S-Phase Cells of Rapidly Growing and Resting Populations

Differences in Response to Methotrexate

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SUMMARY

The effects of methotrexate on L5178Y cells from logarithmically growing cultures and from resting cultures were studied. Methotrexate killed cells 6.7 times faster and produced 20-fold greater inhibition of deoxyuridine incorporation into DNA in the cells from logarithmic as compared to resting cultures. The antifolate also acutely suppressed the incorporation of thymidine into DNA by cells from logarithmic cultures but had no effect on that by cells from resting cultures.

These differences in the effects of methotrexate on the S-phase cells from cultures of different growth rates are interpreted in the light of current hypotheses relating cell kinetics to the cytocidal effects of antimetabolites.

INTRODUCTION

The faster the growth rate of a cell population, the greater is the susceptibility of that population to the cytocidal effects of antimetabolites (1, 2). The cells in the growing fraction of a rapidly expanding population traverse the mitotic cycle more rapidly than do cells in the growing fraction of a resting population (3). Since antimetabolites interfere with synthetic functions which occur only during certain phases of the mitotic cycle, it has been suggested that in the faster-growing population more cells per unit of time will enter that phase in which the drug can interfere with their metabolism. Hence, it has been proposed that under conditions of rapid growth a greater proportion of the growth fraction will be killed per unit time of drug exposure (1, 2).

Implicit in this hypothesis is the assumption that when cells enter a particular drug-sensitive phase of the mitotic cycle, for example, the S-phase, they undertake the same synthetic functions regardless of the over-all proliferative rate of their parent population (4). Therefore, interference with these functions by antimetabolites should produce the same biochemical disturbances.

We have tested this hypothesis by examining the effects of the folic acid antagonist methotrexate on deoxynucleoside incorporation into DNA by cells (presumably S-phase cells) derived from nonsynchronized cultures of different growth rates. Methotrexate inhibits the enzyme dihydrofolate reductase and thus interferes with the maintenance of intracellular pools of reduced folates, in particular $N^{5,10}$-methylenetetrahydrofolate. The latter compound is required in substrate amounts for conversion of deoxyuridylate to thymidylate, which is essential for DNA synthesis

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(5). The effect of the antifolate is therefore to block conversion of dUMP to dTMP, resulting in blockage of DNA synthesis (6). This is believed to be the basis for the cytotoxic effects of methotrexate (5). Ordinarily, the bulk of dTMP is derived from dUMP synthesized *de novo*. However, thymidine kinase can phosphorylate deoxyuridine to dUMP and thymidine to dTMP, and thus these exogenous deoxynucleosides can be utilized and join the main pathway for DNA synthesis (7). In this circumstance the incorporation of UdR into DNA would still be blocked by methotrexate, but if, in the presence of antifolate, the DNA-synthesizing system were otherwise intact, TdR incorporation into DNA should not be affected (5). Thus, if the S-phase cells of populations with a rapid growth rate are identical in their metabolic pathways with the S-phase cells from populations with a slower growth rate, any effect of methotrexate on UdR and TdR incorporation into DNA by these cells should be independent of the growth rates of their respective parent populations. However, the results of our studies indicate that there are differences in the effects of methotrexate on cellular incorporation of these deoxynucleosides into DNA, depending upon the growth rate of the populations from which the cells were sampled. Coincident with these differences in effect on incorporation patterns are striking differences in the effects of the drug on the viability of cells as measured by cloning techniques. Thus, faster-growing populations of cells may be more susceptible to the effects of methotrexate because the individual S-phase cells from such populations may be more susceptible to antimetabolite effects than their counterparts from resting populations.

MATERIALS AND METHODS

Fischer's medium and horse serum were obtained from Grand Island Biological Company; methotrexate (sodium salt), from Lederle; Noble agar, from Difco; and thymidine tritiated in the methyl group (^{3}H-TdR) (specific activity, 1.9 Ci/mmole) and deoxyuridine tritiated in position C-6 (^{3}H-UdR) (specific activity, 3.2 Ci/mmole), from Schwarz BioResearch. The scintillation fluid mixture was prepared by standard methods (8). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, model 3380, with correction for efficiency and quenching with automatic external standardization. Calculations of rates and standard deviations were performed in a table-top Olivetti-Underwood Programma computer, using standard statistical formulae (9).

L5178Y mouse leukemia cells maintained in culture as previously described (10) were grown until the cell number no longer increased with time, then diluted into fresh medium. Just before dilution and at various times thereafter, the cultures were exposed to 10^{-6} M methotrexate for periods up to 6.5 hr. At intervals during drug exposure, the cells were washed free of drug and were studied. Cloning efficiency was measured by the soft agar method (11). Reduction of cloning efficiency caused by methotrexate can be taken as an index of cell death, since the cells cannot be rescued by addition of a mixture of thymidine, serine, and hypoxanthine (12). Hereafter, cell kill will be defined as reduction in cloning efficiency. The rate of incorporation of deoxynucleosides into DNA was determined as follows. After exposure to methotrexate in culture, the lymphoblasts were resuspended in Fischer's medium —10% horse serum to the number of cells per milliliter required for accurate deoxynucleoside incorporation rate determination. The same concentration of methotrexate (10^{-6} M) was maintained. A portion of the suspension was counted in a Coulter counter, model B. Then aliquots were distributed into 10-ml flasks and placed in a Dubnoff incubator with sufficient atmospheric CO_2 to maintain a pH of 7.1–7.3. After 10 min of incubation at 37° with shaking, either ^{3}H-TdR at a final concentration of 6×10^{-7} M or ^{3}H-UdR at a final concentration of 9×10^{-7} M was added. The suspensions were again incubated at 37°, and 5, 10, 15, and 20 min after the addition of deoxynucleoside the

cells and horse serum protein were precipitated with ice-cold 5% perchloric acid. The precipitates were washed free of acid-soluble radioactivity. The radioactivity extractable into 5% perchloric acid by heating at 85° for 15 min was measured by liquid scintillation techniques. The radioactivity was presumed to originate in the thymine of DNA (6).

The rate of incorporation of the deoxynucleoside into DNA was calculated from these samples by plotting counts per minute per million cells against the time of incubation with the deoxynucleoside and obtaining the slope of the regression line. By adjusting the cell concentration in the various suspensions, it was possible to obtain sufficient incorporation of deoxynucleoside into DNA to keep the standard deviation of this slope to less than 10% of the absolute value of the slope. This allowed comparisons of the rates to be made on a logarithmic scale (13). In the UdR experiments, the cell concentration ranged between 3 and 10×10^6/ml. Incorporation rates per million cells did not vary over these ranges.

Control cultures were similarly sampled and studied, but without exposure to methotrexate. The rate of ^{3}H-UdR incorporation by cells exposed to methotrexate was divided by the rate of ^{3}H-UdR incorporation by cells not exposed to the drug, and the quotient was expressed as a decimal fraction, called the fractional ^{3}H-UdR incorporation rate.

RESULTS

Effect of methotrexate on cloning efficiency of cells. After dilution of cells from a resting culture into fresh culture medium, the cloning efficiency increased approximately 3-fold as the growth rate reached a maximum during logarithmic growth, and then declined once more to predilution values as the culture returned to a resting state (growth rate of zero). Coincident with the period of maximum growth rate and maximum cloning efficiency there was a period of maximum susceptibility of the cells to the lethal effects of methotrexate. The results of six separate experiments are summarized in Fig. 1. Thus, a 4.5-hr exposure to 10^{-6} M methotrexate produced a 15-fold greater kill of log culture cells than of resting culture cells.

Effect of methotrexate on ^{3}H-UdR incorporation by cells. When cells from a resting culture were diluted into fresh medium, the ^{3}H-UdR incorporation rate of the cells in the control (untreated) cultures rose approximately 6-fold as the growth rate reached a maximum, and then returned to predilution values as the resting condition was resumed. At the period of maximum rate of ^{3}H-UdR incorporation, methotrexate produced the greatest degree of inhibition of this rate in relative as well as absolute terms. Figure 2 shows the results of two separate experiments, each with duplicate determinations of con-

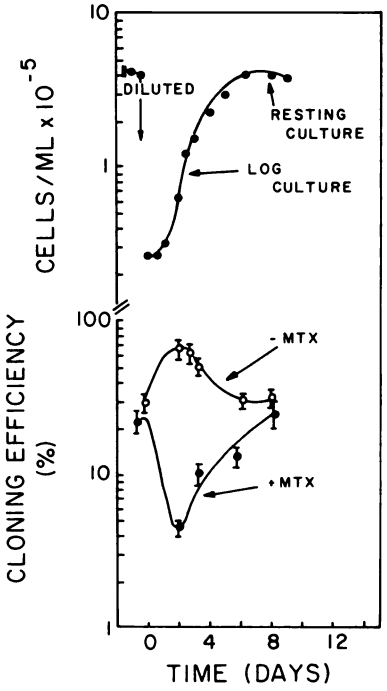

FIG. 1. *Effect of methotrexate (MTX) on cloning efficiency of L5178Y cells*

Cells from a resting culture were diluted into fresh medium at zero time. Then, at intervals, cells were sampled from the new culture, exposed to no drug or to 10^{-6} M methotrexate for 4.5 hr, and washed, and cloning efficiency was measured. Vertical bars represent standard errors of the geometric means.

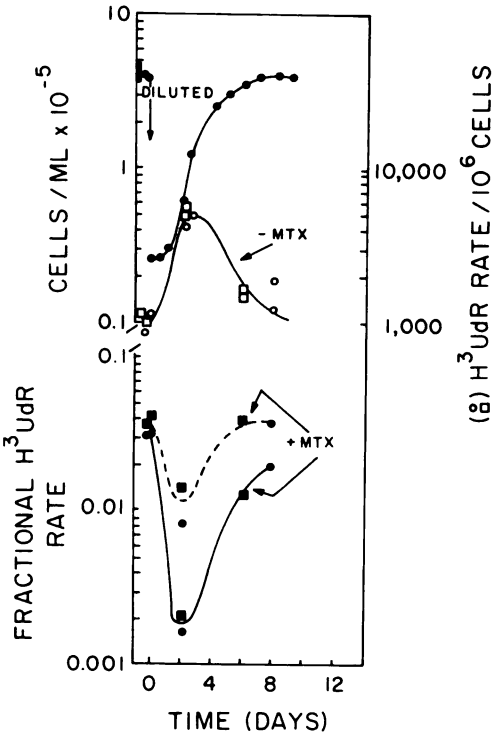

FIG. 2. *Effect of methotrexate (MTX) on incorporation of ^{3}H-UdR into DNA by L5178Y cells*

Experiments were performed as described for Fig. 1, except that instead of cloning efficiency, the rate of incorporation of ^{3}H-UdR into DNA was measured. ● and ■ represent replicate experiments. In each experiment, control rates (no methotrexate) were measured in duplicate (○ and □, double symbols). The fractional ^{3}H-UdR rate is the rate obtained in the presence of methotrexate (and after a 4.5-hr exposure to the drug in culture) divided by the rate obtained in the absence of methotrexate, and is expressed as a decimal fraction. The solid line connects the uncorrected, and the dashed line the corrected, fractional ^{3}H-UdR rates (see DISCUSSION).

trol rates. The rates after exposure to 10^{-6} M methotrexate for 4.5 hr are shown as fractions of the respective control rates.

In cells from resting cultures, exposure to methotrexate reduced ^{3}H-UdR incorporation to 3.5×10^{-2} times the control rate, whereas the same treatment of cells from logarithmic cultures reduced ^{3}H-UdR incorporation to 1.8×10^{-3} times the control rate. Thus, methotrexate produced a 20-fold greater blockade of the incorporation of ^{3}H-UdR into DNA in the cells of logarithmic cultures. This is shown as a solid line in the lower half of Fig. 2. (The derivation of the dashed line will be explained under DISCUSSION.)

Effects of methotrexate on ^{3}H-TdR incorporation. The effects of 10^{-6} M methotrexate on ^{3}HTdR incorporation into DNA and on cloning efficiency were studied over a period of 6.5 hr on samples of cells taken from logarithmic and resting cultures. There was a striking difference in the effect of the antimetabolite on the incorporation of this deoxynucleoside by the cells from the two cultures.

After 30 min of exposure to methotrexate there was a 50% reduction in ^{3}H-TdR incorporation by cells obtained from log phase cultures, and by 2 hr this rate decreased to one-sixth the control value. Then the rate seemed to stabilize, and by 6.5 hr it appeared to rise (upper half of Fig. 3A). In marked contrast, cells from resting cultures showed no significant change in ^{3}H-TdR incorporation during exposure to methotrexate (upper half of Fig. 3B).

Coincident with these differences in effects on ^{3}H-TdR incorporation there was exponential kill of cells in both types of cultures; a higher rate of kill of cells from the logarithmic culture was observed in comparison to cells from the resting cultures. Thus, the cloning efficiency of cells from log cultures decreased at 6.7 times the rate of decline seen when resting culture cells were treated with methotrexate (compare slopes of lines in lower halves of Fig. 3A and B).

DISCUSSION

The effect of methotrexate on the cloning efficiency of cells was clearly related to the growth rate of the culture from which the cells were taken for testing. The rate of kill of the log culture cells by 10^{-6} M methotrexate was 6.7 times that of the cells from the resting culture. This was not unexpected, as differences of this order have been observed for several antimetabolites in model systems (14–18). This relationship has hitherto been explained by differences in duration of the generation time

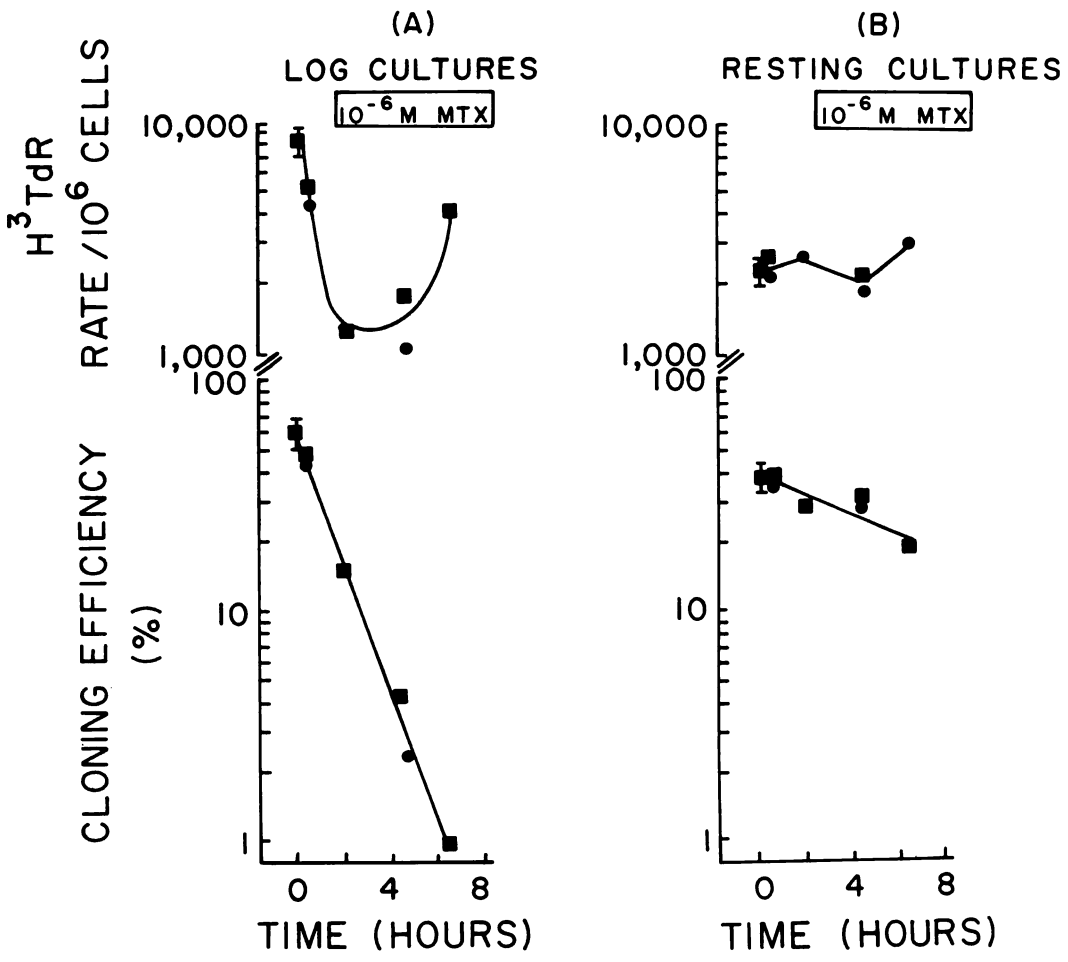

FIG. 3. *Effect of methotrexate (MTX) on ^{3}H-TdR incorporation into DNA and on cloning efficiency of L5178Y cells*

Cells were sampled from log phase cultures (A) and resting cultures (B) and exposed to no drug (controls) or to 10^{-6} M methotrexate for 6.5 hr. At the indicated intervals, cells were removed and the rate of incorporation of ^{3}H-TdR into DNA and cloning efficiency were measured as described in the text. Cells exposed to methotrexate in culture were also exposed to the drug during measurement of the rate of ^{3}H-TdR incorporation. The controls are shown as the zero time values. The results of two experiments are shown (● and ■); in each, controls were done in triplicate.

and of the S-phase between different cell populations. However, for this difference in the rate of kill to be explained strictly in kinetic terms, one would have to postulate that the mean generation time of the viable fraction of the resting culture was 6.7 times longer than that of the logarithmic culture. That is, the rate of entry of cells into the S-phase during drug exposure would have to be 6.7 times slower for the resting culture cells, so that the proportion of the viable cells in the S-phase would be 6.7 times smaller. Thus, per unit time of drug exposure, 6.7 times fewer viable cells would commit themselves to S-phase in the resting culture compared to the log culture. This seems unlikely. First, the deoxynucleoside incorporation rate of log culture cells was only 3.75–5 times greater than that of resting culture cells. Because the incorporation rate takes into account both the percentage of cells in the S-phase and

the rate per cell, and since the rate per cell in logarithmic cultures would be, if anything, higher, the actual percentage of S-phase cells in the log culture would probably be not 3.75–5 times greater but something less than this factor. We have not yet determined radioautographically the ratio of the cells in the S-phase under the two culture conditions. Others (19, 20) have shown that when Ehrlich ascites cells are transferred from a slowly proliferating mature tumor into a fresh host, the percentage of cells in the S-phase increases by a factor of 1.2–1.5 and the generation time shortens by a factor of 2–2.7. Madoc-Jones and Bruce (14) stated that 45% of the cells were in the S-phase in logarithmically growing cultures and virtually none in a resting L cell culture, but did not report on the efficiency of detection of ^{3}H-TdR label in obtaining their radioautographs. One must be cautious of extrapolating these results to the present study, however, in view of the differences in model systems and techniques used.

It appears likely that the greater rate of kill of cells in the logarithmic culture in our experiments was related to factors in addition to kinetic influences. The difference in the effect of methotrexate on deoxynucleoside incorporation by cells from cultures of different growth rates supports this hypothesis.

If we assume that the cells capable of incorporating deoxynucleosides into DNA are the cells in the S-phase, it follows that the effects of methotrexate on this incorporation represent the effects of the drug on the cells in this state. If this is so, then it is clear from the present investigation that the effects of methotrexate on S-phase cells vary according to the growth rate of the culture from which the cells have been taken. The greater the growth rate, the greater is the inhibition of ^{3}H-UdR incorporation into DNA produced by methotrexate. In addition, ^{3}H-TdR incorporation into DNA is inhibited by methotrexate at the most rapid growth rates, but is little affected in cells from resting cultures.

From what is known of the mode of action of methotrexate in biochemical terms, it seems reasonable to conclude that these differences in its effects on deoxynucleoside incorporation by DNA-synthesizing cells are causally related to the differences in the cytocidal effects of the drug. Therefore, it is important to inquire into the basis underlying these variations in the effects of the drug on deoxynucleoside incorporation. It is believed that methotrexate kills bacterial and mammalian cells by inducing a "thymineless state" through blockade of the conversion of dUMP to dTMP (4). However, this would not explain the sudden reduction of ^{3}H-TdR incorporation seen in methotrexate-treated cells from logarithmic cultures. This reduction may simply represent death of cells in the S-phase, with complete cessation of all DNA synthesis, so that even if ^{3}H-TdR were to bypass the blockade of dUMP conversion it could not be incorporated into DNA. If this were so, one would expect changes in ^{3}H-TdR incorporation to parallel changes in cloning efficiency, and it is clear from Fig. 3A and B that this is not the case. However, changes in ^{3}H-TdR incorporation may not accurately reflect changes in DNA synthesis because of complicating alterations in intracellular thymidylate pools (21). If differences in pool size changes are not significant, an alternative explanation may be that methotrexate has at least two effects on DNA synthesis in cells from rapidly growing populations. The first is blockade at the step of dUMP conversion to dTMP occurring in S-phase cells from both log and resting cultures. The second effect, occurring only in cells in the S-phase from log cultures, is not understood, but could involve inhibition of purine biosynthesis *de novo* required for DNA synthesis, since C-2 and C-8 of the purine ring are donated by reduced folates (22). Others (23) have proposed this explanation to account for the observation that administration of methotrexate to humans with acute leukemia results in a decreased rate of ^{3}H-TdR incorporation into DNA by acute lymphoblastic leukemia cells but does not change the rate of ^{3}H-TdR incorporation into acute granulocytic leukemia cells. It

is known that acute lymphoblastic leukemia cells are much more sensitive to antimetabolites in general, and methotrexate in particular, than are acute granulocytic leukemia cells. We are still left with the necessity of explaining why this inhibition occurred only in logarithmic cells or in acute lymphoblastic leukemia cells, because purine biosynthesis *de novo* would presumably also occur in resting culture cells or acute granulocytic leukemia cells and in these instances should also be dependent upon reduced folates. The results of ^{3}H-UdR incorporation studies may provide the explanation.

^{3}H-UdR and ^{3}H-TdR share the same pathway for incorporation into DNA, except that ^{3}H-UdR goes through the additional step of conversion of dUMP to dTMP. When ^{3}H-TdR incorporation is suppressed by methotrexate, ^{3}H-UdR incorporation should likewise be suppressed *in addition* to being blocked at the step of dUMP conversion. Measurement of the blockade of ^{3}H-UdR incorporation into DNA under these circumstances does not provide a measure of the blockade of dUMP conversion. Let us assume that when methotrexate suppressed ^{3}H-TdR incorporation, it also suppressed ^{3}H-UdR incorporation to an *equal* degree by virtue of the same mechanism. If we know the degree of suppression of both ^{3}H-TdR and ^{3}H-UdR incorporation for any given treatment with the drug, it is a simple matter to recalculate the degree of suppression of ^{3}H-UdR incorporation which might be attributable solely to the blockade at the dUMP conversion step. When this is done, as for the data in Fig. 2, the fractional UdR rates obtained are those connected by the dashed lines in the lower half of the figure. The inhibition of ^{3}H-UdR incorporation due to this calculated "blockade" of dUMP conversion still remains more marked in the logarithmic cells than in the resting culture cells. Thus logarithmic cells may be more susceptible to blockade of dUMP conversion to dTMP than cells of resting cultures, and besides are susceptible to the additional action of methotrexate on ^{3}H-TdR incorporation. Perhaps in these cells such profound inhibition of dihydrofolate reductase occurs that not only is dUMP conversion more severely hampered for lack of substrate amounts of reduced folates, but the enzyme is also rendered incapable of providing even the small catalytic amounts of reduced folates required for maintenance of purine biosynthesis. This could occur if transport of the drug into cells at any given methotrexate concentration were greater for logarithmic cells than for resting cells, provided that the enzyme levels and the K_i for methotrexate did not change. There is evidence that the transport of methotrexate is an important factor in conditioning the response of a variety of murine leukemias (24), and perhaps also human leukemias, to therapy with the drug (25), but the data are conflicting (26).

On the other hand, Borsa and Whitmore (27) have recently shown that addition of purines *augments* the killing of L cells by methotrexate. Significantly, this occurred in logarithmically growing cultures but not in resting cultures. Others have shown (28, 29) that inhibition of RNA and protein synthesis protect cells from the "thymineless" death produced by this agent.

An alternative possibility is that in logarithmic cells, but not in resting culture cells, there is a major locus related to DNA synthesis at which methotrexate acts in addition to its effect on dihydrofolate reductase and reduced folate pools. We cannot choose between these various alternatives on the basis of the data presented here.

Whichever mechanism is operative, one point seems clear from the present study: the effect of methotrexate on S-phase L5178Y cells differs according to the growth rate of the population from which the cells are derived, at least in terms of the effect on ^{3}H-TdR and ^{3}H-UdR incorporation into DNA. This suggests that differences in metabolism of S-phase cells may underlie at least some of the differences in susceptibility of cell populations to methotrexate. Thus, in a fast-growing population, not only may a larger part of the growth fraction be at risk during drug exposure, but

each cell at risk may be more profoundly affected.

The phenomena described herein may also occur in cells proliferating *in vivo* as well as *in vitro*, with other cell types, and with other antimetabolites. If so, it will be necessary to revise the current concepts (1, 2) of how the kinetics of proliferation of cells conditions their susceptibility to the cytocidal effects of antimetabolites.

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