the requirement for the natural growth factor (citrovorum factor; 5) or synthetic folinate (8). On the other hand, it was also reported that thymidine was able both to spare and partly to replace folinate (1, 3, 7, 11, 20, 21, 25).

In the synthesis of thymidine, 5,10-methylene-tetrahydropteroylglutamate (5,10- CH_2H_4-PteGlu) participates both as a coenzyme transferring the 1-carbon moiety and as a source of the two hydrogens that reduce the methylene group to the methyl form (2, 18, 24).

In an attempt to elucidate the mechanism by which thymidine is able to spare folinate in the growth of *P. cerevisiae*, we investigated the incorporation of labeled thymidine into cells incubated in the presence and absence of folinate.

MATERIALS AND METHODS

Chemicals. Folinate was obtained from Sigma Chemical Co., St. Louis, Mo. Thymidine-6-T(n), specific activity 3,000 mc/mmole, and amethopterin-3′,5′-T, specific activity 9.17 c/mmole, were products of Radiochemical Centre, Amersham, England. Uracil-*2-*^{14}*C*, specific activity 32 mc/mmole, was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y., and deoxyuridine was obtained from Schwarz Laboratories, Inc., Mount Vernon, N.Y. Fluorodeoxyuridine was a product of Hoffman-La Roche, Basel, Switzerland. Uracil, thymine, and unlabeled thymidine were products of Nutritional Biochemicals Corp., Cleveland, Ohio. Aminopterin was obtained from Mann Research Laboratories, Inc., New York, N.Y., and amethopterin was obtained from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

Growth of the organism. P. cerevisiae ATCC 8081 was maintained in yeast-tryptone stock medium (9), and stock cultures were transferred monthly. The microorganism from the stock culture was transferred to 5 ml of liquid medium (9) supplemented with folinate (150 $\mu\mu$g/ml—an amount supporting half-maximal growth). The overnight culture was used as inoculum for obtaining logarithmically growing cells (generation time, about 120 min). Growth was estimated by measuring the turbidity in a Klett-Summerson photoelectric colorimeter with a red filter or in a Coleman Junior spectrophotometer at 650 mμ.

The log-phase cells were harvested by centrifugation, washed three times with saline, and diluted to 4.0×10^{10} cells per ml; 0.1 ml of the cell suspension, corresponding to 4.0 mg (dry weight), was used in the reaction mixture.

Reaction mixture. The reaction mixture contained, in a total volume of 2 ml: potassium phosphate buffer

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(*p*H 6.5), 40 μmoles; glucose, 20 μmoles; cells, 4.0 mg (dry weight); and ^{3}H-thymidine, 5×10^{-5} μmoles (0.1 μc). The mixture was incubated with gentle shaking for 2 min in a water bath at 35 C, and the reaction was stopped by immersing the tubes in an ice bath. The cells were removed by centrifugation in a Sorvall centrifuge at 14,000 × *g* at 0 C. For kinetic measurements, samples were rapidly filtered through membrane filters (type HA; diameter, 25 mm; pore size, 0.45 μ; Millipore Corp., Bedford, Mass.), and were washed with ice-cold saline.

Extraction and estimation of cellular nucleic acids. The cold and hot trichloroacetic acid fractions were separated according to Schneider's method (22). The washed cells were resuspended in 2 ml of ice-cold 5% (w/v) trichloroacetic acid and centrifuged; the precipitate was resuspended in the same volume of ice-cold 5% trichloroacetic acid. Both supernatant fluids were combined to yield the cellular thymidine pool (cold trichloroacetic acid fraction). To obtain the nucleic acid fraction, the sediment was extracted at 90 C for 20 min with 5% trichloroacetic acid (hot trichloroacetic acid fraction). Since thymidine is incorporated into deoxyribonucleic acid (DNA), the hot trichloroacetic acid fraction represents the radioactivity of the DNA. Portions (0.2 ml) of the various extracts were added to 10.5 ml of a scintillation fluid consisting of dioxane-toluene (23).

The uptake of uracil-*2-^{14}C* into DNA was measured according to Hanawalt's modification (10) of the Schmidt and Thannhauser procedure. After removal of the cold trichloroacetic acid fraction, the residue was resuspended in 1 M KOH for 2 hr at 35 C. The DNA was precipitated from the extract by the addition of ice-cold 10% trichloroacetic acid and was collected on a Millipore filter. The filters were washed with cold 5% trichloroacetic acid containing 10 μg of unlabeled uracil per ml. They were then dried and counted in vials with 10 ml of toluene (3.0 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1,000 ml of toluene.

Radioactivity measurements were carried out in a Tri-Carb liquid scintillation counter, model 3314 (Packard Instrument Co., Inc., Downers Grove, Ill.).

RESULTS

Effect of temperature and glucose on thymidine uptake. The reaction mixtures were incubated in a water bath at 35 C and at 0 C for 2 min, and the reaction was terminated as described in Materials and Methods.

The results presented in Table 1 show that thymidine uptake was temperature-dependent. Cells incubated at 0 C showed only 16.6% of the amount of radioactivity in cells incubated at 35 C.

Thymidine uptake was also shown to be dependent on an exogenous energy source; when the system was incubated in the absence of glucose, the uptake of labeled thymidine was only 10% of that found in the presence of glucose (Table 2).

Kinetics of thymidine uptake. Figure 1 demonstrates the effect of time on the uptake of thymidine by resting cells incubated at 35 C with buffer and glucose. On short incubation (about 30 sec), a similar increase of radioactivity in both cold and hot trichloroacetic acid fractions was obtained. However, as incubation proceeded, the radioactivity of the thymidine pool decreased while that of the nucleic acids fraction (DNA) increased, reaching a peak value after 5 min; at that time, the radioactivity of the pool dropped almost to the zero-time level.

TABLE 1. *Effect of temperature on thymidine uptake by Pediococcus cerevisiae*[a]

Temp	Counts/min	Uptake
C		%
0	1,000	2.7
35	6,000	16.0

[a] The reaction mixture, consisting of phosphate buffer, glucose, thymidine (5×10^{-5} μmole), and bacteria as described in Materials and Methods, was incubated for 2 min in a water bath at 35 C or in an ice bath at 0 C. The reaction was stopped by immersing the tubes in an ice bath, centrifugation of the cells, and rapid washing with ice-cold saline. The radioactivity was estimated after extraction of whole cells with hot trichloroacetic acid.

TABLE 2. *Effect of glucose on thymidine uptake*[a]

Glucose	Counts/min	Uptake
μmoles		%
—	580	1.5
20	6,000	15.5

[a] The cells were incubated in the presence and absence of glucose. Other experimental conditions were as in Table 1.

Effect of pH on thymidine uptake. As shown in Fig. 2, thymidine uptake into DNA was optimal at a *p*H range between 6.5 and 8.0; that of the thymidine pool (cold trichloroacetic acid) had a somewhat narrower *p*H optimum.

Effect of iodoacetate on thymidine uptake. Since thymidine uptake has been shown to be energy-dependent, the effect of iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, was investigated (17). The experiment presented in Fig. 3 shows that iodoacetate (10^{-2} M) caused a 50% inhibition of thymidine incorporation into cellular DNA.

Thymidine uptake by cells preincubated with folinate. The effect of preincubation of resting cells of *P. cerevisiae* with folinate on the uptake of thymidine is shown in Fig. 4. Preincubation for 10 min with folinate caused a 75% inhibition of thymidine incorporation into the DNA and a slightly lower inhibition (65%) of the incorporation into the cold trichloroacetic acid fraction.

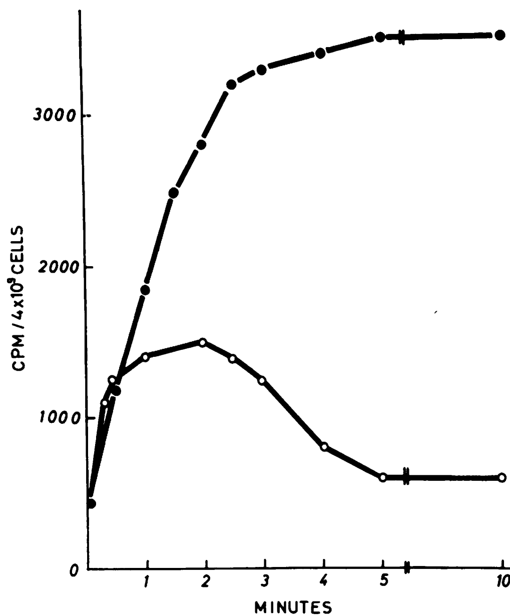

FIG. 1. *Kinetics of thymidine uptake. The reaction mixture consisting of Pediococcus cerevisiae cells (4 mg, dry weight), glucose, phosphate buffer, and* ^{3}*H-thymidine* (5×10^{-5} *μmole) was incubated at 35 C. The reaction was stopped by rapid filtration through Millipore filters; hot and cold trichloroacetic acid fractions were separated as described in Materials and Methods. Symbols:* ●, *hot trichloroacetic acid fraction;* ○, *cold trichloroacetic acid fraction.*

In Fig. 5, the effect of increasing concentrations of folinate on the uptake of labeled thymidine is shown; the highest inhibition of thymidine uptake (75%) was caused by 2×10^{-3} μmole of folinate.

The uptake of thymidine by growing cells of *P. cerevisiae* is shown in Fig. 6. Cells grown in the presence of folinate hardly incorporated any thymidine into their DNA as compared with those grown without added folinate (see legend to Fig. 6); under the latter conditions, within 30 min as much as 2.1 mμmoles of thymidine per mg (dry weight) of cells was incorporated.

Effect of 5-fluorodeoxyuridine (FUdR) on the inhibition of thymidine uptake by cells preincubated with folinate. The folinate-induced inhibition of thymidine uptake by *P. cerevisiae* cells (Fig. 4–6) suggested that the vitamin stimulates the de novo synthesis of thymidine. To test this assumption, the influence of FUdR, a potent inhibitor of thymidylate synthetase (6, 19), was examined.

Figure 7 shows that FUdR abolished the folinate effect; in the presence of 0.2 mg of the analogue, the incorporation of thymidine into cellular DNA was the same as in cells incubated in the absence of folinate.

Inhibition of thymidine uptake by cells preincubated with deoxyuridine or uracil. To elucidate

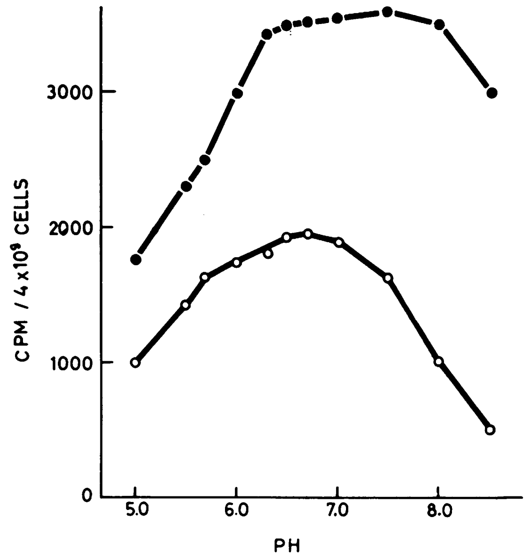

FIG. 2. *Effect of pH on thymidine uptake. Cells were incubated for 2 min in a reaction mixture containing glucose,* ^{3}*H-thymidine, and potassium phosphate buffer (100 μmoles). The reaction was stopped by cooling and centrifugation. Symbols:* ●, *hot trichloroacetic acid fraction;* ○, *cold trichloroacetic acid fraction.*

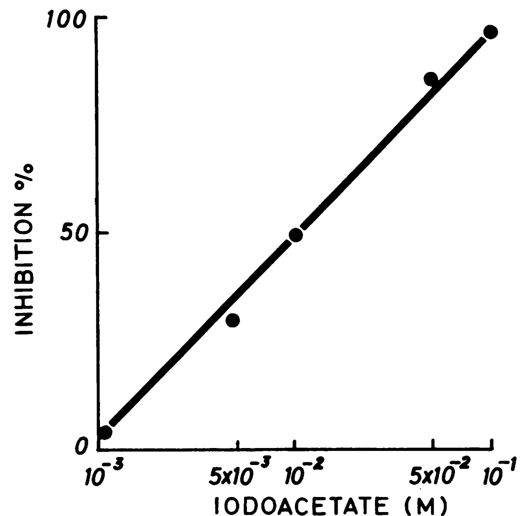

FIG. 3. *Effect of iodoacetate on thymidine uptake. Reaction mixture as in Fig. 1 and with increasing concentrations of iodoacetate. After incubation for 2 min, the reaction was stopped by cooling and cells were removed by centrifugation. The radioactivity of the nucleic acid was measured after separation from the cold trichloroacetic acid fraction.*

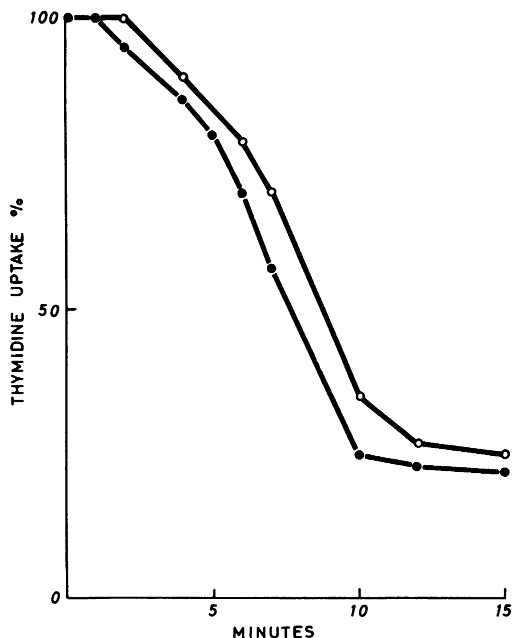

FIG. 4. *Effect of preincubation time with folinate on thymidine uptake by resting cells of Pediococcus cerevisiae. The cells were preincubated at 35 C, for varying time intervals, with folinate (2 × 10^{-3} μmole), glucose, and phosphate buffer (pH 6.5). Thereafter, thymidine was added and the reaction mixtures were incubated for an additional 2 min. The radioactivities of the cold and hot trichloroacetic acid fractions were measured. Symbols:* ●, *hot trichloroacetic acid fraction;* ○, *cold trichloroacetic acid fraction.*

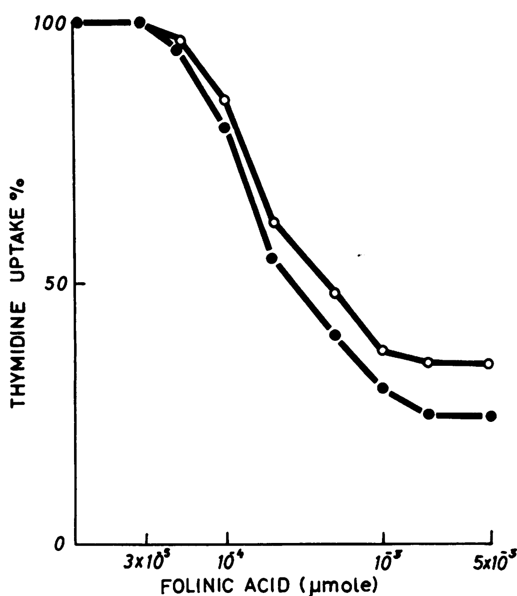

FIG. 5. *Thymidine uptake after preincubation with varying amounts of folinate. The reaction mixtures, as described in Fig. 4, were preincubated for 10 min at 35 C with increasing amounts of folinate. After addition of thymidine, the system was further incubated for 2 min. The radioactivities of cold and hot trichloroacetic acid fractions were determined. Symbols:* ●, *hot trichloroacetic acid fraction;* ○, *cold trichloroacetic acid fraction.*

further the role of folinate, the effect of possible thymidine precursors (deoxyuridine and uracil) on the uptake of labeled thymidine into cells preincubated with folinate was tested. As is apparent from Table 3, the inhibition of thymidine incorporation into DNA was more pronounced in cells preincubated with deoxyuridine and folinate than in cells preincubated with folinate alone. The effect of uracil was less marked.

Effect of preincubation with folinate on the uptake of uracil-2-^{14}*C.* The enhancement by uracil of the folinate effect (Table 3) can be interpreted as being due to the conversion of the former into deoxyuridine-monophosphate, the immediate precursor of thymidylate, and the subsequent incorporation of the latter into the DNA. As shown in Table 4, cells incubated with labeled uracil and folinate showed a threefold increase of the radioactivity in the DNA in comparison with cells preincubated without folinate.

Uptake of ^{3}*H-thymidine by cells preincubated with unlabeled thymidine or thymine.* In addition to the experiments with FUdR and uracil, the effect of unlabeled thymidine on the uptake of the radioactive substrate was tested. The results presented in Table 5 show that preincubation with unlabeled thymidine (4.0 × 10^{-2} μmole) prevented almost entirely the incorporation of the labeled compound. On the other hand, even a 20-fold higher amount of thymine (8.0 × 10^{-1} μmole) had no effect on the uptake of thymidine.

Effect of aminopterin and amethopterin on thymidine uptake by cells preincubated with folinate. Since it was shown by Kisliuk and Levine (13) and by Mathews and Sutherland (16) that aminopterin and amethopterin inhibit thymidylate synthetase from *E. coli*, the effect of these analogues on the folinate-induced inhibition of thymidine uptake was tested. Table 6 demonstrates that cells preincubated with folinate and either of the analogues incorporated thymidine at a level equal to that of cells preincubated without folinate. It was found that, to prevent completely the folinate effect, the analogue has to be given in a 10-fold higher concentration than the metabolite.

Effect of preincubation time with aminopterin on the folinate-induced inhibition of thymidine uptake. For further elucidation of the analogue effect

on the inhibition of thymidine uptake by cells treated with folinate, varying preincubation times with equimolar concentrations of the analogue were tested (*see* Fig. 8). Preincubation with the analogue for 8 to 10 min prevented the folinate effect.

Effect of folinate on the uptake of ³H-amethopterin. It was shown that amethopterin or aminopterin, or both, prevented the inhibition of thymidine uptake by cells preincubated with folinate (Table 6 and Fig. 8). This effect of the analogue could have resulted from the inhibition of thymidylate synthetase or from a competition between the analogue and the metabolite for uptake sites within the cell. To discriminate between these two possibilities, the uptake of ³H-amethopterin by cells preincubated with folinate was tested. As shown in Table 7, the uptake of amethopterin was not affected by preincubation of the cells with folinate, whether at equimolar or higher concentrations of the vitamin. It is therefore suggested that the amethopterin effect is due to a direct action on thymidylate synthetase and not to competition for the uptake sites of the cell.

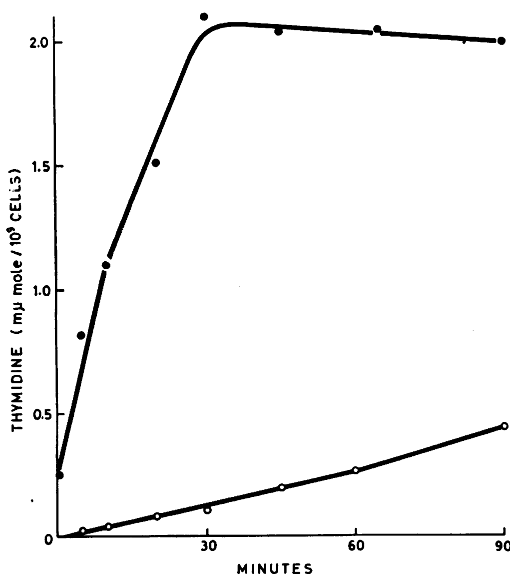

Fig. 6. *Thymidine uptake by cells grown in the presence and absence of folinate. A logarithmic-phase culture of Pediococcus cerevisiae supplemented with folinate (300 μμg/ml) was distributed into two series of tubes, each containing 3 ml of culture. The vitamin from one series of cultures was removed by centrifugation and the cells were resuspended in fresh medium without folinate. Thymidine (5×10^{-3} μmole) was added to both series of tubes and incubation was continued for varying time intervals as indicated in the figure. The reaction was stopped by cooling and centrifugation. The radioactivity was measured in the hot trichloroacetic acid fraction (DNA). Symbols: ○, thymidine uptake in the presence of folinate; ●, thymidine uptake in the absence of folinate.*

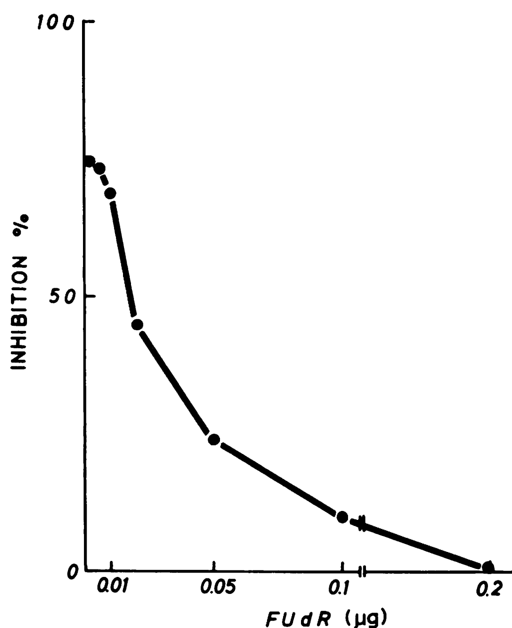

Fig. 7. *Effect of FUdR on the inhibition of thymidine incorporation into DNA of cells preincubated with folinate. Reaction mixtures were preincubated for 10 min with folinate and increasing amounts of FUdR; after the addition of thymidine, incubation was continued for 2 min. The radioactivity was estimated in the hot trichloroacetic acid fraction (see Materials and Methods).*

Table 3. *Effect of deoxyuridine and uracil on the incorporation of thymidine into DNA*[a]

Folinate (2.0×10^{-3} μmole)	Additions (10^{-1} μmole)	Counts/min
−	—	3,500
+	—	1,000
−	Deoxyuridine	3,300
+	Deoxyuridine	250
−	Uracil	3,600
+	Uracil	830

[a] Cells (4 mg dry wt) were preincubated for 10 min in phosphate buffer, glucose with or without deoxyuridine or uracil and in presence or absence of folinate; thereafter, ³H-thymidine was added and incubation was continued for 2 min. The radioactivity of the hot trichloroacetic acid fraction was counted.

Discussion

The experiments described show that the uptake of thymidine by resting cells of *P. cerevisiae*

TABLE 4. *Effect of preincubation with folinate on the incorporation of uracil-2-^{14}C into the DNA of Pediococcus cerevisiae*[a]

Folinate (2×10^{-3} μmole)	Counts/min
−	510
+	1,560

[a] The cells were preincubated for 10 min at 35 C with folinate, phosphate buffer, and glucose; after the addition of uracil (3×10^{-2} μmole/0.5 μc) they were incubated for an additional 10 min. The radioactivity was measured in the DNA after removal of ribonucleic acid by hydrolysis (*see* Materials and Methods).

TABLE 5. *Uptake of* ^{3}*H-thymidine by cells preincubated with unlabeled thymidine or thymine*[a]

Thymine (8.0×10^{-1} μmole)	Thymidine (4.0×10^{-2} μmole)	Counts/min
−	−	8,000
−	+	400
+	−	8,000

[a] The cells were preincubated with cold thymidine or thymine for 2 min; after the addition of labeled thymidine, the incubation was continued for 2 min. The radioactivity was measured after extraction of the cells with hot trichloroacetic acid.

is markedly influenced by the temperature of incubation and is dependent on glucose as an energy source. Iodoacetate, an inhibitor of the glycolytic cycle, inhibited thymidine uptake. A similar inhibitory effect was caused by sodium fluoride (*unpublished data*), which may be ascribed to inhibition of enolase activity (15) or of the DNA-polymerase (14), or both.

In resting cells of *P. cerevisiae*, the addition of folinate to the reaction mixture resulted in a considerably diminished uptake of thymidine (up to 75% inhibition). In growing cultures, the folinate-induced effect was even more striking; almost no thymidine was incorporated.

It is assumed that the inhibitory effect induced by folinate results from its participation, after the conversion to the coenzymatic form 5,10-CH_2-H_4PteGlu (2, 18, 24), in the de novo synthesis of thymidylate from cellular percursors.

The following findings support this assumption. (i) Fluorodeoxyuridine, a specific inhibitor of thymidylate synthetase (6, 19), prevented the folinate-induced inhibition of thymidine uptake. (ii) An augmented inhibition of thymidine uptake was obtained when cells were preincubated with folinate and deoxyuridine or uracil (poten-

TABLE 6. *Thymidine incorporation into DNA of cells preincubated with folinate and either aminopterin or amethopterin*[a]

Amethopterin	Aminopterin	Folinate (2×10^{-3} μmole)	Inhibition of thymidine uptake
μmoles	*μmoles*		%
—	—	−	0
—	—	+	75
—	2×10^{-3}	+	45
—	2×10^{-2}	+	0
—	2×10^{-2}	−	0
2×10^{-3}	—	+	50
2×10^{-2}	—	+	0
2×10^{-2}	—	−	0

[a] The reaction mixtures containing phosphate buffer, glucose, and cells (4 mg, dry weight), were preincubated for 10 min with folinate and either aminopterin or amethopterin as indicated; after addition of ^{3}H-thymidine, the incubation continued for 2 min. The radioactivity was measured in the hot trichloroacetic acid fraction (DNA).

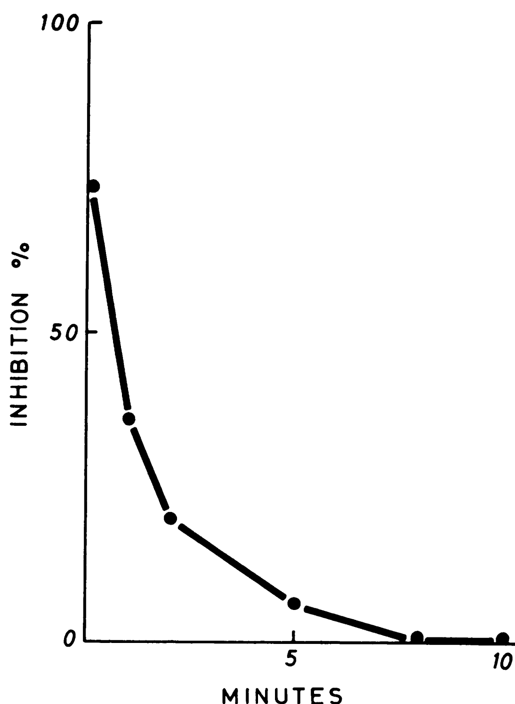

FIG. 8. *Aminopterin-folinate interaction and its effect on thymidine uptake. This was a three-stage incubation experiment: (a) cells (4 mg, dry weight) were preincubated with aminopterin (2×10^{-3} μmole) for varying time intervals as indicated; (b) folinate (2×10^{-3} μmole) was added and incubation was continued for 10 min, and (c)* ^{3}*H-thymidine was added and incubation was resumed for an additional 2 min. The incorporation into DNA (hot trichloroacetic acid fraction) was measured.*

TABLE 7. *Effect of folinate on the uptake of ^{3}H-amethopterin*[a]

H^3-amethopterin (10^{-3} μmole)	Folinate	Counts/min
	μmoles	
+	—	360
+	10^{-2}	380
+	2×10^{-2}	370
+	10^{-1}	370

[a] The reaction mixtures, containing phosphate buffer, glucose, cells (3 mg, dry weight), and folinate as indicated, were incubated for 10 min. The uptake of ^{3}H-amethopterin (0.1 μc) was tested after additional incubation for 10 min. The radioactivity was measured in a trichloroacetic acid extract of the washed cells.

tial precursors of thymidine). (iii) Preincubation with folinate increased the incorporation of labeled uracil into DNA, presumably after prior conversion to thymidylate. (iv) Finally, preincubation with unlabeled thymidine inhibited the uptake of the labeled substrate.

The concentration of the internally synthesized thymidylate was apparently sufficiently high to saturate rapidly the available sites of both resting and growing cells. The phenomenon of saturation was reflected by the inhibition of the uptake of external thymidine.

The folinate effect on thymidine uptake could be counteracted by aminopterin or amethopterin. For complete reversal, the concentration of the analogue has to be considerably higher than that of folinate. However, the folinate effect could be fully annulled also at equimolar concentrations of the metabolite and analogue, provided the system was preincubated with the analogue for as long as 8 to 10 min. Since the uptake of ^{3}H-amethopterin was not influenced by preincubation with folinate, the antagonistic effect of the analogue was not due to competition for the same sites within the cell. Thus, the reversal of the folinate effect by aminopterin (and amethopterin) is apparently due to the inhibition by these analogues of thymidylate synthetase activity (13, 16). Were *only* dihydrofolate reductase affected by aminopterin, addition of the tetrahydroform (folinate) should have immediately annulled the effect, which was not the case.

Thymidine is known to exhibit a sparing effect on folinate for the growth of *P. cerevisiae* (5, 8). This sparing by thymidine may be explained by the rapid and efficient de novo synthesis of thymidylate. In this reaction, the coenzyme is irreversibly consumed, and for each molecule of thymidylate formed a molecule of 5,10-CH_2-H_4-PteGlu is oxidized to H_2-PteGlu (2, 18, 24). Externally added thymidine may therefore spare folinate for other biosynthetic functions. The reports that thymidine may substitute for folinate as a growth factor (1, 3, 7, 11, 20, 21, 25; Kisliuk, *personal communication*) may be due to the use of different strains with increased ability to store or with varying degrees of leakiness in the synthesis of this vitamin.

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