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Inhibitors of DNA Precursor Metabolism in *Dictyostelium discoideum*

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The effects of various inhibitors of DNA precursor metabolism were studied on *Dictyostelium discoideum* growing in a defined axenic medium. Fluorodeoxyuridine was an effective inhibitor of growth at 20 μg/ml; this inhibition was not reversed by thymidine, suggesting that in this organism fluorodeoxyuridine is not acting on thymidylate synthetase alone. Removal of the required nutrient, folic acid, from the medium resulted in a lower maximum level of growth than in the control. The inclusion of adenine, guanine, serine, and thymidine in the minus-folic acid medium allowed the final growth level to approach that of the control. Methotrexate, a folic acid analog and dihydrofolate reductase inhibitor, blocked growth completely at 200 μg/ml; its effect was partly reversed by the addition of adenine, guanine, serine, and thymidine. Aminopterin, another folic acid analog, had only a temporary effect on cell multiplication, followed by a return to exponential growth. Trimethoprim was ineffective up to 200 μg/ml. Hydroxyurea blocked growth in the concentration range of 150 to 300 μg/ml. These results indicate that several of these inhibitors are effective for altering thymidine monophosphate synthesis in *D. discoideum* and hence may be useful for studies of DNA replication and repair and for the isolation and characterization of thymidine-requiring mutants.

The cellular slime mold *Dictyostelium discoideum* is used as a model for differentiation in lower eucaryotes (13) and for studies of DNA repair (7). An understanding of DNA precursor metabolism is essential for the study of DNA replication and repair and for effective radioisotopic labeling of DNA. Currently, little is known of these pathways in *D. discoideum*, or of their susceptibility to blockage by various metabolic inhibitors. Therefore, we have investigated the effects of several widely used antimetabolites on the growth of this organism: fluorodeoxyuridine (FUdR), methotrexate (amethopterin; MTX), aminopterin, trimethoprim, and hydroxyurea. The effect of removing the required precursor, folic acid, from the growth medium was also studied. In addition, we present results on the effectiveness of certain precursors for bypassing these metabolic blocks. The totally defined axenic medium of Franke and Kessin (10) facilitates these experiments and their interpretation.

MATERIALS AND METHODS

D. discoideum strain A3 (here designated A3K since some genetic differences may distinguish this strain from other A3 strains) obtained from R. Kessin (Harvard University) was used for most experiments. For some comparisons, other strains were occasionally used as indicated. Spores stored at −70°C were germinated in HL-5 complex axenic medium (18, except with peptone and yeast extract [Difco Laboratories] instead of Oxoid components), and then the amoebae were transferred to the defined medium (D-medium) of Franke and Kessin (10) several days before the start of the inhibition experiments. All growth was in 10 ml of medium in 250-ml Erlenmeyer flasks at 23°C, with rotation at 200 rpm. Cell counts were determined with a Coulter Counter. Cultures were already in exponential growth at the time of addition of inhibitors or supplements or both. The inhibitors were obtained as follows and dissolved in D-medium shortly before dilution into the cultures: FUdR, aminopterin, and hydroxyurea (Calbiochem), MTX (Lederle Laboratories), and trimethoprim (Sigma Chemical Co.).

RESULTS

FUdR. Figure 1 shows growth curves for *D. discoideum* A3K in D-medium containing FUdR. Growth was significantly reduced at 5 μg/ml, whereas 20 μg/ml allowed only a very limited increase in cell number, followed by a gradual decline. FUdR at 50, 100 or 200 μg/ml yielded lesser initial increases, followed by even sharper declines in cell number (data not shown).

The growth inhibition by FUdR was not reversed by adding thymidine at 50 or 250 μg/ml (Fig. 1), or by thymidine monophosphate (dTMP) at 250 μg/ml (data not shown). Howev-

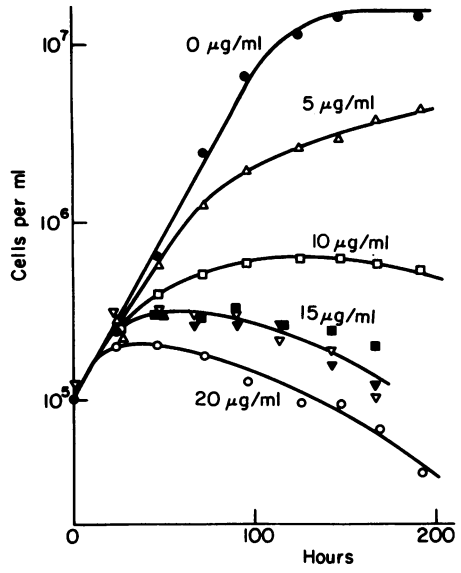

FIG. 1. Growth of *D. discoideum* A3K in D-medium at 23°C in the presence of FUdR added at zero time. Concentrations of FUdR: ●, 0 μg/ml; ▼, 5 μg/ml; ■, 10 μg/ml; ▽, 15 μg/ml; ○, 20 μg/ml. Thymidine was added in the following concentrations at zero time to the culture with 15 μg of FUdR per ml: △, 50 μg/ml; □, 250 μg/ml. (15 μg of FUdR per ml is 6.1×10^{-5} M.)

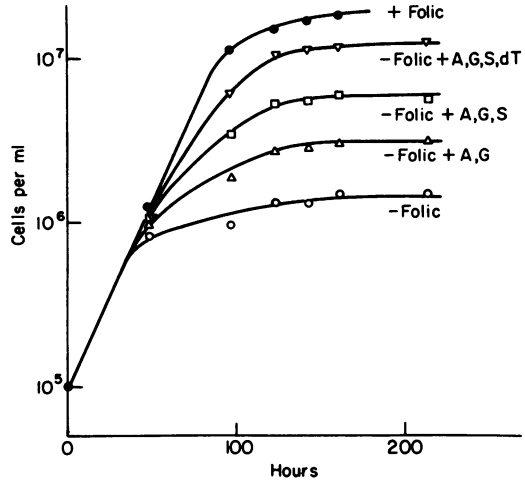

FIG. 2. Growth in the presence or absence of folic acid, with supplementation as indicated. ●, D-medium; ○, D-medium minus folic acid; △, minus folic acid + 50 μg each of adenine (A) and guanine (G) per ml; □, minus folic acid + 50 μg each of adenine and guanine per ml and 300 μg of serine (S) per ml; ▽, minus folic acid + 50 μg each of adenine and guanine per ml, 300 μg of serine per ml, and 300 μg of thymidine (dT) per ml.

er, in other experiments, a small enhancement of growth in 20 μg of FUdR per ml was observed with 100 μg each of thymidine, uracil, uridine, and orotic acid per ml, although neither of the latter three alone with thymidine was effective.

In experiments using HL-5 complex growth medium, 3.3-fold-greater FUdR concentrations were required to give the same inhibition as in D-medium; for example, FUdR at 50 μg/ml in HL-5 gave the same inhibition as 15 μg/ml in D-medium. Strain HPS104, a *radC* mutant defective in excision repair (20, 21), was inhibited to the same extent as strain A3K. In HL-5, 5 to 10 μg of FUdR per ml had little, if any, effect on the growth of A3K or HPS104 strain.

During growth on *Escherichia coli* B/r, FUdR at 20 μg/ml had no effect on *D. discoideum* growth (data not shown).

Withholding folic acid. Figure 2 and Table 1 show results for growth of strain A3K in the absence of folic acid, with or without supplementation with adenine and guanine, serine or thymidine, or both serine and thymidine. Other amino acids whose synthesis might depend upon the folic acid pathway, glycine, histidine, and methionine, were already present in D-medium. For these experiments, cells growing in D-medium were centrifuged, washed, and resuspended at zero time in D-medium minus folic acid. Supplements, when included, were added at this time. Figure 2 shows that considerable growth ensued after the removal of the folic acid, possibly due to residual endogenous pools of folate derivatives which required time for depletion. Growth stopped at 1.1×10^6 cells per ml in the absence of folic acid. The final growth plateau was higher in the presence of various growth

TABLE 1. Partial bypass of requirement for folic acid[a]

Growth conditions	Maximal growth (units of 10^6 cells per ml)			
	+ dT (0)	+ dT (3)	+ dT (30)	+ dT (300)
+ Folic acid + A, G (0) + S (0)	19.0			
− Folic acid + A, G (0) + S (0)	1.1	1.1	1.1	1.4
− Folic acid + A, G (0) + S (300)	1.6			3.9[b]
− Folic acid + A, G (50) + S (0)	2.6	3.0		8.6
− Folic acid + A, G (50) + S (3)	3.3	5.7	8.0	13.0
− Folic acid + A, G (50) + S (300)	6.0	7.0	11.5	12.0

[a] Folic acid, 0.4 μg/ml. A, Adenine; G, guanine; S, serine; dT, thymidine. Numbers in parentheses indicate concentration in micrograms per milliliter. Inoculum, 0.1×10^6 cells per ml.

[b] Two hundred micrograms of thymidine per ml.

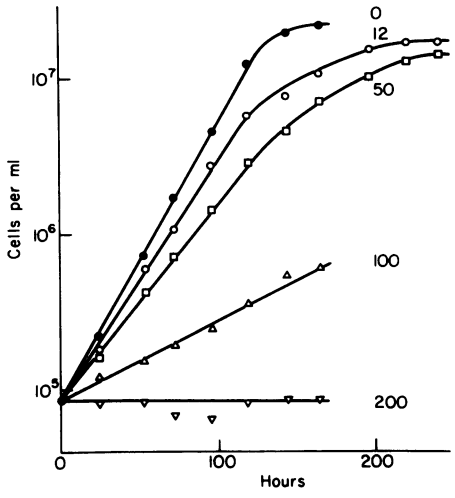

FIG. 3. Effect of different concentrations of MTX on growth. ●, Control; ○, 12 μg/ml; □, 50 μg/ml; △, 100 μg/ml; ▽, 200 μg/ml (200 μg of MTX per ml is 4.4 × 10^{-4} M).

supplements (Fig. 2, Table 1). Thymidine alone was relatively ineffective in bypassing the folic acid requirement (Table 1). Although some improvement in the final level of growth was achieved with adenine, guanine, or serine, and combinations of these, nearly maximal growth (1.2 × 10^7 to 1.3 × 10^7 cells per ml compared with the control of 1.9 × 10^7 cells per ml) was attained only with the combination of purines, serine, and thymidine.

MTX. Figure 3 shows the growth-inhibitory effects of various MTX concentrations. Exponential growth, at a rate which decreased with increasing MTX concentrations, was observed; 200 μg/ml completely inhibited growth. Figure 4 demonstrates that the inhibitory effect of MTX was partly reversed by supplementation with adenine, guanine, serine, and thymidine. MTX was less inhibitory in HL-5 medium than in D-medium (data not shown).

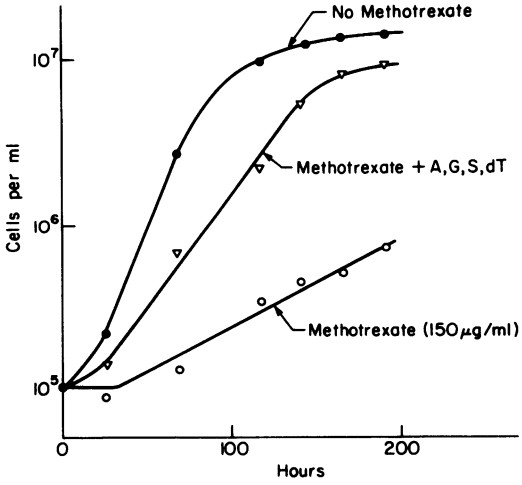

FIG. 4. Partial reversal of MTX inhibition. ●, Control; ○, MTX at 150 μg/ml; ▽, 150 μg of MTX per ml + adenine (A) and guanine (G) at 50 μg/ml each, 300 μg of serine (S) per ml, and 300 μg of thymidine (dT) per ml.

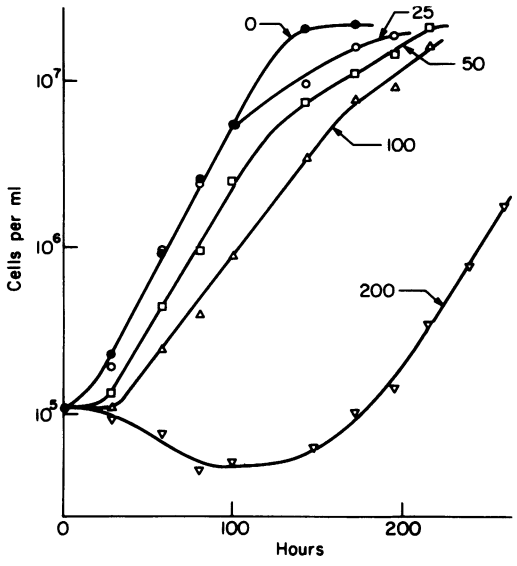

FIG. 5. Effect of different concentrations of aminopterin on growth. ●, Control; ○, 25 μg/ml; □, 50 μg/ml; △, 100 μg/ml; ▽, 200 μg/ml. (200 μg of aminopterin per ml is 4.5 × 10^{-4} M.)

Aminopterin. Figure 5 shows the effect of aminopterin on the growth of strain A3K in D-medium. The response differed from that due to MTX in that inhibition was only temporary; for example, 100 μg/ml produced a growth lag of about 30 h, followed by a return to nearly normal exponential growth. Even at 200 μg/ml, growth resumed after an extended lag of about 150 h.

Trimethoprim. This drug had no effect on the growth of strain A3K in D-medium up to 200 μg/ml.

Hydroxyurea. Growth of strain A3K in D-medium was inhibited by hydroxyurea (Fig. 6); significant effects were observed at concentrations greater than 100 μg/ml, with almost complete inhibition at 300 to 400 μg/ml.

DISCUSSION

FUdR. Subsequent to phosphorylation, FUdR is an effective inhibitor of thymidylate synthetase (4, 9). In addition, metabolites derived from this analog can interfere with RNA metabolism (4). Our results show that FUdR in the concentration range of 5 to 20 μg/ml is effective for

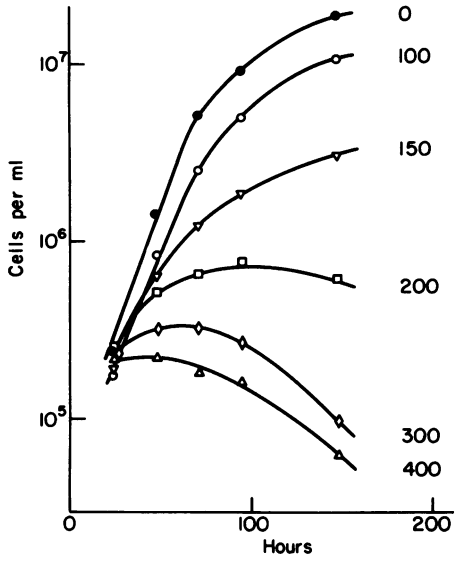

FIG. 6. Effect of hydroxyurea on growth. Concentrations in μg/ml were as indicated in the figure (300 μg of hydroxyurea per ml is 3.9×10^{-3} M).

inhibiting *D. discoideum* growth in D-medium. The inhibition was not reversed by thymidine up to 250 μg/ml, or by dTMP at this concentration. *D. discoideum* contains thymidine kinase (14) and is capable of utilizing exogenous thymidine in a recently isolated thymidine-requiring mutant; in the latter studies there is no evidence that thymidine at 250 μg/ml is either significantly degraded in the medium or inhibitory to the growth of *D. discoideum* over the time range used here (G. Podgorski, R. Guyer, T. Ohnishi, and R. A. Deering, unpublished data). If FUdR were phosphorylated by the thymidine kinase and inhibited thymidylate synthetase only, thymidine might be expected to provide a bypass of the block via the traditional "salvage pathway." That such was not the case suggests that FUdR also exerts an inhibitory effect on RNA synthesis as observed in other organisms, possibly via fluorouracil, an enzymatic breakdown product of FUdR (4). This conclusion is supported by observations that RNA precursors, when added at high concentrations with thymidine, yield a partial reversal of the FUdR inhibition (12; this report).

In complex axenic medium, our results show that a greater concentration of FUdR is necessary for inhibition. In addition, Loomis (12) showed that on the order of 200 μg of FUdR per ml was required to inhibit the growth of *D. discoideum* A3 in a complex, undefined, axenic medium. The reduced effectiveness of FUdR in the complex axenic medium probably results from the bypass-allowing properties of the complex mixture of nucleic acid precursors present in the medium. For example, HL-5 medium may contain as much as 6 μg of thymidine per ml, as measured by the supplementation of growth of *E. coli* 15T$^-$ (W. Ford and R. A. Deering, unpublished data). However, since strain 15T$^-$ growth is also supported by thymine, the actual content of thymidine may be considerably less. Thymine is not utilized by *D. discoideum* (11; G. Podgorski, unpublished data). Other nucleic acid precursors are also present in unknown amounts.

The FUdR concentrations required to inhibit the growth of *D. discoideum* in D-medium are high compared with those required to inhibit mammalian cells (5), but not with those that are inhibitory in *E. coli* (8). Since sensitivity depends upon transport and potentially complex metabolic conversions, as well as the nature of the target molecules, it is difficult to reach useful conclusions from these comparisons at present.

Although unsuccessful, the attempts to reverse the FUdR-induced inhibition by supplementation with dTMP are worthy of comment. Although dTMP does not usually readily enter cells, yeast mutants which can utilize dTMP to supplement thymidylate synthetase deficiencies are known (3). In addition, the growth of our thymidine-requiring mutant can be supplemented with dTMP, although at much higher concentrations than that required for thymidine (Podgorski et al., unpublished data). This may not be surprising since *D. discoideum* in axenic medium may, at least in part, take up exogenous soluble molecules by engulfing tiny droplets of solution. Therefore, the lack of dTMP effect on the FUdR inhibition may be a corollary to the lack of thymidine effect, supporting the possibility that FUdR exerts a large effect on RNA synthesis in *D. discoideum*.

The observed delay in the appearance of an FUdR effect on growth may be due to many factors such as poor entry or transport of precursors, low rate of phosphorylation of FUdR, or large internal competing pools. The possibility that the FUdR is also acting at the level of mitochondrial DNA synthesis cannot be excluded (see below); this might lead to a delayed growth response. A delay in inhibition of *E. coli* growth by FUdR has also been observed (8).

Another property of *D. discoideum*, that about 30% of the total cellular DNA is mitochondrial, has implications in DNA precursor metabolism and inhibitor effects. Kielman and Deering (11) have shown that although [^{3}H]adenine in HL-5 medium is incorporated into mitochondrial DNA (m-DNA) and nuclear DNA (n-DNA) at about equal specific activities, [^{3}H]thymidine is taken up into the m-DNA at 8- to 10-fold the specific activity in n-DNA. The overall uptake of label is not very efficient in these non-thymi-

dine-requiring strains. When uptake of [^{3}H]adenine into m- and n-DNA was measured in the presence or absence of 20 μg of FUdR per ml, using CsCl gradient resolution of these DNAs, m-DNA synthesis was more seriously depressed than was n-DNA synthesis (C. A. Michrina and R. A. Deering, unpublished data). Furthermore, we have shown that m-DNA undergoes a greater density shift than does n-DNA when bromodeoxyuridine is supplied to the cells at 100 μg/ml in axenic medium. These results all indicate that exogenous thymidine and its analogs, FUdR and bromodeoxyuridine, are processed more efficiently into m-DNA than into n-DNA, possibly due to different effective precursor pool sizes, differential transport, differences in thymidine kinase activities in the pathways to these DNAs, or all three of the above. It has been shown that both mitochondrial and cytoplasmic thymidine kinase activities are found in *D. discoideum* (14).

During growth of strain A3K on *E. coli* B/r, 20 μg of FUdR per ml was not inhibitory. Under these circumstances, when the *D. discoideum* nucleic acid precursors are obtained from the degraded bacterial nucleic acids, the endogenous conversion of deoxyuracil monophosphate to dTMP via thymidylate synthetase, and the synthesis of RNA precursors are probably not mandatory for *D. discoideum* nucleic acid synthesis and growth.

Withholding folic acid. For maximal growth, *D. discoideum* requires folic acid (0.4 μg/ml used here) in D-medium (10). Since a product of the folic acid pathway, 5,10-methylene tetrahydrofolate, is involved in the conversion of deoxyuracil monophosphate to dTMP by thymidylate synthetase and in other metabolic pathways, notably for purine synthesis and in the synthesis of certain amino acids (4), we studied some of the growth responses to the deletion of folic acid from D-medium. The combination of thymidine, adenine and guanine, and serine could largely replace the requirement for folic acid in D-medium (Fig. 2, Table 1). These results are consistent with the involvement of folic acid in the same pathways observed in other organisms—dTMP synthesis, purine synthesis, and amino acid synthesis (4). The requirement for high concentrations of the precursors probably relates to poor uptake of exogenous components from solution, a mechanism that may be of limited utility to an organism that usually feeds by engulfing particulate material, for example bacteria and other microorganisms.

MTX. The folic acid analog MTX is an inhibitor of dihydrofolate reductase; consequently, it interferes with those metabolic pathways that depend upon 5,10-methylene tetrahydrofolate (4). The inhibitory effect of MTX on *D. discoideum* in D-medium (Fig. 3) was partly reversed by supplementation of the medium with adenine and guanine, serine, and thymidine (Fig. 4). This result is consistent with the results of the previous section in which these same nutrients could alleviate the reduced growth resulting from the absence of folic acid. Thus, MTX appears to act as expected in blocking the metabolism of folic acid in *D. discoideum* by the inhibition of dihydrofolate reductase.

The reduced effectiveness of MTX in complex HL-5 medium is probably due to the presence in this medium of precursors such as purines, amino acids, and DNA precursors, which facilitate bypass of the block in folate utilization.

The concentration of MTX required to inhibit the growth of *D. discoideum* is much higher than that required to inhibit mammalian cells (2), but lower than that required to inhibit yeast (22) and many bacteria (15). Since cell sensitivity depends upon numerous factors including enzyme sensitivity, mechanism of uptake, and metabolic activation or degradation (19), these comparisons between different organisms, although interesting, do not allow specific conclusions.

Aminopterin. Inhibition of growth of *D. discoideum* by this folic acid analog was only temporary. After an initial lag, growth resumed at nearly the control rate. The duration of the lag increased with increasing aminopterin concentration. These results suggest that aminopterin is being metabolically degraded by these cultures, a phenomenon also observed with some bacteria and yeast (4, 15, 19). A greater resistance of MTX to degradation compared with aminopterin has also been observed in some cases (19) and may account for the differential effects of these two analogs, as observed here.

Trimethoprim. Trimethoprim acts as an inhibitor of dihydrofolate reductase in many bacteria, but is much less effective in mammalian cells (6). The lack of an effect on the growth of *D. discoideum* suggests that the dihydrofolate reductase of this organism, like those of the higher eucaryotes, is relatively resistant to this analog. Alternatively, uptake into the amoeboid cells of *D. discoideum* may be limiting.

Hydroxyurea. Hydroxyurea blocks ribonucleotide reductase in the cells of many organisms (17). It was effective in *D. discoideum* in the concentration range of 150 to 300 μg/ml. These concentrations are lower than those required for inhibition of *E. coli* or yeast and only slightly higher than that required to inhibit mammalian cells (1, 16, 17). Our results indicate that hydroxyurea can act as an effective growth inhibitor for *D. discoideum*; we do not yet know if it has a preferential effect on semiconservative DNA replication relative to repair replication (17).

D. discoideum responds to the antimetabolites

used here in a manner consistent with their expected effects on DNA precursor metabolism. They may be useful in this organism for studies of DNA replication and repair and in protocols for the isolation and characterization of thymidine-requiring mutants.

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