Studies Relating to the Mode of Action of Methotrexate

II. Studies on Sites of Action in L-Cells *in Vitro*

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SUMMARY

The methotrexate-induced inhibition of thymidylate and of purine synthesis has been studied in L-cells in tissue culture under conditions which permitted the separation of the two types of inhibition. Data are presented which suggest that under the conditions employed methotrexate may have sites of action in addition to its inhibition of folate and dihydrofolate reductase. These findings are discussed in relation to the strengths and weaknesses of the noe-site-of-action hypothesis.

INTRODUCTION

Methotrexate has proven useful as a chemotherapeutic agent for the treatment of various neoplastic diseases (1), primarily choriocarcinoma (2), Burkitt's lymphoma (3), and acute leukemia of children (4). In the majority of cases, however, it is either ineffective or of limited use. If chemotherapy is to progress rationally to new and better drugs, the mode of action of agents such as MTX[2] that offer limited success must be thoroughly understood, for such knowledge might suggest desired characteristics for new drugs and enable the more efficient use of those presently available.

Methotrexate has been studied intensively, and a tenable picture of its mode of action has emerged which finds wide acceptance today. While the presently accepted model, henceforth referred to as the one-site-of-action hypothesis and summarized below, satisfactorily accounts for many of the experimental observations, there are a few well documented findings that seem discordant with it.

Methotrexate is an analogue of the vitamin folic acid. Folic acid itself is not biologically active and must be reduced by folate and dihydrofolate reductase to dihydrofolic acid, and thence to tetrahydrofolic acid, which accepts 1-carbon fragments from various sources to generate the folate coenzymes. The latter are utilized as 1-carbon group donors in a variety of reactions, including the synthesis of the methyl group of thymidylic acid, the insertion of the C-2 and C-8 atoms of the purine skeleton, and the synthesis of the β-carbon of serine. [For a review of folic acid metabolism, see Stokstad and Koch (5).] The one-site-of-action hypothesis postulates that the biological effects of MTX are due entirely to its inhibition of the reductase. [For a review of the properties of MTX, see Jukes and Broquist (6).] Inhibition of the reductase prevents generation of tetrahydrofolate and ultimately results in a cessation of the reactions requiring folate coenzymes.

In this report we present experimental results which suggest that the one-site-of-

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[2] The abbreviation used is: MTX, methotrexate.

action hypothesis is not completely satisfactory and that in the L-cell system other sites of action may be more sensitive than inhibition of the reductase. In the accompanying publication (7), additional independent evidence for one of the proposed new sites of action will be presented to substantiate the conclusions derived from the growth inhibition experiments described here.

MATERIALS AND METHODS

All of the experiments described in this paper were carried out on a subline of Earle's L-cells, L60T, as described elsewhere (8). These were grown in suspension culture in medium CMRL 1066 (9), from which the coenzymes and all of the nucleosides were omitted, supplemented with 10% (v/v) dialyzed fetal calf serum. This medium will be referred to as "growth medium" throughout this paper. The serum was dialyzed for three successive 24-hr intervals against 20 volumes of fresh, phosphate-buffered 0.9% NaCl (10) at 4°.

Experiments were performed on various volumes of cell suspension. For those experiments requiring repeated sampling from a single culture, 200-ml suspension cultures were used. For experiments requiring a single determination of total cell number at some time following a specific treatment, replicate 10-ml suspension cultures containing 6×10^4 cells/ml were set up in 17×10 mm Hi-Temp plastic tubes (Falcon Plastics, Los Angeles). The cell cultures were treated some 20 hr after they had been set up. At this time, the cultures were in exponential growth with a doubling time (T_D) of 15–16 hr and contained about 1×10^5 cells/ml. Cell suspensions were assayed for total cell number (usually after 48 hr of post-treatment incubation) by means of an electronic particle counter (Coulter Electronics, Hialeah, Florida). The number of viable cells in cell suspensions was assayed by means of the plating technique of Puck and Marcus (11), using a plating medium consisting of CMRL 1066 supplemented with 12% (v/v) horse serum and 12% fetal calf serum.

Most of the experiments to be reported here required the addition of various substances to the cell cultures at some point. Nucleosides were purchased from General Biochemicals and were dissolved in either 1066 N^-Co^- or phosphate-buffered 0.9% NaCl prior to addition to the cultures. Methotrexate and N^5-formyltetrahydrofolate were purchased from Lederle Products Department, Cyanamid of Canada, Ltd., Montreal, and were dissolved in sterile phosphate-buffered 0.9% NaCl.

RESULTS

Inhibition by MTX of L-cell proliferation and reversal of such inhibition with N^5-formyltetrahydrofolate or thymidine plus deoxyadenosine. In order to investigate the response of our tissue culture system to MTX, spinner cultures of L-cells were exposed to various concentrations of MTX. Figure 1 shows the resulting growth curves. For concentrations of MTX below 10^{-9} g/ml, growth was exponential and the growth curves were indistinguishable from those of the control culture. Higher concentrations of MTX resulted in various degrees of inhibition of the rate of cellular proliferation.

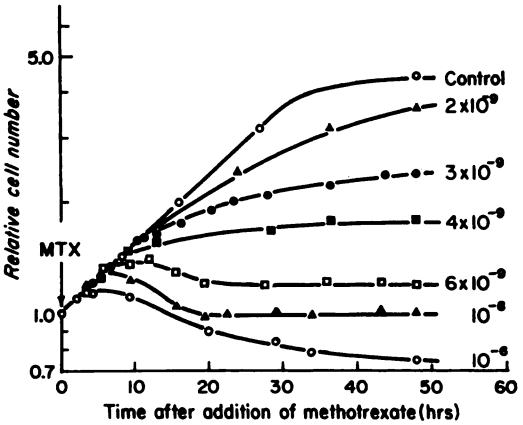

FIG. 1. *Growth curves of L-cells after addition of various concentrations of MTX to the growth medium*

Exponentially growing suspension cultures of L-cells, in spinners of 200-ml capacity, were treated with the indicated concentrations of MTX (in grams per milliliter). Then the total number of cells per milliliter in each cell culture was determined as a function of time and plotted as a multiple of the number initially present.

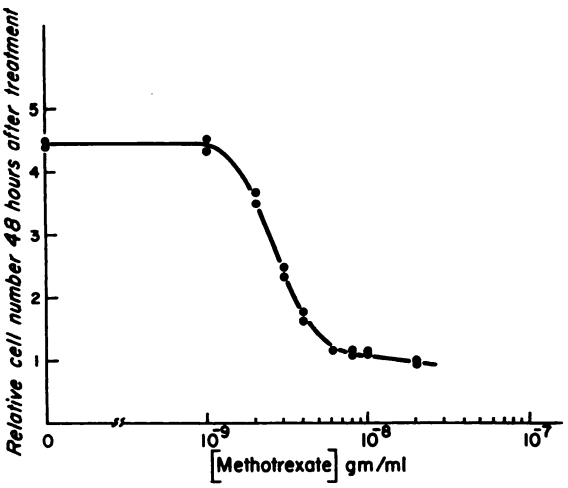

FIG. 2. *Effect of MTX concentration on inhibition of L-cell proliferation in growth medium*

Exponentially growing suspension cultures of L-cells (10 ml each) were treated with the indicated concentrations of MTX. Following 48 hr of incubation at 37°, the total number of cells per milliliter in each culture was determined and plotted as a multiple of the number initially present. The double points are values of duplicate cultures.

To investigate the dependence of the inhibition of cellular proliferation on MTX concentration, a series of 10-ml cultures of L-cells were exposed to various concentrations of MTX. Figure 2 shows the ratio of the cell number per milliliter after a 48-hr incubation period to the number present at the start of the incubation as a function of MTX concentration. From Figs. 1 and 2 it can be seen that in the concentration range of 10^{-9} to 6×10^{-9} g/ml the rate of cellular proliferation varied inversely with the MTX concentration. At higher concentrations of MTX, the inhibition of cellular proliferation was complete following a brief period of normal growth, which was probably due to the presence of pools of metabolites whose synthesis is inhibited by MTX.

Because MTX prevents the conversion of folic acid through tetrahydrofolate to the active coenzyme form (12, 13), it should be possible to prevent the inhibitory action of MTX by addition of either N^5-formyltetrahydrofolate (as a source of folate coenzymes) or those metabolites

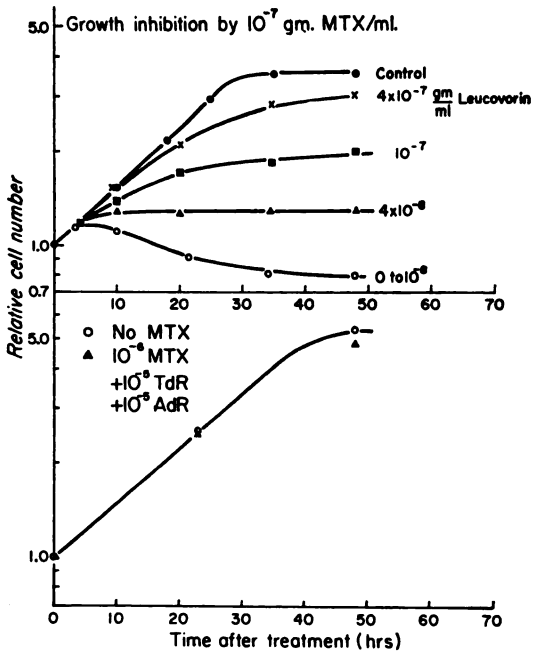

FIG. 3. *Upper: reversal of MTX inhibition of L-cell proliferation in growth medium by N^5-formyltetrahydrofolate (leucovorin)*

Exponentially growing suspension cultures of L-cells were treated with 10^{-7} g of MTX per milliliter, together with the indicated concentrations of N^5-formyltetrahydrofolate (in grams per milliliter). The cultures were incubated at 37°, and the total number of cells per milliliter in each culture was determined as a function of time. The ratio of cell number per milliliter observed at the indicated times to that initially present is plotted. Control cultures received no MTX.

Lower: reversal of MTX inhibition of L-cell proliferation in growth medium by thymidine (TdR) and deoxyadenosine (AdR)

Exponentially growing suspension cultures of L-cells were treated as follows: ○, no additions; ▲, 10^{-6} g of MTX, 10^{-5} g of thymidine, and 10^{-5} g of deoxyadenosine per milliliter. The total number of cells per milliliter in each culture was determined after 24 hr of incubation at 37° and again after 48 hr. The ratio of number of cells observed at the indicated times to that initially present is plotted.

whose synthesis is dependent on the folate coenzymes. The upper half of Fig. 3 shows the growth curves for cell cultures to which MTX was added at a concentration of 10^{-7} g/ml together with various concentrations of N^5-formyltetrahydrofolate. For concen-

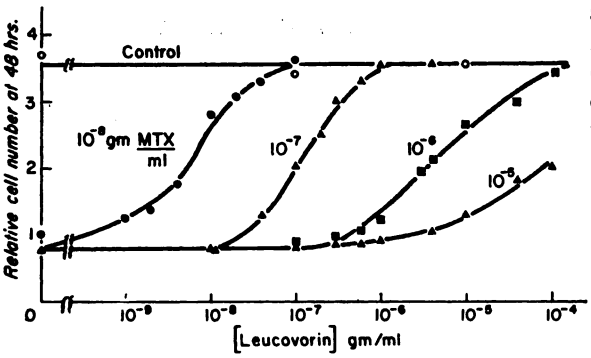

FIG. 4. *Competitive nature of reversal by N^5-formyltetrahydrofolate (leucovorin) of the MTX inhibition of L-cell proliferation in growth medium*

Exponentially growing suspension cultures (10 ml each) of L-cells were treated with MTX at the concentrations shown (in grams per milliliter). Controls received no MTX. For each concentration of MTX, a series of replicate cultures was treated with various concentrations of N^5-formyltetrahydrofolate. Following 48 hr of incubation at 37°, the total number of cells per milliliter in each culture was determined and plotted as a multiple of the number initially present.

trations of N^5-formyltetrahydrofolate less than about 10^{-8} g/ml, cellular proliferation was completely inhibited, but at a concentration of about 10^{-6} g/ml the growth curve was indistinguishable from the control with no MTX. The lower portion of Fig. 3 shows the growth curve resulting from the addition of 10^{-6} g of MTX/per milliliter along with 10^{-5} g/ml each of thymidine and deoxyadenosine. The open circles show the proliferation in the presence of 10^{-5} g/ml each of thymidine and deoxyadenosine without MTX. Both deoxynucleosides, when present at sufficient concentrations, completely overcame the MTX-induced inhibition of cell proliferation. These data indicate that under our cell culture conditions MTX exerts its biological effects exclusively through inhibition of thymidylate and purine synthesis. N^5-Formyltetrahydrofolate can reverse this inhibition, as can the addition of thymidine (a source of thymidylate) plus deoxyadenosine (a purine source).

Characteristics of reversal by N^5-formyltetrahydrofolate of MTX-induced inhibition of cell cultures in growth medium. The ability of N^5-formyltetrahydrofolate to reverse the MTX-induced inhibition of L-cell proliferation was studied as a function of the MTX concentration used to produce the inhibition, since the interaction between metabolite and antimetabolite over a wide concentration range of both could shed considerable light on the mode of action of the antimetabolite (14, 15). Replicate 10-ml cultures were treated with various concentrations of MTX; for each MTX concentration, a series of N^5-formyltetrahydrofolate concentrations was used. The change in cell number after a 48-hr incubation was measured (Fig. 4). The presence of N^5-formyltetrahydrofolate alone ("control") at any of the concentrations tested did not significantly affect cellular proliferation, but in its absence, with MTX present at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} g/ml, cellular proliferation was completely inhibited. For any particular concentration of MTX, the inhibition of cellular proliferation was gradually overcome as the N^5-formyltetrahydrofolate concentration was increased past some threshold value. The concentration of N^5-formyltetrahydrofolate needed to reverse the MTX-induced inhibition of cellular proliferation was roughly proportional to the concentration of MTX producing the inhibition.[3] This type of reversal suggests a competition between MTX and N^5-formyltetrahydrofolate (or derivatives of either or both) for some folate-binding site essential for cell proliferation.[4] Because N^5-formyltetra-

[3] "Competitive reversal," as used in this paper, describes this type of relationship between N^5-formyltetrahydrofolate and MTX. "Noncompetitive reversal" refers to the situation where the amount of N^5-formyltetrahydrofolate, or other reversing agent, required to reverse the biological effect of MTX is independent of MTX concentration, except where the inhibition by MTX is incomplete in the absence of reversing agent.

[4] To save space, we refer to a competition between MTX and N^5-formyltetrahydrofolate even though the actual competition may arise between MTX (or a derivative) and one or another of the folate coenzymes to which N^5-formyltetrahydrofolate is converted intracellularly

TABLE 1

Reversal of MTX-induced inhibition of cellular proliferation by addition of various nucleosides to the growth medium

All nucleosides were added to a final concentration of 10^{-5} g/ml.

Nucleosides added	Relative No. of cells after 48 hr	
	No MTX	10^{-6} g/ml of MTX
None	4.48	0.56
Thymidine	4.38	0.49
Deoxyadenosine	4.67	0.98
Adenosine	4.21	0.75
Deoxyguanosine	3.86	0.75
Deoxyadenosine +deoxyguanosine	4.20	1.01
Thymidine + deoxyadenosine	4.08	4.31
Thymidine + adenosine	4.24	4.43
Thymidine+ deoxyguanosine	3.69	0.85
Thymidine + guanosine	3.63	0.78
Thymidine + deoxyadenosine + deoxyguanosine	3.92	3.69

hydrofolate represents a metabolite distal to the known inhibition of folate and dihydrofolate reductase, the competitive nature of the reversal is consistent with the interpretation either that MTX and N^5-formyltetrahydrofolate compete for entry into the cell or that there are additional sites of MTX action distal to the reductase block. Both possibilities are discussed below.

Comparison of ability of various purine nucleosides to reverse growth inhibition by MTX. The ability of various purine nucleosides to act as a source of purines for L-cells in the presence of MTX was investigated. A series of exponential cultures was treated with 10^{-6} g of MTX per milliliter, together with various combinations of thymidine (10^{-5} g/ml) and deoxyadenosine, adenosine, deoxyguanosine, or guanosine (each at 10^{-5} g/ml). After 48 hr of incubation, the number of cells present relative to that at the start of the incubation was determined. Table 1 shows that only deoxyadenosine or adenosine could serve as an efficient purine source in overcoming the depletion due to MTX inhibition of synthesis *de novo*. Both deoxyguanosine and guanosine were completely ineffective. The combination of deoxyadenosine and deoxyguanosine offered no advantage over deoxyadenosine or adenosine alone.

Comparison of inhibition by MTX of the thymidylate- and purine-synthetic pathways. The data of Fig. 3 (lower) indicate that under our culture conditions the effects of MTX on cell growth were due entirely to effects on either or both the thymidylate- and purine-synthetic pathways. These effects could be separated and their individual effects on cell growth determined by making the culture dependent on only one of the two vulnerable pathways through adding an excess of the product of the other pathway. As an initial step, the sensitivity of the two pathways to inhibition by MTX was determined (Fig. 5). For equivalent inhibition of the two pathways, as measured by this growth assay, the purine-biosynthetic pathway requires approximately a 10-fold greater concentration of MTX than does the thymidylate-biosynthetic pathway. The existence of two such well-defined sensitivities suggests that more than one locus of action may exist, although the greater inhibition of dTMP synthesis may arise from the cyclic nature of the role of folic acid in dTMP synthesis (17). How-

(5). There is no evidence for any significant metabolism of MTX (16), and in our tissue culture system the various impurities present in the commercial preparation used in these studies were inactive (7).

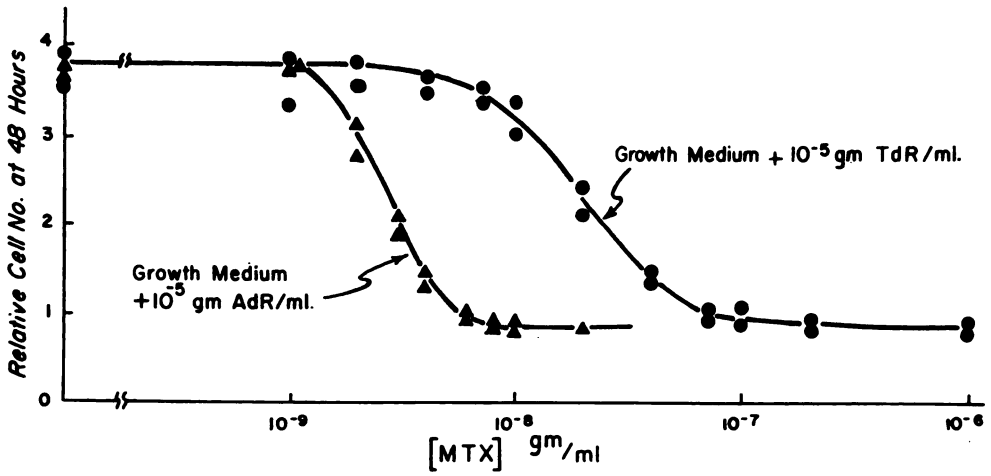

FIG. 5. *Comparison of sensitivities of thymidylate and purine synthesis de novo to inhibition by MTX*

Exponentially growing suspension cultures of L-cells were treated by addition of the indicated concentrations of MTX together with a 10^{-5} g/ml concentration of either thymidine (TdR) or deoxyadenosine (AdR). After 48 hr of incubation at 37°, the total number of cells per milliliter in each culture was determined and plotted against the number initially present. The deoxyadenosine-supplemented cultures reflect the sensitivity of thymidylate synthesis to inhibition by MTX; those supplemented with thymidine reflect the sensitivity of purine synthesis *de novo* to inhibition by MTX.

ever, we believe we have evidence against the latter possibility.

Reversal of MTX-induced inhibition of thymidylate synthesis. The growth inhibition caused by MTX inhibition of thymidylate synthesis can be reversed by addition of either thymidine or N^5-formyltetrahydrofolate. A series of experiments (Figs. 6 and 7) were therefore carried out to determine the characteristics of

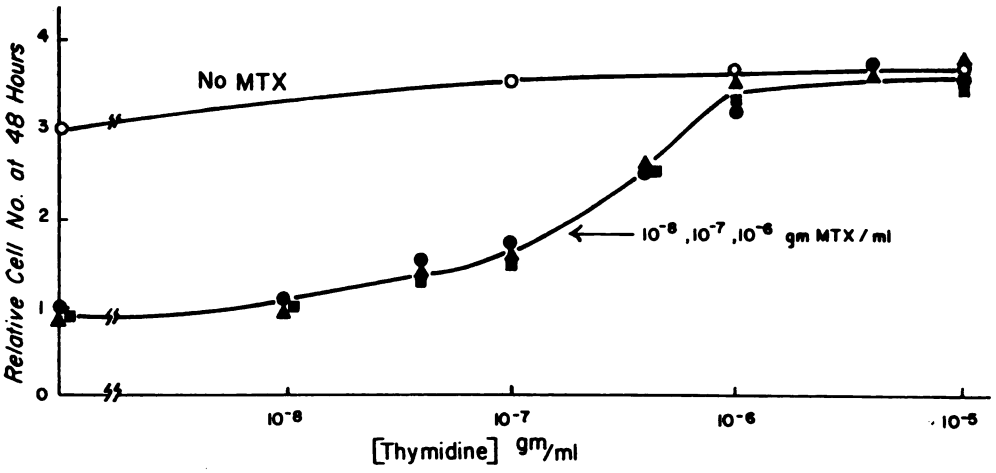

FIG. 6. *Reversal of inhibition by MTX of L-cell proliferation due to inhibition of thymidylate synthesis by addition of thymidine*

Exponentially growing suspension cultures of L-cells were treated with either 0, 10^{-8}, 10^{-7}, or 10^{-6} g of MTX per milliliter, together with 10^{-5} g of deoxyadenosine per milliliter. For each concentration of MTX a series of replicate cultures was treated with various concentrations of thymidine. Following 48 hr of incubation at 37°, the total number of cells per milliliter was determined for each culture and plotted as a multiple of the number initially present.

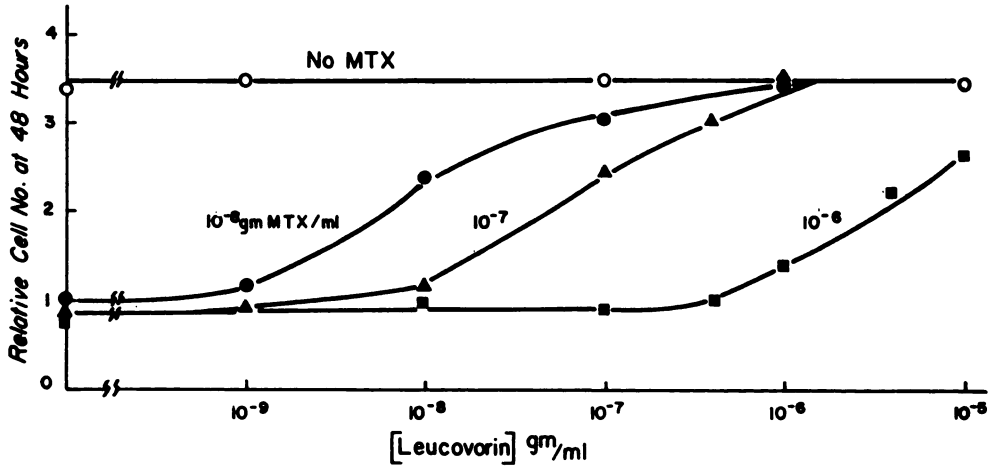

FIG. 7. *Reversal of inhibition by MTX of L-cell proliferation by N^5-formyltetrahydrofolate (leucovorin) in the presence of excess deoxyadenosine*

Exponentially growing suspension cultures of L-cells were treated with either 0, 10^{-8}, 10^{-7}, or 10^{-6} mg of MTX per milliliter, together with 10^{-5} g of deoxyadenosine per milliliter. For each concentration of MTX a series of replicate cultures was treated with various concentrations of N^5-formyltetrahydrofolate. Following 48 hr of incubation at 37°, the total number of cells per milliliter in each culture was determined and plotted as a multiple of the number initially present.

reversal of MTX inhibition by these two compounds. The results shown in Fig. 6 indicate that sufficient thymidine could completely overcome the MTX-induced inhibition of cellular proliferation regardless of MTX concentration, and that over the MTX concentration range employed, the inhibition of cellular proliferation resulted entirely from lack of thymidylate.

Figure 7 shows that N^5-formyltetra-

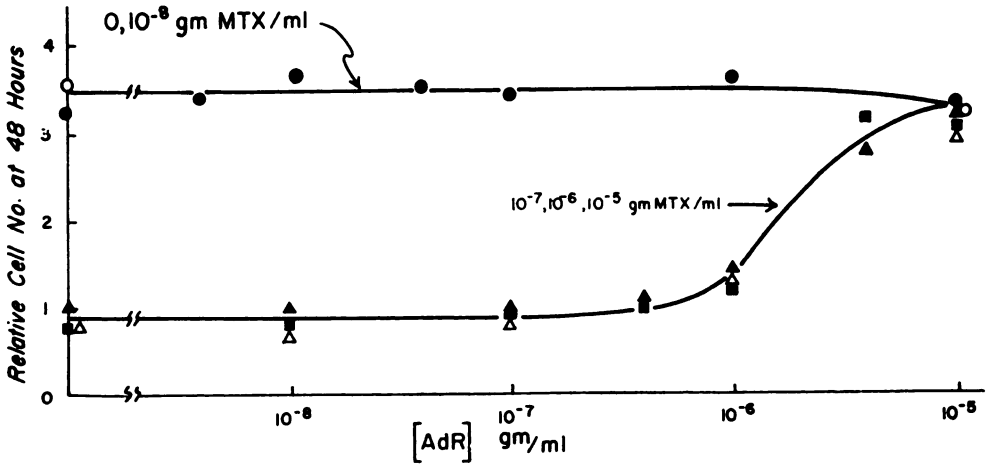

FIG. 8. *Reversal of inhibition by MTX of L-cell proliferation by addition of deoxyadenosine (AdR) in the presence of excess thymidine*

Exponentially growing suspension cultures of L-cells were treated with either 0, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} g of MTX per milliliter, together with 10^{-5} g of thymidine per milliliter. For each concentration of MTX a series of replicate cultures was treated with various concentrations of deoxyadenosine. Following 48 hr of incubation at 37°, the total number of cells per milliliter in each culture was determined and plotted as a multiple of the number initially present.

hydrofolate, as well as thymidine, can prevent the inhibitory action of MTX in these cultures and, furthermore, that the reversal of the effects of MTX is competitive. This competitive reversal indicates that MTX may have a site of direct action along the pathway leading to the synthesis of dTMP distal to the site of generation of tetrahydrofolate (i.e., distal to dihydrofolate reductase).

Reversal studies of MTX-induced inhibition of purine synthesis. To determine whether or not MTX was capable of interfering with the utilization of N^5-formyltetrahydrofolate in purine synthesis, a series of experiments was carried out to determine the characteristics of the reversal by both deoxyadenosine and N^5-formyltetrahydrofolate of the growth inhibition resulting from MTX inhibition of purine synthesis. In the presence of deoxyadenosine (Fig. 8), a 10^{-8} g/ml concentration of MTX failed to inhibit purine synthesis significantly as measured by this growth assay. The results for 10^{-7}, 10^{-6}, and 10^{-5} g/ml are virtually indistinguishable. This indicates that over the MTX concentration range employed, the inhibition of growth observed was due entirely to MTX-induced inhibition of the synthesis of purines. Furthermore, the noncompetitive nature of the reversal indicates that MTX does not interfere with the utilization of endogenous purines.

Figure 9 shows the results of an analogous experiment, in which N^5-formyltetrahydrofolate rather than deoxyadenosine was used to reverse the inhibition of cellular proliferation by MTX in the presence of 10^{-5} g of thymidine per milliliter. At all concentrations of MTX tested, the inhibition was overcome by N^5-formyltetrahydrofolate, and, contrary to the case of thymidylate synthesis, the reversal was *noncompetitive*. Under these conditions, therefore, MTX *cannot* interfere with utilization of N^5-formyltetrahydrofolate in purine-biosynthetic reactions. These data are consistent with the interpretation that growth inhibition under these conditions results from inhibition of dihydrofolate reductase.

Effect of deoxycytidine on MTX-induced inhibition of purine synthesis. The presence of deoxycytidine in the culture medium exerts a profound influence on the inhibition of purine synthesis by MTX. Figure 10 shows the results of an experiment in which various levels of MTX were added to 10-ml cultures of exponentially growing cells in growth medium supplemented either with 10^{-5} g of thymidine per milliliter, or with a 10^{-5} g/ml concentration each of thymidine and deoxycytidine. The presence of 10^{-5} g of deoxycytidine per milliliter sensitizes the purine-biosynthetic pathway to inhibition by MTX. At 10^{-8} g of MTX per milliliter, inhibition of growth is complete in the presence of 10^{-5} g of deoxycytidine per milliliter but is essentially unaffected in the absence of the deoxynucleoside.

Reversal of the MTX-induced inhibition of purine synthesis was also studied in the presence of 10^{-5} g each of deoxycytidine and thymidine per milliliter. When purines were used to effect the reversal, the results were identical with those obtained in the absence of deoxycytidine (Fig. 8). Thus, in spite of the change in sensitivity due to deoxycytidine, the inhibition of growth still resulted entirely from a lack of purines. This would rule out the possibility that deoxycytidine was blocking the conversion of thymidine to dTMP, thereby making the cells dependent on the conversion of dUMP to dTMP as a source of thymidylate. Figure 11 shows that N^5-formyltetrahydrofolate reversed the MTX-induced inhibition, in the presence of 10^{-5} g each of deoxycytidine and thymidine per milliliter, in a *competitive* manner. Hence the presence of 10^{-5} g of deoxycytidine per milliliter causes the purine-biosynthetic pathway to become more sensitive to inhibition by MTX, and the nature of the reversal effected by N^5-formyltetrahydrofolate becomes competitive. It would appear that in the absence of deoxycytidine the inhibition of purine synthesis is indirect, resulting from inhibition of folate and dihydrofolate reductase, while in the presence of 10^{-5} g of deoxycytidine per milliliter the inhibition occurs as a result of MTX action at a site where it can interfere with the utilization of the folate coenzymes.

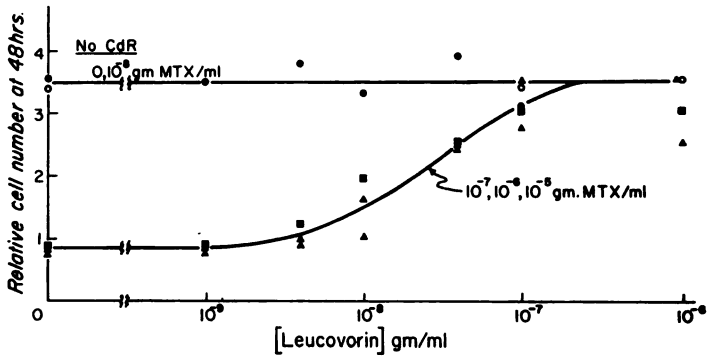

FIG. 9. *Reversal of inhibition by MTX of L-cell proliferation by N^5-formyltetrahydrofolate (leucovorin) in the presence of excess thymidine*

Exponentially growing cultures of L-cells were treated with either 0, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} g of MTX per milliliter, together with 10^{-5} g of thymidine per milliliter. For each concentration of MTX a series of replicate cultures was treated with various concentrations of N^5-formyltetrahydrofolate. Following 48 hr of incubation at 37°, the total number of cells per milliliter in each culture was determined and plotted as a multiple of the number initially present.

The concentration dependence of the deoxycytidine effect was determined (Fig. 12). Increasing concentrations of the deoxynucleoside resulted in sensitization of the pathway to MTX inhibition, with a plateau of maximum sensitization being reached at a deoxycytidine concentration of approximately 5×10^{-6} g/ml.

The specificity of the effect was also studied. Cytosine, cytidine, uridine, and deoxyuridine were tested for their ability to affect inhibition of cellular proliferation by 10^{-8} g of MTX per milliliter in the presence of excess thymidine. All these metabolites were ineffective at a concentration of 10^{-5} g/ml.

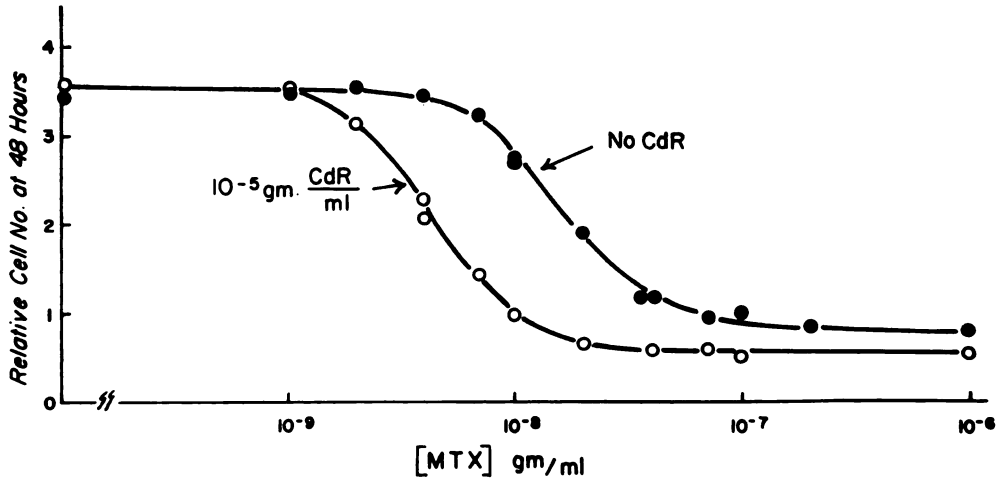

FIG. 10. *Effect of deoxycytidine (CdR) on MTX inhibition of L-cell proliferation in the presence of excess thymidine*

Exponentially growing cultures of L-cells were treated with a series of concentrations of MTX, together with either thymidine (10^{-5} g/ml) or thymidine plus deoxycytidine (10^{-5} g/ml each). Following 48 hr of incubation at 37°, the total number of cells per milliliter in each culture was determined and plotted as a multiple of the number initially present.

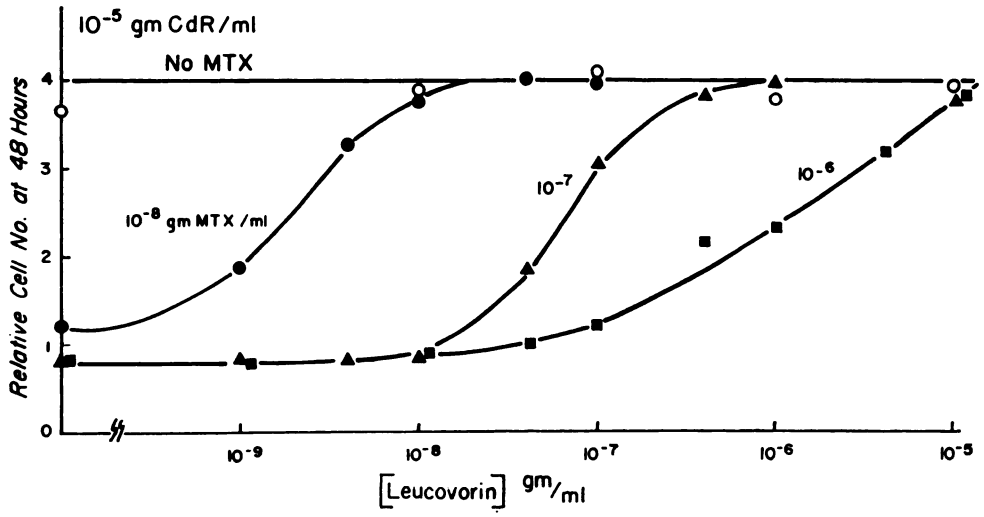

FIG. 11. *Reversal of MTX inhibition of L-cell proliferation by N^5-formyltetrahydrofolate (leucovorin) in the presence of excess thymidine and of 10^{-5} g of deoxycytidine (CdR) per milliliter*

Exponentially growing suspension cultures of L-cells were treated with either 0, 10^{-8}, 10^{-7}, or 10^{-6} g of MTX per milliliter, together with 10^{-5} g each of thymidine and deoxycytidine per milliliter. For each concentration of MTX a series of replicate cultures was treated with various concentrations of N^5-formyltetrahydrofolate. Following 48 hr of incubation at 37°, the total number of cells per milliliter in each culture was determined and plotted as a multiple of the number initially present.

Inhibition of thymidylate synthesis by MTX in the presence and absence of deoxycytidine was tested by the growth assay, and no effect of the deoxynucleoside at 10^{-5} g/ml could be detected.

Relation of reversal of MTX-induced inhibition of thymidylate and purine synthesis to colony-forming ability of MTX-treated cells. In the first paper of this series (8), data were presented (Fig. 3

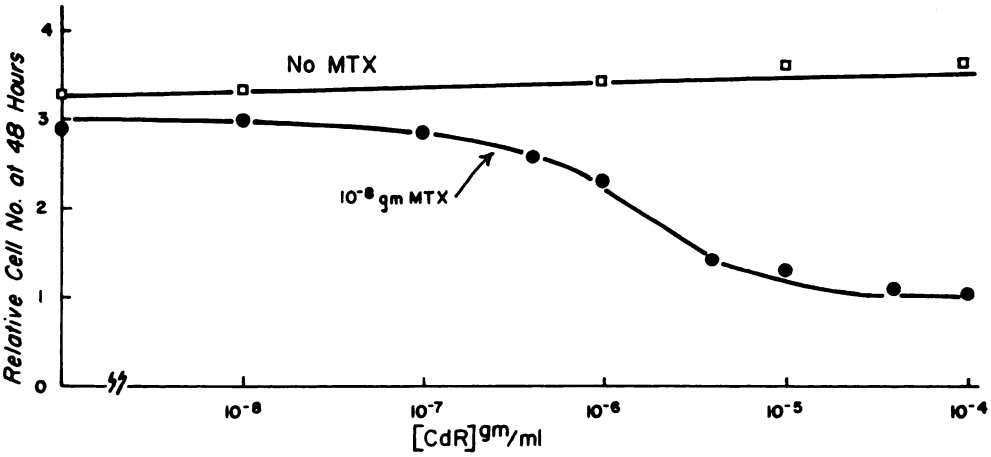

FIG. 12. *Dependence of cell proliferation on concentration of deoxycytidine (CdR) in the presence of 0 or 10^{-8} g of MTX per milliliter*

Exponentially growing suspension cultures of L-cells were treated with MTX as specified, together with, thymidine (10^{-5} g/ml) and various concentrations of deoxycytidine. Following 48 hr of incubation at 37° the total number of cells per milliliter in each culture was determined and plotted as a multiple of the number initially present.

in that paper) which indicated that, when cell cultures were exposed for 72 hr to 10^{-6} g of MTX per milliliter in the presence of various concentrations of N^5-formyltetrahydrofolate, there was a pronounced minimum in survival of colony-forming ability at an N^5-formyltetrahydrofolate concentration of 10^{-7} g/ml. Since it was also shown that the presence of purines increases cell killing in the absence of thymidylate, the presence of this minimum was interpreted as indicating that the inhibition of purine synthesis by MTX was reversed at a lower concentration of N^5-formyltetrahydrofolate than was the inhibition of thymidylate synthesis. This hypothesis was tested directly by comparing the reversal data in Figs. 7 and 9. Under the conditions used (no deoxycytidine present) inhibition of purine synthesis by 10^{-6} g of MTX per milliliter (Fig. 9) was released at a much lower concentration of N^5-formyltetrahydrofolate than was thymidylate synthesis (Fig. 7). At an N^5-formyltetrahydrofolate concentration of 10^{-7} g/ml, which corresponds to the observed minimum in cell survival (8), purine synthesis was essentially uninhibited whereas thymidylate synthesis was still essentially completely blocked. This observation is consistent with the hypothesis advanced to explain the observed minimum in survival.

DISCUSSION

Experimental data which have been interpreted as support for the one-site-of-action hypothesis include the following: (a) MTX prevents the conversion of folate to tetrahydrofolate derivatives *in vivo* (12); (b) the biological effects of MTX can be prevented or reversed by administration of tetrahydrofolate derivatives such as N^5-formyltetrahydrofolate (18), whereas folic acid is effective only if administered prior to MTX administration (19); (c) the reductase has been shown to be very effectively inhibited by MTX *in vitro* (13); (d) there appears to be a correlation between the drug dose per animal necessary to inhibit the reductase completely and that necessary to produce either toxic or antitumor effects (20); and (e) resistance in some cell lines is characterized by increased levels of the reductase (21).

Although this evidence seems formidable, there are certain observations not readily accounted for by the one-site-of-action hypothesis.

1. The biological effects of MTX, both *in vivo* (18) and in tissue culture (22, data presented in this paper) are reversed by tetrahydrofolate derivatives in a competitive manner. Since such derivatives are utilized beyond the reaction catalyzed by the reductase, they should reverse MTX effects noncompetitively if MTX inhibited only the reductase. Our data indicate that competition occurs at some point of action along the route to dTMP biosynthesis. Such competition is observed over very large variations in concentration of MTX and N^5-formyltetrahydrofolate, which would preclude the possibility that the competition was arising between MTX and N^5-formyltetrahydrofolate-derived dihydrofolate for dihydrofolate reductase.[5] Competition is also observed at some point along the purine-biosynthetic pathway *de novo,* but only in the presence of excess deoxycytidine.

2. The antileukemic effect of MTX in mice is reversed by dihydrofolate (23). Bertino *et al.* (24) have demonstrated that under certain conditions *in vitro* dihydrofolate and MTX interact competitively for the reductase. This observation could be extrapolated to the situation *in vivo* to account for the anti-MTX effect of

[5] This possibility could be positively ruled out by using as a reversing agent a 1-carbon derivative of tetrahydrofolate labeled in the C-1 moiety. If the competition referred to here arises between MTX and dihydrofolate derived from the reversing agent, no label should be incorporated from the C-1 group into cellular DNA. On the other hand, if our interpretation of the observed competition is correct, and if above some minimum concentration N^5-formyltetrahydrofolate (considerably lower than the maximum concentration used in our reversal studies) dTMP synthesis is independent of dihydrofolate reductase, label would be incorporated from the C-1 group of the reversing agent into cellular DNA.

dihydrofolate. However, the relevance of this observation is brought into question in the following paragraph.

3. In mice MTX does not appear to affect significantly the conversion of dihydrofolate to tetrahydrofolate derivatives, even at very high dosages (25), although folic acid reduction is blocked at low doses. Since dihydrofolate reduction to tetrahydrofolate is catalyzed by dihydrofolate reductase, this, along with the observations *in vitro* cited above (24), raises the question whether this enzyme is inhibited sufficiently *in vivo* to account for the biological effect of the drug.

4. Deoxycytidine introduces qualitative and quantitative changes into the inhibition of purine synthesis by MTX, but not into dTMP synthesis in cells in tissue culture.

5. There are marked specificities in inhibition of the various folate coenzyme-requiring reactions. *In vivo*, the synthesis of dTMP is very markedly inhibited relative to the synthesis of purines (26) or of serine (27), and the inhibition of C-2 incorporation into the purine skeleton is markedly greater than the inhibition of C-8 incorporation. If the inhibition resulted solely from folate coenzyme depletion, such marked specificity might not occur, since the various coenzyme forms are readily interconverted and the same coenzyme form is involved in the synthesis of both dTMP and serine.

On the basis of our data we propose that MTX has direct sites of action in addition to its inhibition of folate reductase. Thymidylate synthetase and, in the presence of excess deoxycytidine, the transformylase enzymes involved in purine synthesis *de novo* are probably two such sites.

From our data it is not possible to distinguish between methotrexate's inhibition of the folate coenzyme interconversion enzymes (tetrahydrofolate formylase, cyclohydrolase, 5,10-methylenetetrahydrofolate dehydrogenase, serine hydroxymethylase, etc.) and of the folate coenzyme-utilizing enzymes (thymidylate synthetase and the transformylase enzymes). We favor the latter as possible sites of action, however, because the existence of multiple pathways between N^5-formyltetrahydrofolate and the coenzyme form utilized for thymidylate synthesis (5) would require the simultaneous inhibition of all the alternative routes between N^5-formyl- and 5,10-methylenetetrahydrofolate.

Our argument for the multiple-sites-of action hypothesis relies heavily on the observed competition between MTX and N^5-formyltetrahydrofolate, which might arise at the level of cellular transport. Two considerations militate strongly against this possibility: (a) competition is observed when N^5-formyltetrahydrofolate is used to reverse the inhibition of dTMP synthesis (Fig. 7) but not of purine synthesis (Fig. 9); (b) in the accompanying paper (7) the incorporation of deoxyuridine-6-^{3}H into cellular DNA, used as an assay for thymidylate synthesis, is inhibited by MTX and the inhibition is reversed by addition of N^5-formyltetrahydrofolate, as is growth inhibition. Competition between MTX and N^5-formyltetrahydrofolate is observed whether the latter is added prior to or simultaneously with MTX, indicating that MTX can interfere with the utilization of existing reduced folates and that the observed competition is not at the transport level. It now remains to be established whether a multiple-sites-of-action hypothesis is in agreement with the observations used to support the single-site-of-action hypothesis and with the observations that are difficult to reconcile with this hypothesis.

The observed anti-MTX effect of dihydrofolate in mice (23) and the observation by Condit and Mead (25) that in mice the conversion of dihydrofolate to tetrahydrofolate derivatives appears to be unaffected by MTX pose no difficulty, since the biological effect of MTX need not arise from inhibition of dihydrofolate conversion to tetrahydrofolate if other sites of inhibition are present. Similarly, the marked differences observed in inhibition of the various folate coenzyme-requiring pathways (26–30) are readily explained as reflecting differing affinities between

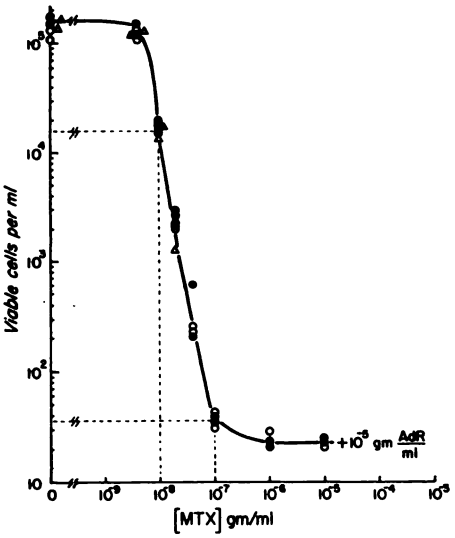

FIG. 13. *Cell killing as a function of MTX concentration*

Exponentially growing suspension cultures of L-cells in growth medium supplemented with 10^{-5} g of deoxyadenosine per milliliter were treated with the indicated concentrations of MTX. After 72 hr of incubation at 37°, the number of viable cells per milliliter was determined. The initial cell concentration was 1×10^5/ml. CdR, deoxycytidine.

MTX and the various enzymes involved.

One of the principal arguments for the single-site-of-action hypothesis is that antitumor or toxic activity seems to correlate with inhibition of folate reductase. As indicated below, the multiple-site theory would not rule out such a correlation even if the inhibition of thymidylate synthetase were a more sensitive site of action.

The indication that MTX inhibits enzymes beyond the level of tetrahydrofolate in addition to inhibition of the reductase suggests that *synergism* might occur between two sites of action (each only partially inhibited) to produce the observed biological effect. For example, the inhibition of folate reductase would serve to keep the intracellular level of reduced folates low so that the inhibition of enzymes distal to the reductase would be more effective. Dependence of cell killing on synergism is entirely consistent with the observed correlation between toxicity to tumor or animal and effect on folate reductase. Under conditions in which synergism was responsible for an observed effect, one would expect a correlation between the drug dose required to produce the effect and that required to inhibit the *least* sensitive of the sites involved in the synergism. Some evidence for this scheme of cell killing is provided by a comparison of cell killing and inhibition of the reductase and thymidylate synthetase enzymes as functions of MTX concentration. Figure 13 shows the results of some cell killing experiments, reported separately (8), in which cell survival after a 72-hr exposure to MTX was measured. Comparison of Fig. 13 with Fig. 5, which shows the sensitivity of the dTMP- and purine-biosynthetic pathways to inhibition by MTX, reveals that at an MTX concentration of 10^{-8} g/ml, thymidylate synthetase, as measured by the growth assay, is inhibited while the more resistant reductase is still essentially uninhibited. This results in less than a decade of cell kill. At 10^{-7} g of MTX per milliliter, a concentration which, by our interpretation of our data, results in inhibition of folate reductase as well as of thymidylate synthetase, cell killing reaches a maximum. We can conclude that maximum cell killing is correlated with inhibition of the reductase, in agreement with other authors. The findings that increased levels of reductase are sometimes found in MTX-resistant cell lines (21) is consistent with this model. Similarly, the addition of dihydrofolate effectively eliminates one of the two sites of action, and the remaining block, being competitively reversed by tetrahydrofolate derivatives, cannot sustain the biological effect (cell killing) alone.

From the results on growth inhibition, in which the effect appears to be dependent on only a single site of action (i.e., inhibition of either thymidylate synthetase, folate and dihydrofolate reductase, or glycinamide ribotide and/or 5-amino-4-imidazolecarboxamide ribotide transformylases), it must be concluded that growth inhibition by MTX is not dependent on the synergism postulated above. This can be readily understood if it is assumed that cell growth

requires more dTMP than is needed for prevention of cell killing, an assumption supported by comparisons of cell killing data (8) with the cell proliferation data of Fig. 6.

The effect of deoxycytidine on the sensitivity of purine biosynthesis to inhibition by MTX poses some interesting questions. A tentative hypothesis is that deoxycytidine serves as an allosteric activator of the transformylase enzyme(s) involved in purine biosynthesis. In its absence, the enzyme may be in a configuration which prevents MTX from binding to the active site, although the folate coenzyme can bind. In the presence of excess deoxycytidine, the enzyme molecule may undergo a steric transition which removes the hindrance to MTX binding, allowing it to compete for the binding site involved. This model indicates the possibility of a control mechanism which regulates the rate of purine synthesis, depending on the concentration of deoxycytidine within the cell.

The model presented here for the mode of action of MTX was deduced primarily from experiments in which the effect of MTX on growth was examined. Such experiments are readily performed in tissue culture; they can be efficiently used, under the proper conditions, to check for inhibitory effects of an agent like MTX on various metabolic pathways, and may even be useful in localizing the site of direct action of the antimetabolite. However, it was considered essential to obtain additional independent evidence for at least one of the proposed new sites of action of MTX. In the accompanying paper evidence is presented suggesting a direct action of MTX in the inhibition of thymidylate synthetase, both inside the living cell and in cell-free extracts. Consideration will also be given to the discrepancy between the present results and those published studies which indicate that folic acid antagonists fail to inhibit this enzyme.

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