Depression of Thymidylate Synthetase Activity in Response To Cytosine Arabinoside

DeWayne Roberts and Ellen V. Loehr

St. Jude Children's Research Hospital, Memphis, Tennessee 38101 [D. R., E. V. L.] and Department of Pharmacology, University of Tennessee Medical Units, Memphis, Tennessee 38101 [D. R.]

SUMMARY

Depression of thymidylate synthetase activity by cytosine arabinoside in CCRF-CEM cells was correlated with a blocking of precursor incorporation into RNA and with cell lysis. With increasing concentrations of cytosine arabinoside in the culture media, a proportional decrease of uridine incorporation into RNA and decrease in thymidylate synthetase activity occurred and was accompanied by lysis of the cells. These effects continued to develop with drug concentrations in excess of that required to block thymidine incorporation into DNA. The addition of deoxycytidine at concentrations that failed to reverse inhibition by cytosine arabinoside of thymidine incorporation into DNA reversed the drug effects on uridine incorporation, thymidylate synthetase activity, and cell lysis. Inhibition of precursor incorporation into RNA was also observed with isotopically labeled glycine and hypoxanthine. Cytidine could not be substituted for deoxycytidine to reverse drug effects on uridine incorporation and thymidylate synthetase. Deoxycytidine blocked intracellular accumulation of cytosine arabinoside, while cytidine was ineffective in blocking the drug uptake. We conclude that depression of thymidylate synthetase activity by cytosine arabinoside is a secondary drug action requiring drug concentrations in excess of that required to inhibit thymidine incorporation into DNA and is attributed to an inhibition of RNA synthesis that results from the action of the drug.

INTRODUCTION

Cytosine arabinoside, 1-β-D-arabinofuranosylcytosine, is an analog of both deoxycytidine and cytidine. The functional relationship to deoxycytidine has been emphasized by the inhibition of DNA synthesis (2, 6, 7, 9, 15, 17, 21–24, 32, 35) and the inhibition of DNA polymerase (14, 16, 25, 29) incorporation into DNA (6, 7, 16, 29, 35, 38), and because its cytotoxicity and oncolytic activity can be reversed by deoxycytidine (4, 5, 12, 15, 31). Cytolysis by cytosine arabinoside is dependent on conversion to a nucleotide in a reaction catalyzed by deoxycytidine kinase (6, 10, 19, 30, 36). The structural analogy with cytidine is expressed by incorporation into RNA (6, 7, 35).

Cytosine arabinoside induces remission of acute granulocytic leukemia (11). After the administration of cytosine arabinoside to patients, changes in the enzyme activity pattern of leukemic leukocytes occur (32). After drug administration, a decrease in thymidylate synthetase activity is observed as early as 1 hr and persists for 24 hr in some patients. A concentration-dependent decrease in thymidylate synthetase activity also follows the addition of cytosine arabinoside to CCRF-CEM cultures (33).

Methotrexate elevates thymidylate synthetase activity in leukocytes from patients with acute leukemia (32), in rat liver and transplantable tumors (27), and in cells of L1210 or CCRF-CEM cultures (32, 33). The simultaneous addition of cytosine arabinoside and methotrexate to CCRF-CEM cultures modulated the effect of each drug on thymidylate synthetase activity (33). Intermediate levels of thymidylate synthetase activity, between the level observed for each drug alone, developed in cultures receiving both methotrexate and cytosine arabinoside. The level of enzyme activity can be altered by changing the ratio of the 2 drugs. The methotrexate-induced elevation of thymidylate synthetase activity in leukocytes correlates with recovery of the capacity by these drug-intoxicated cells to incorporate deoxyuridine into DNA (32). By blocking the methotrexate-induced elevation of thymidylate synthetase activity with a second drug, such as cytosine arabinoside, we considered that the inhibition of DNA synthesis by methotrexate might be prolonged and the oncolytic action of the drug might be increased.

The CCRF-CEM lymphoblast line provides an experimental system in which the response of thymidylate synthetase to cytosine arabinoside and methotrexate resembles these drug-induced changes in patients' leukocytes (33). The present studies examine the molecular basis for cytosine arabinoside depression of thymidylate synthetase activity.

MATERIALS AND METHODS

The CCRF-CEM cell line with lymphoblastic morphology originated from the blood specimen of a child with lymphosarcoma that converted to acute lymphocytic leukemia (13). Cells were grown in suspension or spinner cultures as previously described (33). Drugs in aqueous solution were added in 0.4-ml aliquots to 40 ml of Eagle's minimal essential medium plus 10% fetal calf serum at the time of inoculation with approximately 2 X 10⁷ cells, and, unless otherwise
indicated, these suspension cultures were incubated for 20 hr at 37°C. At the end of this time, aliquots of the cultures were removed for measurement of thymidine-methyl-3H incorporation into DNA; uridine-5-3H, glycine-1-14C, and hypoxanthine-8-3H incorporation into nucleic acids or RNA; and thymidylate synthetase activity. The thymidylate synthetase activity in homogenates of the cells was assayed as previously described (33). Thymidine incorporation into DNA was assayed with 2 μM concentration, which saturated the rate-limiting reaction in these cells (33).

Uridine incorporation into RNA was measured by the same general technique as thymidine incorporation into DNA, except that 1.25 μCi of uridine-5-3H, 26.1 Ci/mMole, were added in 25-μl aliquots to 2-ml aliquots of the cultures. The trichloroacetic acid-insoluble radioactivity was collected on 0.45-μm MF Millipore cellulose ester filters and counted by liquid scintillation technique. This was a tracer level of uridine; and although incorporation was linear for more than 30 min, the rate-limiting step in its utilization was not saturated with substrate. In control cultures, less than 10% of the trichloroacetic acid-insoluble product remained insoluble after treatment with 1 N NaOH for 2 hr at 37°C.

Hypoxanthine incorporation into nucleic acid was assayed with 1 μM hypoxanthine-8-3H, 0.42 μCi, by the same technique as that used for uridine and thymidine. With cytosine arabinoside, total intracellular pool and incorporation into trichloroacetic acid-insoluble pool were measured. Two ml of cell suspension were incubated at 37° with 10 μM cytosine arabinoside-5-3H, 2.92 μCi. The radioactivity retained after the cell suspension was washed with 0.9% NaCl solution on a Millipore filter and after an additional trichloroacetic acid wash was assayed as the total intracellular pool and the acid-insoluble pool, respectively, by liquid scintillation counting. The acid-soluble pool was calculated from the difference between these 2 measured pools.

For the double labeling experiment, incorporation of glycine-1-14C and hypoxanthine-8-3H into RNA was assayed by a different technique. The cells were collected by centrifugation at 500 x g and washed with 0.9% NaCl solution and cold 5% trichloroacetic acid. The isotope incorporated into RNA was released into solution by suspension of the acid-insoluble precipitate in 1 N NaOH for 2 hr at 37°C. The DNA and protein were reprecipitated by addition of an equivalent volume of 1 N HCl to neutralize the NaOH at 0°C and then addition of trichloroacetic acid to give a final concentration of approximately 7%. After centrifugation at 500 x g for 5 min at 4°C, aliquots of the supernatant fluid were removed for liquid scintillation counting. Correction was made for 14C spillover into the 3H counts.

RESULTS

Time Course of Cytosine Arabinoside Inhibition of Thymidine Incorporation. Cytosine arabinoside inhibition of thymidine incorporation into DNA by CCRF-CEM cells is time and concentration-dependent (Chart 1). The time coordinate represents the period between addition of cytosine arabinoside, 0.01 to 10 μM, to freshly inoculated cultures of CCRF-CEM cells and the addition of labeled thymidine. For
the "zero" time point, approximately 15 min elapsed at room temperature between addition of drug and initiation of the 30-min incubation with tritiated thymidine. Incomplete inhibition of thymidine incorporation was observed with these zero-time studies. A 3-hr lag occurred after transfer of the cells before the control cultures began to increase their rate of thymidine incorporation. An earlier and more complete inhibition of thymidine incorporation was observed with higher concentrations of cytosine arabinoside. With 0.01 μM cytosine arabinoside, the capacity to incorporate thymidine into DNA was recovered; initiation of recovery was noted at 9 hr for cultures exposed to 0.03 μM cytosine arabinoside.

**Cytosine Arabinoside Lysis of Cells and Inhibition of DNA Synthesis.** Recovery of the capacity to incorporate thymidine into DNA and the effect on growth of cultures exposed to cytosine arabinoside, 0.01 to 10 μM, were examined in greater detail at 3 and 20 hr after drug addition to freshly inoculated cultures (Chart 2A). After 20 hr with 0.01 or 0.02 μM cytosine arabinoside, cultures incorporated thymidine at 69 and 46% of the rate observed for the control, although at 3 hr parallel cultures had incorporated 10 and 6%, as much substrate as the control, respectively. With 0.1 to 10 μM cytosine arabinoside, thymidine incorporation remained completely inhibited at 20 hr.

Cytosine arabinoside, 0.01 to 0.04 μM, did not completely inhibit growth of the culture (Chart 2B). At higher drug concentration, 0.1 to 10 μM, partial lysis of the inoculum occurred, and after 20 hr of exposure to 10 μM cytosine arabinoside the cultures contained only 20% of the cells added in the inocula. Cell lysis became more pronounced as the drug concentration was increased above the level required to inhibit thymidine incorporation into DNA. The latter was completely inhibited at 3 and 20 hr by 0.1 μM cytosine arabinoside.

**Correlation between Cell Lysis, Depression of Uridine Incorporation, and Decrease in Thymidylate Synthetase Activity after Cytosine Arabinoside.** Chart 3, A, B, C, and D, shows the effects of various concentrations of cytosine arabinoside on uridine incorporation into RNA, thymidylate synthetase activity, and the correlation between the effect of the drug on these 2 parameters and cell lysis. As the concentration of cytosine arabinoside was increased, uridine incorporation into RNA was more effectively inhibited; 75%
Inhibition occurred at 10 μM concentration (Chart 3A). At 0.1 μM cytosine arabinoside, an elevation of uridine incorporation into RNA occurred. This has been repeatedly observed.

Thymidylate synthetase activity was more effectively depressed at higher concentrations of cytosine arabinoside (Chart 3B). An elevation of thymidylate synthetase activity was not observed with 0.1 μM cytosine arabinoside, although in other studies a slight stimulation was observed with lower concentrations of this drug (28). Uridine incorporation and thymidylate synthetase activity increased together. As the concentration of cytosine arabinoside was lowered from 10 to 1 μM and then to between 1 and 0.4 μM, the incorporation of uridine was inhibited less than was thymidylate synthetase activity (Chart 3C). These changes in thymidylate synthetase activity and uridine incorporation into RNA occurred at drug concentrations in excess of that required completely to inhibit thymidine incorporation into DNA but correlated with cell lysis by the drug (Chart 3D).

**Table 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Uridine-5-3H (cpm/million cells)</th>
<th>Hypoxanthine-8-3H (cpm/million cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosine arabinoside inhibition of precursor incorporation into RNA</td>
<td>663</td>
<td>18</td>
</tr>
<tr>
<td>Drug-treated</td>
<td>3563</td>
<td>9689</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual product following alkaline hydrolysis</td>
<td>165</td>
<td>1772</td>
</tr>
</tbody>
</table>

**Chronological Sequence of Cytosine Arabinoside Effects.**

Inhibition of uridine incorporation into RNA was observed within 2 hr after the addition of 10 μM cytosine arabinoside to CCRF-CEM cultures (Chart 4A). Lysis of cells, when assayed by decrease in cell number, required slightly longer for expression and was observed between 4 and 8 hr after drug addition (Chart 4B). Inhibition of uridine incorporation into RNA began to level off 4 hr after drug addition. Maximum cell lysis occurred between 4 and 8 hr after drug addition and dropped only slightly thereafter. Cellular debris was observed in cultures as early as 2 hr after drug addition, although the decrease in cell number occurred later.

**Cytosine Arabinoside Inhibition of Precursor Incorporation into Nucleic Acid.** Drug inhibition of precursor incorporation into RNA was more dramatically shown with hypoxanthine than with uridine (Table 1). Cytosine arabinoside, 10 μM, inhibited 80% of the uridine incorporation and all incorporation of hypoxanthine into nucleic acids. Incubation for 2 hr at 37° in 1 N NaOH resulted in hydrolysis of 95% of the trichloroacetic acid-insoluble uridine products in the control cultures and 82% of the hypoxanthine products. From these data, the conclusion was drawn that 95 and 80% of the trichloroacetic acid-insoluble products of uridine and hypoxanthine, respectively, were incorporated into RNA by the control cultures. The trichloroacetic acid-insoluble products in the control cultures, which were not hydrolyzed by NaOH, presumably represent incorporation into DNA. As expected, cytosine arabinoside completely inhibited the incorporation of uridine and hypoxanthine into DNA. The continued, although reduced, incorporation of pyrimidine nucleotide precursor into RNA is attributed to an exchange of terminal cytidine nucleotides of tRNA. Resolution of cytoplasmic RNA by sucrose gradient showed that cytosine arabinoside more effectively inhibited uridine incorporation into rRNA than that into the slower sedimenting fractions.

**Concentration Curve for Hypoxanthine Incorporation into Nucleic Acid.** Cytosine arabinoside inhibition of precursor incorporation into RNA was more dramatically shown with hypoxanthine than with uridine (Table 1). Cytosine arabinoside, 10 μM, inhibited 80% of the uridine incorporation and all incorporation of hypoxanthine into nucleic acids. Incubation for 2 hr at 37° in 1 N NaOH resulted in hydrolysis of 95% of the trichloroacetic acid-insoluble uridine products in the control cultures and 82% of the hypoxanthine products. From these data, the conclusion was drawn that 95 and 80% of the trichloroacetic acid-insoluble products of uridine and hypoxanthine, respectively, were incorporated into RNA by the control cultures. The trichloroacetic acid-insoluble products in the control cultures, which were not hydrolyzed by NaOH, presumably represent incorporation into DNA. As expected, cytosine arabinoside completely inhibited the incorporation of uridine and hypoxanthine into DNA. The continued, although reduced, incorporation of pyrimidine nucleotide precursor into RNA is attributed to an exchange of terminal cytidine nucleotides of tRNA.
incorporation into RNA was also observed when hypoxanthine was substituted for uridine. An extracellular concentration of 6 µM hypoxanthine saturated the cellular capacity for incorporation into the nucleic acids (Chart 5). The rate-limiting reaction for incorporation has not been identified. Preincubation of cultures with cytosine arabinoside for 2.5 hr prior to addition of the hypoxanthine reduced incorporation into the nucleic acids, and saturation of the rate-limiting reaction was observed with 2 µM hypoxanthine. If the cytosine arabinoside inhibition of hypoxanthine incorporation were the result of a larger acid-soluble purine nucleotide pool, then higher, rather than lower, concentrations of hypoxanthine should have been required to equilibrate isotonically with the acid-soluble pool.

Development of Cytosine Arabinoside Inhibition of Hypoxanthine and Glycine Incorporation into DNA. In a double labeling study with glycine-1-14C and hypoxanthine-8-3H, CCRF-CEM cultures were preincubated for 0 to 5 hr with 0.01, 0.1, or 10 µM cytosine arabinoside prior to addition of the isotopically labeled precursors (Chart 6, A and B). The rate of glycine incorporation initially increased in the control cultures and then decreased to slightly below pretreatment rate. Pretreatment for 2 hr with cytosine arabinoside, 0.01 µM, reduced glycine incorporation to approximately 70% of the pretreatment rate where the rate remained almost constant for the remaining 3 hr. Preincubation with 0.1 and 10 µM cytosine arabinoside reduced glycine incorporation to approximately one-fourth the pretreatment rate.

In this same study, the rate of hypoxanthine incorporation by control cultures increased approximately 50% with 1 hr of preincubation before substrate addition and remained approximately constant throughout the remainder of the study. With 0.01, 0.1, and 10 µM cytosine arabinoside, the hypoxanthine incorporation rate after preincubation for 5 hr was approximately 10% of the initial rate. Glycine and hypoxanthine incorporation into RNA was inhibited by concentrations of cytosine arabinoside lower than those for the inhibition of uridine.

Deoxycytidine Reversal of Cytosine Arabinoside Action. The simultaneous addition of deoxycytidine at concentrations between 0.2 and 10 µM did not reverse the inhibitory effect of 10 µM cytosine arabinoside on thymidine incorporation (Chart 7A). In control cultures, which lacked cytosine arabinoside, high concentrations of deoxycytidine lowered the incorporation of exogenous thymidine into DNA. Although in the assay the initial extracellular concentration of thymidine was 2 µM, there is no assurance that deoxycytidine inhibition of thymidine incorporation may not be the result of the conversion of deoxycytidine to either deoxyuridine or dUMP. Deoxycytidine would compete with thymidine for conversion to nucleoside monophosphate by thymidine kinase. dUMP, if normally rate limiting in the de novo synthesis of TMP, could increase synthesis by the de novo pathway and dilute the exogenous precursor of DNA.

Deoxycytidine, 0.2 to 10 µM, produced very little effect on the growth rate of control cultures (Chart 7B). As shown earlier, a 20-hr exposure of the cultures to 10 µM cytosine arabinoside resulted in cell lysis. The addition of deoxycytidine reversed the lytic action of 10 µM cytosine arabinoside, and the reversal was proportional to the concentration of deoxycytidine. As would be expected in the absence of thymidine incorporation into DNA, no increase in cell number was observed following the addition of deoxycytidine to cultures containing cytosine arabinoside.
Chart 7. The observed metabolic changes in CCRF-CEM cultures after growth for 20 hr in the presence of 10 μM cytosine arabinoside (CA) and various concentrations of deoxycytidine (CdR) were: A, the absence of a reversal of the drug inhibition of thymidine incorporation into DNA; B, reversal of the drug-induced cell lysis; C, reversal of drug-depressed uridine incorporation into RNA; D, reversal of drug-depressed thymidylate synthetase activity; and E, relationship between deoxycytidine reversal of cytosine arabinoside depression of uridine incorporation and thymidylate synthetase activity.

Deoxycytidine caused a slight inhibition of uridine incorporation when the deoxycytidine concentration was 10 μM but produced no effect at lower concentrations (Chart 7C). Deoxycytidine reversed cytosine arabinoside inhibition of uridine incorporation into RNA. The reversal was biphasic with respect to deoxycytidine concentration. In the presence of cytosine arabinoside, a stimulation of the utilization of uridine was noted with the higher concentrations of deoxycytidine.

The addition of deoxycytidine to the control cultures produced no effect on thymidylate synthetase activity (Chart 7D). As the concentration of deoxycytidine was increased in the cultures to which cytosine arabinoside had been added, the level of thymidylate synthetase activity increased. At 10 μM deoxycytidine, an elevation of thymidylate synthetase activity was observed in the presence of cytosine arabinoside.

Deoxycytidine reversed cytosine arabinoside inhibition of uridine incorporation and depression of thymidylate synthetase activity.
Chart 8. The inability of cytidine to reverse cytosine arabinoside inhibition of uridine incorporation. The CCRF-CEM cells were incubated for 20 hr in medium containing 10 μM cytosine arabinoside (CA) and various concentrations of cytidine before the addition of uridine-5-3H.

synthetase activity (Chart 7E). At between 0.02 and 2 μM deoxycytidine, the reversal of cytosine arabinoside inhibition of uridine incorporation and the reduction of thymidylate synthetase activity occurred in parallel. However, at higher concentrations of deoxycytidine, 4 and 10 μM, which stimulated 1 or both parameters in the presence of cytosine arabinoside, a change in the relationship between these 2 parameters was observed. These effects by deoxycytidine on cytosine arabinoside-induced cell lysis, inhibition of uridine incorporation, and the depression of thymidylate synthetase activity occurred without reversal of the inhibition of thymidine incorporation into DNA.

Twenty hr elapsed between the addition of deoxycytidine to the cultures and the collection or removal of cells for study. This period of time could have permitted a conversion of deoxycytidine to ribosides, ribotides, or other products that possibly could have reversed the effect of cytosine arabinoside on RNA synthesis and in turn could have led to the responses attributed to deoxycytidine. Cytidine and its nucleotides would presumably be among the products of these reactions.

Cytidine Effect on Cytosine Arabinoside Action. The addition of 10 to 100 μM cytidine blocked uridine incorporation into RNA by control cultures (Chart 8). Presumably, this occurred as the result of cytidine deamination, although no information was obtained to support this assumption. With the combination of 10 μM cytosine arabinoside and cytidine, 10 to 100 μM, a more effective inhibition of uridine incorporation was observed than with cytosine arabinoside alone. With lower cytidine concentration, 0.4 to 4 μM, uridine incorporation into RNA was close to the rate observed for the control cultures. These cultures incorporated uridine at the same rate as cultures with only cytosine arabinoside alone. With lower cytidine concentration, 0.4 to 4 μM, uridine incorporation into RNA was close to the rate observed for the control cultures. These cultures incorporated uridine at the same rate as cultures with only cytosine arabinoside alone. Cytidine produced no effect on the level of thymidylate synthetase activity in cells from control cultures or from cytosine arabinoside-inhibited cultures.

Uptake of Cytosine Arabinoside. Although drug uptake was not saturated by 10 μM cytosine arabinoside-5-3H, uptake of the drug was linear for more than 3 hr. Radioactivity was linearly distributed with time into 2 pools. The trichloroacetic acid-insoluble pool comprised slightly less than 20% of the total radioactivity retained by cells that received only a 0.9% NaCl solution wash.

The simultaneous addition of 10 μM deoxycytidine with 10 μM cytosine arabinoside reduced the drug content retained by the washed cells to approximately 10% of the level observed with drug alone (Table 2). The simultaneous addition of 10 μM cytidine with cytosine arabinoside produced no effect on intracellular accumulation of radioactivity. A competition for transport between cytosine arabinoside and both cytidine and deoxycytidine was observed with a cytosine arabinoside-resistant subline of L1210 (20). Competition between cytidine and cytosine arabinoside was not observed in the present study. The 10 μM concentration of drug and of nucleosides, which was lower than the levels used in the earlier study, or differences in the relative rates of uptake and phosphorylation may be the basis for the variation observed with the 2 cell types.

Cytosine arabinoside was not detected in the acid-soluble labeled pool, which was resolved into 3 components by chromatography (39) with 6 volumes 0.5 N NH₄OH plus 10 volumes isobutyric acid on Whatman No. 1 paper. Deoxycytidine severely inhibited the accumulation of radioactivity into these 3 components. The present evidence suggests that deoxycytidine reversal of drug effects on CCRF-CEM cells resulted from a competition for
With higher concentrations of cytosine arabinoside, greater than 0.1 \( \mu M \), the depression of thymidylate synthetase activity became more pronounced as the drug concentration was increased. In addition, an increase in cell lysis and a decrease in precursor incorporation into RNA were observed. The depression of thymidylate synthetase activity, uridine incorporation into RNA, and increase in cell lysis were correlated. These correlations developed at concentrations of cytosine arabinoside that were in excess of that required to block thymidine incorporation.

Inhibition of precursor incorporation into RNA developed more slowly than inhibition of thymidine incorporation. This might be expected from the requirement for higher drug concentrations to inhibit the former and from the observation that drug uptake was linear for 3 hr with 10 \( \mu M \) drug. Incorporation of isotope from the drug into an acid-insoluble product was also linear for 3 hr and presumably represents cytosine arabinoside or its deaminated product, uracil arabinoside (8), since mammalian cells are apparently unable to cleave the glycoside linkage.

Inhibition of uridine incorporation into RNA was incomplete after 20 hr of preincubation with 10 \( \mu M \) cytosine arabinoside. The residual uridine incorporation was into low-molecular-weight RNA and may have represented terminal exchange on tRNA. Hypoxanthine incorporation was inhibited 95% by this drug concentration in 5 hr, while glycine incorporation was inhibited approximately 75%.

The correlation between the increased cell lysis and decreased uridine incorporation with elevation of drug concentration may have been the primary result of an altered cell membrane, but no information is available to substantiate this consideration. The possibility that depression of precursor incorporation resulted from increases in nucleotide pool size or from greater \emph{de novo} synthesis appears unlikely. The biphasic effect of cytosine arabinoside on uridine incorporation is compatible with the suggestion that inhibition of \emph{de novo} pyrimidine biosynthesis may have occurred at lower drug concentrations, an occurrence which would explain the observation of increased uridine incorporation at these drug concentrations. As the drug concentration was increased, this effect was masked by an inhibition of exogenous uridine utilization.

If an increase in \emph{de novo} purine nucleotide synthesis by cytosine arabinoside diluted the exogenous hypoxanthine, then an increased utilization of glycine should have been observed, rather than its inhibition. Increases in the size of the purine nucleotide pools should have been reflected by an increase in the hypoxanthine concentration required to saturate the rate-limiting step if this reaction were beyond the pool. If the pool were beyond the rate-limiting step, then similar saturation kinetics would have been expected. The observation that lower concentrations of hypoxanthine were required to saturate the cytosine arabinoside depressed incorporation is incompatible with these possibilities.

The observation that depression of uridine incorporation, thymidylate synthetase activity, and lysis of cells by cytosine arabinoside were prevented by deoxycytidine is compatible with the hypothesis of a 2nd drug action (7, 38) although Graham and Whitmore (16) suggest that some of these secondary drug effects should be attributed to inhibition of DNA polymerase. Deoxycytidine decreased the conversion of cytosine arabinoside to nucleotides, as expected (19, 30, 34). However, the decrease in nucleotide formation was inadequate, under the present conditions, to reverse the inhibition of thymidine incorporation. These results are compatible with the observations that cytidine could not block the effect of cytosine arabinoside on thymidylate synthetase activity or precursor incorporation into RNA. Deoxycytidine and cytosine arabinoside are converted to their respective nucleoside monophosphate form by deoxycytidine kinase. Cytidine is not a substrate for this enzyme.

The pharmacological action of cytosine arabinoside has been generally attributed to an inhibition of DNA synthesis. Dosage scheduling with repeated small dosage of drug at spaced intervals (26, 37) or prolonged infusions have been used to maintain intracellular drug concentrations for inhibition of all cells entering the S phase during the course of therapy (11). The very favorable therapeutic indices observed for the rodent systems with these schedules have probably been achieved to some degree because of certain known biological and biochemical variations between normal and neoplastic cells. The presumptions are: (a) stem cell pools of normal, nondividing, or "resting" cells are insensitive to the drug until "stimulated" to synthesize DNA, while the general neoplastic population may resume cell division soon after completion of mitosis; and (b) at low doses of cytosine arabinoside, the greater drug activation by sensitive, neoplastic cells permits attainment of inhibitory intracellular drug levels at lower extracellular drug concentrations (20, 21). The rapid blood clearance and inactivation of cytosine arabinoside (1, 9) requires repeated drug administration at short intervals to maintain optimal extracellular drug concentrations and to expose cells to drug as they enter the S phase. Two sites of cytosine arabinoside action have been postulated (7, 38). The relationship of inhibition of DNA synthesis and incorporation into DNA to these 2 sites of action has not been evaluated with respect to drug incorporation into RNA or drug interference with cytosine nucleotide function in intermediary metabolism. The rapid lysis of cells, which is associated with higher concentrations of cytosine arabinoside, indicates the
presence of a 2nd biochemical lesion in this lymphoblastic cell line of human origin. The only present correlation between the cell culture studies and the response of leukemic leukocytes following cytosine arabinoside on a twice-weekly schedule to patients is the observation that cytosine arabinoside depressed thymidylate synthetase activity in some patients and in cultures.

In conclusion, the addition of cytosine arabinoside to CCRF-CEM cultures altered a number of parameters in addition to DNA synthesis. The assumed 2nd locus of drug action on this cell line is less sensitive than DNA synthesis to drug inhibition. Whether this secondary effect of cytosine arabinoside can be attributed to drug incorporation into DNA and termination of chain elongation (38) or to incorporation into RNA (7) is not known. The effect of these higher concentrations of cytosine arabinoside results in a rapid cell lysis and is accompanied by changes in the synthesis of RNA and level of thymidylate synthetase activity, as well as the level of other enzymes (34). The present results are compatible with the interpretation that high concentrations of cytosine arabinoside interfere with RNA synthesis, an interference which in turn leads to a cessation of thymidylate synthetase synthesis.

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