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Studies on the Phosphorylation of Cytosine Arabinoside in Mammalian Cells

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SUMMARY

The effect of cytosine arabinoside 5′-triphosphate (araCTP) on the phosphorylation of deoxycytidine or cytosine arabinoside by deoxycytidine kinase and the phosphorylation of deoxyadenosine by deoxyadenosine kinase has been studied. AraCTP was a potent inhibitor of the phosphorylation of cytosine arabinoside and deoxyadenosine, but not of deoxycytidine. The inhibition produced by araCTP appeared to be competitive with respect to the phosphate donor, ATP, for both deoxycytidine kinase and deoxyadenosine kinase.

The phosphorylation of cytosine arabinoside was measured in sonic extracts of HeLa cells obtained from different phases of the cell cycle. The rate of phosphorylation of cytosine arabinoside appeared to increase significantly during the S phase of the cell cycle.

INTRODUCTION

Cytosine arabinoside (1-β-D-arabinofuranosylcytosine) is a very potent inhibitor of the reproduction of mammalian cells (1, 2). Studies with mammalian cells have suggested that the lethal effects produced by cytosine arabinoside may be due to the inhibition of DNA synthesis (1) and the limited incorporation of this analogue into nucleic acids (3–6). In order to be active as an inhibitor, cytosine arabinoside must first be phosphorylated (7) to its 5′-mono-, -di-, and -triphosphates; araCTP[1] is the predominant form of this anitmetabolite in mammalian cells (8, 9). Enzymatic studies with DNA polymerase partially purified from calf thymus have shown that araCTP is a potent competitive inhibitor with respect to dCTP of this enzyme (10) and that araCTP is also incorporated into the DNA template (11).

Kinetic data with mammalian deoxycytidine kinase suggest that this enzyme catalyzes the phosphorylation of both deoxycytidine and cytosine arabinoside (12, 13). The importance of the mechanisms involved in the phosphorylation of cytosine arabinoside were demonstrated with mutant leukemic cells resistant to the lethal effects of this drug. These mutant cells had a decreased capacity to phosphorylate cytosine arabinoside (7, 8, 14), presumably because of a deficiency in deoxycytidine kinase.

In this paper we have studied the effects of araCTP on the phosphorylation of cytosine arabinoside by deoxycytidine kinase in order to determine whether the feedback inhibition of this enzyme by araCTP is an important factor in preventing excessive accumulation of the phosphorylated derivatives of cytosine arabinoside in mam-

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[1] The abbreviation used is: araCTP, cytosine arabinoside 5′-triphosphate.

malian cells. Also, since the S phase of the cell cycle of mammalian cells is more sensitive to the lethal effects of cytosine arabinoside (15, 16), it was of interest to determine the capacity of cells in the different phases of the cell cycle to phosphorylate cytosine arabinoside.

EXPERIMENTAL PROCEDURE

Materials. The radioactive nucleosides were obtained from Schwarz/Mann and New England Nuclear Corporation. Other nucleosides and nucleotides were obtained from P-L Laboratories and Sigma Chemical Company. The radioactive nucleosides were purified by descending chromatography on Whatman No. 3MM paper for 48 hr in 86% 1-butanol–concentrated ammonium hydroxide (94.5:5.5). The purified nucleosides were eluted from the paper with 50% ethanol, evaporated to dryness, and immediately dissolved in water. Deoxycytidine kinase and deoxyadenosine kinase were purified about 100-fold from calf thymus as described previously (12, 17). It was not possible to separate these two enzyme activities completely by means of the purification procedures used. Deoxycytidine kinase (fraction VI) and deoxyadenosine kinase (fraction VIII) had specific activities of about 5 and 70 units/mg, respectively. AraCTP was synthesized enzymatically from cytosine arabinoside using deoxycytidine kinase (12), dCMP kinase (18), and nucleoside diphosphokinase (19). The araCTP formed was purified by column chromatography on DEAE-cellulose (Serva), using a linear gradient of triethylammonium bicarbonate, pH 8, desalted under vacuum, and dissolved in water.

Kinase assay. The enzyme assay measures the conversion of ^{3}H-nucleosides to ^{3}H-nucleotides by the binding of the latter compounds to DEAE-cellulose discs (20). The composition of the reaction mixture is given in the legend for each figure and table. Following the addition of enzyme, the mixture was incubated at 37° for the time indicated, and the reaction was terminated by heating at 100° for 1 min. The mixture was diluted with 5 ml of water and centrifuged at 1500 × *g* for 10 min to remove the denatured protein. The supernatant fluid was permitted to flow by gravity through 2.5-cm-diameter DEAE-cellulose discs that had been washed with 2 ml of 0.01 N HCl and 15 ml of water. The discs were then washed with 30 ml of water, dried, and placed in vials containing 10 ml of toluene-based scintillation fluid. The efficiency of counting of tritium under these conditions was about 3%. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the conversion of 1 nmole of nucleoside to nucleotide per minute under these assay conditions.

Synchronous cultures. HeLa cells were grown as monolayers at 37° in roller bottles containing Eagle's minimal essential medium supplemented with 5% fetal calf serum. Synchronous cultures were obtained by selective detachment of mitotic cells from the monolayer (21). Mitotic cells were harvested at 30-min intervals and accumulated at 0° for up to 3 hr. Upon rapid warming to 37°, the mitotic cell suspension proceeded through the cell cycle with unimpaired synchrony (22). Under these conditions the length of the cell cycle of the HeLa cells was about 16 hr. The rate of DNA synthesis during different phases of the cell cycle was determined by pulse-labeling 2-ml aliquots of synchronous culture (3 × 10^5 cells) with ^{3}H-deoxythymidine (15 mCi/μmole) at a concentration of 1 μCi/ml for 20 min. The cells were collected on a nitrocellulose filter (Millipore GSWP, 25 mm, 0.22 μ), washed with cold 0.15 M NaCl and cold 5% trichloracetic acid, dried, and assayed for radioactivity in toluene scintillation fluid.

For the kinase assay, aliquots of about 5 × 10^7 cells were obtained at intervals throughout the cell cycle by centrifugation at 2000 × *g* for 10 min. The cell pellet was washed twice with 0.12 M NaCl containing 0.02 M sodium phosphate buffer, pH 7.0. The washed cell pellet was resuspended in buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 20% glycerol, and 10 mM 2-mercaptoethanol. The ratio of buffer volume to packed cell volume was 3:1. The cells were disrupted by sonication and then centrifuged at 100,000 × *g* for 30 min, and the supernatant fraction was stored at −90° prior to kinase assay. All the steps in the preparation were carried out at about 5°.

The protein content in the supernatant fraction was determined by the method of Lowry *et al.* (23).

RESULTS

Studies with araCTP. The effect of different concentrations of araCTP on the phosphorylation of deoxycytidine and cytosine arabinoside by deoxycytidine kinase is shown in Table 1. Low concentrations of araCTP did not inhibit the phosphorylation of deoxycytidine, whereas a higher concentration of 40 μM produced only 6% inhibition. As reported previously with dCTP (12), the phosphorylation of cytosine arabinoside appeared to be more sensitive to the inhibitory effects of the end product, in this case araCTP. A concentration of 20 μM araCTP produced only 6% inhibition of the phosphorylation of deoxycytidine, in contrast to the 46% inhibition observed when cytosine arabinoside was used as the phosphate acceptor.

Kinetic studies were performed with araCTP, deoxycytidine kinase, and deoxyadenosine kinase in order to determine the type of inhibition produced by this antimetabolite on these enzymes. The effect of araCTP on the phosphorylation of different concentrations of cytosine arabinoside by deoxycytidine kinase is shown in Fig. 1. The data have been plotted according to the method of Lineweaver and Burk (24). The apparent K_m value for cytosine arabinoside was 30 μM, and is similar to the values reported earlier (12, 13, 25). The presence of araCTP appeared to produce a mixed type of inhibition, since the plotted lines did not intersect on the horizontal axis (26).

In Fig. 2 the effect of araCTP on the phosphorylation of cytosine arabinoside by deoxycytidine kinase in the presence of different concentrations of ATP was studied. The apparent K_m value for ATP was 0.15 mM, and is similar to the values reported previously (12–14). The inhibition produced by araCTP appeared to be competitive with respect to ATP, with an apparent K_i value of 2.2 μM. A similar type of inhibition was observed with dCTP (12, 13).

In Fig. 3 the effect of araCTP on the phosphorylation of deoxyadenosine in the presence of different concentrations of ATP

TABLE 1

Effect of different concentrations of araCTP on phosphorylation of deoxycytidine and cytosine arabinoside

The incubation mixture (0.1 ml) contained 10 μmoles of Tris-HCl (pH 8.0), 1.0 μmole of $MgCl_2$, 0.1 μmole of ATP, 0.5 μmole of 2-mercaptoethanol, 0.1 unit of deoxycytidine kinase (fraction VI), and 5 nmoles (4×10^4 cpm) of ^{3}H-deoxycytidine or ^{3}H-cytosine arabinoside, as indicated. The mixture was incubated for 10 min and assayed as described under EXPERIMENTAL PROCEDURE.

Addition	Concentration	Activity with various phosphate acceptors	
		Deoxycytidine	Cytosine arabinoside
	μM	%	%
None		100	100
AraCTP	2	100	85
AraCTP	10	100	70
AraCTP	20	94	54
AraCTP	40	94	44

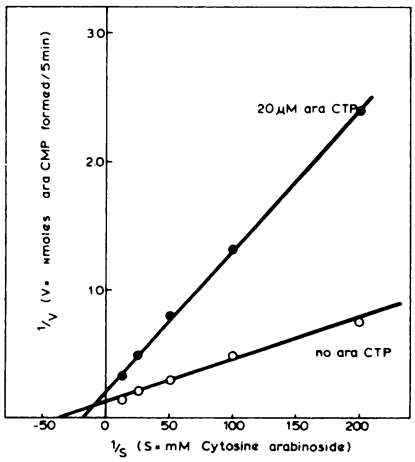

FIG. 1. *Effect of araCTP on phosphorylation of different concentrations of cytosine arabinoside*

The incubation mixture (0.1 ml) contained 10 μmoles of Tris-HCl (pH 8.0), 1.0 μmole of $MgCl_2$, 0.1 μmole of ATP, 0.5 μmole of 2-mercaptoethanol, 0.3 unit of deoxycytidine kinase (fraction VI), and the indicated concentrations of ^{3}H-cytosine arabinoside (5×10^4 cpm) and araCTP. The mixture was incubated at 37° for 5 min and assayed as described under EXPERIMENTAL PROCEDURE. AraCMP, cytosine arabinoside 5′-monophosphate.

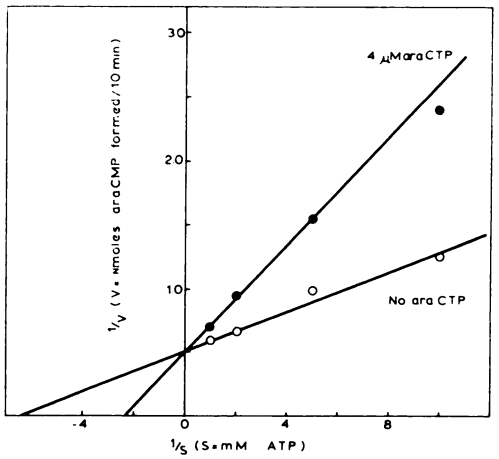

FIG. 2. *Effect of araCTP on phosphorylation of cytosine arabinoside in the presence of different concentrations of ATP*

The incubation mixture (0.1 ml) contained 10 μmoles of Tris-HCl (pH 8.0), 1.0 μmole of $MgCl_2$, 0.5 μmole of 2-mercaptoethanol, 10 nmoles of ^{3}H-cytosine arabinoside (5×10^4 cpm), 0.2 unit of deoxycytidine kinase (fraction VI), and the indicated concentrations of ATP and araCTP. The mixture was incubated at 37° for 10 min and assayed as described under EXPERIMENTAL PROCEDURE. AraCMP, cytosine arabinoside 5′-monophosphate.

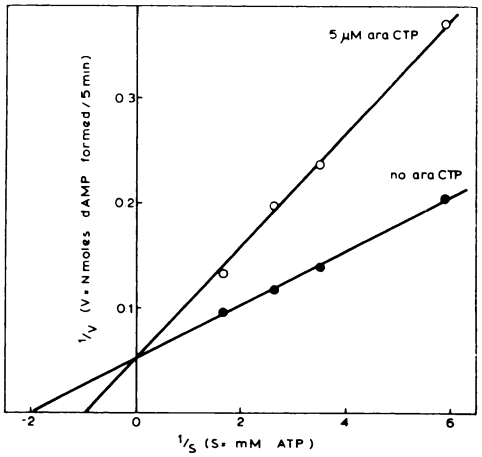

FIG. 3. *Effect of araCTP on phosphorylation of deoxyadenosine in the presence of different concentrations of ATP*

The incubation mixture (0.1 ml) contained 10 μmoles of Tris-HCl (pH 8.0), 1.0 μmole of $MgCl_2$, 1.0 μmole of 2-mercaptoethanol, 167 nmoles of ^{3}H-deoxyadenosine (3.3×10^4 cpm), 0.3 unit of deoxyadenosine kinase (fraction VIII), and the indicated concentrations of ATP and araCTP. The mixture was incubated at 37° for 5 min and assayed as described under EXPERIMENTAL PROCEDURE.

was studied. As observed with deoxycytidine kinase, the inhibition produced by araCTP appeared to be competitive with respect to ATP. The apparent K_m for ATP was 0.5 mM, and the apparent K_i for araCTP was 4.0 μM. A similar pattern of inhibition was reported previously for dCTP on deoxyadenosine kinase (17).

Studies with synchronous cells. The rate of phosphorylation of cytosine arabinoside during the cell cycle of HeLa cells is shown in Fig. 4. In this figure the different phases of the cell cycle occupy the following time intervals: the G_1 phase, from 0 to 7 hr; the S phase, from 7 to 15 hr; and the G_2 phase, from 15 to 17 hr. The capacity of cell extracts to catalyze the phosphorylation of cytosine arabinoside fluctuated in a manner similar to the incorporation of ^{3}H-deoxythymidine into the cellular DNA. The kinase activity was low in the G_1 phase, increased significantly in the S phase, and decreased in the G_2 phase. The peak of kinase activity occurred about 2 hr later than the maximal incorporation of ^{3}H-deoxythymidine into DNA. A similar pattern was reported by Brent (27) for the phosphorylation of deoxycytidine during the cell cycle of HeLa cells.

DISCUSSION

In general, most nucleoside antimetabolites must first be phosphorylated in order to be active as inhibitors in mammalian cells. In the case of cytosine arabinoside, evidence to support this statement has been obtained from studies on mutant leukemic cells resistant to this analogue and enzymatic studies with phosphorylated derivatives of cytosine arabinoside. Mutant leukemic cells resistant to cytosine arabinoside appeared to have an impaired capacity to phosphorylate this drug (7, 8, 14). Studies *in vitro* with mammalian DNA polymerase demonstrated that araCTP was a competitive inhibitor with respect to dCTP (10), and araCTP was also a substrate for this enzyme (11). These

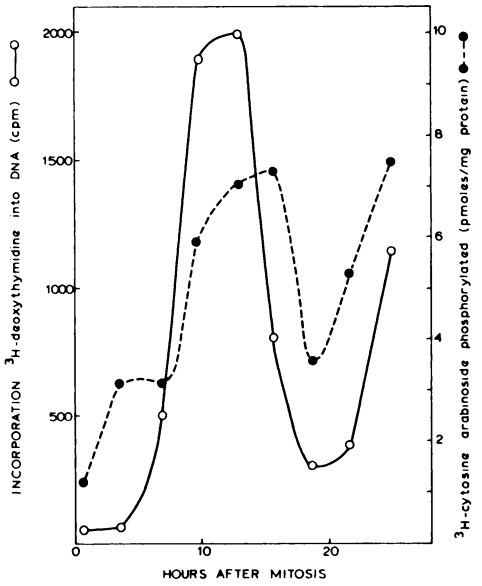

FIG. 4. *Phosphorylation of cytosine arabinoside during cell cycle of HeLa cells*

Mitotic cells were harvested from monolayers of HeLa cells and cultured in medium at 37°. Aliquots of 2 ml (3×10^5 cells) were removed at the times indicated and incubated with 2 μCi of ^{3}H-deoxythymidine (15 mCi/μmole) at 37° for 20 min. The radioactivity incorporated into DNA was determined as described under EXPERIMENTAL PROCEDURE. For the kinase assay, larger aliquots (5×10^7 cells) were collected, washed, and sonicated in Tris-KCl-glycerol buffer. The sonic extract was centrifuged, and 10 μl of the supernatant fluid were added to a reaction mixture (Table 1) containing 100 pmoles of ^{3}H-cytosine arabinoside (1×10^5 cpm) and incubated at 37° for 20 min.

observations, along with the cellular studies showing that cytosine arabinoside was incorporated into nucleic acids (3–6), strongly support the proposal that the active inhibitor of this antimetabolite is a phosphorylated compound, most probably ara-CTP.

Three major factors appear to influence the rate of phosphorylation of cytosine arabinoside in mammalian cells: (a) the amount of deoxycytidine kinase, the enzyme that catalyzes the phosphorylation of cytosine arabinoside (12, 13); (b) the presence of inhibitors of deoxycytidine kinase, such as dCTP (12, 13); and (c) the rate of deamination (28, 29) or glycosidic bond cleavage (28) of cytosine arabinoside.

Previous studies have shown that dCTP is a potent inhibitor of the phosphorylation of both deoxycytidine and cytosine arabinoside by deoxycytidine kinase (12, 13), the phosphorylation of the latter nucleoside being more sensitive to the inhibitory effects of dCTP (12). Similar results are reported in Table 1, which shows that araCTP was a more potent inhibitor of the phosphorylation of cytosine arabinoside than the phosphorylation of deoxycytidine. A concentration of 20 μM araCTP inhibited the phosphorylation of cytosine arabinoside and deoxycytidine by 46% and 6%, respectively. At equimolar concentrations the inhibition produced by dCTP (12) was much greater than the inhibition produced by araCTP. Schrecker and Urshel (8) reported that following the incubation of L1210 murine leukemic cells with 10 μM cytosine arabinoside for 20 min, a concentration of about 100 μM araCTP accumulated in these cells. Assuming that deoxycytidine kinase of the L1210 cells is just as sensitive to the inhibitory effects of araCTP as the calf thymus enzyme used in this study, an ara-CTP concentration in the cells of 100 μM should produce significant inhibition of the phosphorylation of cytosine arabinoside.

The mode of inhibition of deoxycytidine kinase by araCTP appears to be complex. The inhibition produced by araCTP appeared to be competitive with respect to ATP (Fig. 2) and mixed with respect to the phosphate acceptor, cytosine arabinoside (Fig. 1). These observations suggest that araCTP binds to both the phosphate donor and phosphate acceptor sites on deoxycytidine kinase, the binding to the phosphate acceptor site being much weaker than the binding to the phosphate donor site. One possible explanation for the observation that cytosine arabinoside is more sensitive to inhibitory effects of araCTP than deoxycytidine is that the binding of araCTP to the phosphate donor site may produce a slight configurational change in the enzyme at the phosphate acceptor site and thus affect the binding of the unnatural substrate, cytosine arabinoside, to this latter site more than the

binding of the natural substrate, deoxycytidine.

Krygier and Momparler (17) have reported that dCTP is a potent inhibitor of deoxyadenosine kinase, the inhibition produced by dCTP being competitive with respect to ATP and noncompetitive with respect to deoxyadenosine. In this paper the inhibition produced by araCTP appeared to be competitive with respect to ATP for deoxyadenosine kinase, and was similar to the results reported for dCTP. The biological significance of the inhibition of deoxyadenosine kinase by araCTP is not apparent at this time, since the only known function of this enzyme is in the salvage pathway (30). However, this fact may be important in combination chemotherapy in which analogues of deoxyadenosine are used in conjunction with cytosine arabinoside.

Young and Fischer (15) and Karon and Shirakawa (16) reported that the S phase of the cell cycle of mammalian cells is more sensitive than the other phases to the lethal effects of cytosine arabinoside. Since DNA synthesis is the major characteristic of the S phase, it is tempting to assume that the lethal effects produced by cytosine arabinoside implicate DNA replication. However, the data shown in Fig. 4 suggest that the rate of phosphorylation of cytosine arabinoside in the different phases of the cell cycle may be an important factor in determining which phase is most sensitive to the lethal effects of this antimetabolite. It appears from these results that there is a significant rise in the rate of phosphorylation of cytosine arabinoside during the S phase, probably because of an increase in the level of deoxycytidine kinase in this phase of the cell cycle. However, the other factors that affect the phosphorylation of cytosine arabinoside, as discussed above, should also be taken into consideration. Since RNA synthesis takes place during the S phase of the cell cycle (21), it is not possible to conclude from the data on the S phase specificity of cytosine arabinoside that only DNA synthesis is involved in the lethal effects produced by this analogue without considering the variations in its rate of phosphorylation during the cell cycle. The lethal effects produced by cytosine arabinoside on mammalian cells may be due to the accumulation of high intracellular concentrations of araCTP in the S phase of the cell cycle. The araCTP is incorporated into both RNA (3–6, 31) and DNA (3–6, 11) and inhibits DNA polymerase (10). More information is needed to determine the relative importance of these biochemical parameters with respect to the mechanism by which cytosine arabinoside produces its lethal effects on mammalian cells.

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