Effect of 1-β-D-Arabinofuranosylcytosine on DNA Synthesis

I. In Normal Rabbit Kidney Cell Cultures

ALBERT S. KAPLAN, MCKAY BROWN, AND TAMAR BEN-PORAT

Department of Microbiology, Research Laboratories, Albert Einstein Medical Center, Philadelphia, Pennsylvania 19141

(Received August 21, 1967)

SUMMARY

Low concentrations of 1-β-D-arabinofuranosylcytosine (Ara-C) inhibit the incorporation of cytidine and thymidine but stimulate the incorporation of deoxycytidine into the DNA of rabbit kidney cells. This stimulation is correlated with an increase in the level of activity of deoxycytidine kinase and with a consequent increase in the intracellular pool of phosphorylated deoxycytidine derived from the nucleoside in the medium.

Ara-C does not prevent the reduction of cytidine diphosphate to deoxycytidine diphosphate in rabbit kidney cells incubated in medium free of deoxycytidine. However, when small amounts of deoxycytidine are supplied to these cells, the drug is effective in inhibiting this reduction, probably as a result of an increase in the intracellular pool of deoxycytidine triphosphate and an ensuing negative feedback inhibition.

In agreement with observations made with other systems, in rabbit kidney cells the inhibition of DNA synthesis by the drug can be overcome by deoxycytidine. This reversal is due primarily to a successful competition of deoxycytidine with Ara-C at the level of phosphorylation, thereby preventing the accumulation of the effective inhibitor of DNA synthesis.

INTRODUCTION

Ara-C[1] inhibits DNA synthesis in a variety of systems, an inhibition that is reversed competitively by CdR (1–4). However, although there is general agreement that Ara-C is a specific inhibitor of DNA synthesis, the mechanism by which this inhibition occurs is not yet clearly established. Chu and Fischer (1) showed by experiments performed *in vivo* with L5178Y tumor cells that the drug inhibits the reduction of CDP to dCDP and concluded that the primary inhibitory effect of Ara-C on DNA synthesis is an interference with the formation of dCDP. This finding was not substantiated, however, when the effect of the phosphorylated derivatives of Ara-C on CDP reductase (from Novikoff and Ehrlich tumor cells) was tested *in vitro* (5).

The phosphorylated derivatives of Ara-C have been reported to be incorporated into DNA (2, 6, 7); this incorporation could be the basis for the inhibitory action of the drug on DNA synthesis. However, the incorporation of the drug into nucleic acid has not been proved unequivocally and has

[1] *Abbreviations:* Ara-C, 1-β-D-arabinofuranosylcytosine; Ara-CMP, Ara-C monophosphate; Ara-CTP, Ara-C triphosphate; CR, cytidine; CdR, deoxycytidine; CDP, cytidine diphosphate; dCDP, deoxycytidine diphosphate; dCMP, deoxycytidine monophosphate; dCTP, deoxycytidine triphosphate; TdR, thymidine; dTTP, thymidine triphosphate; RK, rabbit kidney; PCA, perchloric acid.

not been accepted universally (8). It also has been reported that Ara-CTP inhibits DNA polymerase derived from mammalian cells (8).[2]

Ara-C has been used with some success as an antiviral agent and has been reported to be effective in the control of herpes keratitis (9, 10). During the course of a study on the mode of the antiviral action of this drug, we made some observations which clarify some of the effects of Ara-C on the nucleic acid metabolism of normal RK cells. These experiments constitute the subject of the present paper.

MATERIALS AND METHODS

Cells and Media

Primary RK cells were prepared as described previously (11) and were grown in 90-mm petri dishes in Earle's saline containing 0.5% lactalbumin hydrolyzate and 5% bovine serum. The cultures were washed twice with Earle's saline prior to being used, and the experiments were performed in Eagle's synthetic medium (12) containing 3% dialyzed serum (EDS). The experiments were carried out with semiconfluent cultures containing approximately 2×10^6 cells each.

Enzyme Assays

The preparation of cell extracts has been described previously (13).

CdR kinase. Two-tenths milliliter of cell extract (approximately 1 μg of protein) was added to 0.15 ml of reaction mixture containing 2.5 μmoles of ATP, 25 μmoles of Tris, pH 7.5, 5 μmoles of $MgCl_2$, and 6 mμmoles of ^{3}H-CdR (specific activity, 0.21 μC/mμmole). The samples were incubated for 2 hr at 37°. The reaction was linear for at least 3 hr with all enzyme preparations used.

Dephosphorylation of dCMP. Two-tenths milliliter of cell extract (approximately 1 μg of protein) was added to 0.55 ml of reaction mixture containing 25 μmoles of Tris pH 7.5, 5 μmoles of $MgCl_2$, and 10 mμmoles of ^{3}H-dCMP (specific activity, 0.125 μC/mμmole). The samples were incubated for 30 min at 37°.

Both reactions were terminated by boiling for 2 min, the protein precipitate was removed by centrifugation, and dCMP was separated from CdR on Dowex 1-HCl (1×4 cm), as described previously (14).

Reduction of CDP to dCDP in vivo. The method described by Chu and Fischer (1) was used with some modifications. The cells were incubated with ^{3}H-CR for 3 hr, washed once with phosphate-buffered saline, and scraped into cold saline. (Five cultures were used per sample.) The samples were acidified and extracted twice with cold 0.2 N PCA. The cold, acid-soluble fraction was boiled for 10 min in 1 N PCA to convert the pyrimidine di- and triphosphate derivatives to monophosphates. Perchlorate was removed as the potassium salt; CMP and dCMP (0.5 mg each) were added to the samples as carrier, and the nucleotides were separated on Dowex 50, as described by Reichard (15). The radioactivity associated with each nucleotide was measured in a Packard spectrometer. The radioactivity associated with the peak of dCMP was found to consist of approximately 98% dCMP and 2% TdR, as determined after the separation of the 4 deoxyribonucleotides on Dowex 1-formate columns, as described previously (16).

Phosphorylation of ^{3}H-CdR in vivo. RK cultures were incubated with ^{3}H-CdR for 3 hr. (Two cultures were used per sample.) The cold acid-soluble fraction was prepared as described above and neutralized with KOH. CdR was separated from its phosphorylated derivatives on Dowex 1, as described previously (14). Ninety-five percent of the phosphorylated compounds consisted of phosphorylated CdR determined, as described previously (16), by their behavior on Dowex 1-formate columns.

Phosphorylation of ^{3}H-Ara-C in vivo. RK cells were incubated with ^{3}H-Ara-C for 3 hr. (Two cultures were used per sample.) The acid-soluble fraction was prepared as described above and neutralized with KOH.

[2] J. J. Furth and S. S. Cohen, personal communication.

^{3}H-Ara-C was separated from its phosphorylated derivatives on Dowex 1-HCl columns. Unlabeled Ara-C and Ara-CMP (0.5 mg each) were used as reference compounds. In all experiments, at least 90% of the phosphorylated compounds consisted of diphosphates or triphosphates; a maximum of 10% consisted of monophosphates. Since the level of activity of CdR deaminase is very low in the system used (see section above on the phosphorylation of ^{3}H-CdR *in vivo*), the assumption that the phosphorylated compounds consisted of phosphorylated Ara-C seems justified. No phosphorolysis of the nucleoside was detected, as determined by the method of Decker (17).

Incorporation of radiochemicals into DNA. After an appropriate incubation period with the radiochemical, the cells were harvested, precipitated with 0.2 N PCA, and the DNA and RNA present in the precipitate were separated (18). The amount of radioactivity associated with the DNA fraction was determined, after extensive washing with cold 0.2 N PCA, in a Packard spectrometer.

Chemicals. ^{3}H-CdR, ^{3}H-CR, ^{14}C-TdR, and ^{3}H-Ara-C were purchased from Schwarz BioResearch Inc. (radiopurity > 99%). Ara-C was a gift of Wayne Magee, The Upjohn Co.; Ara-CMP was a gift of William Wechter, The Upjohn Co.

RESULTS

Effect of Ara-C on the Incorporation of Tritiated Nucleosides into the DNA of RK Cells

Figure 1 shows the effect of various concentrations of Ara-C on the ability of RK cells to incorporate ^{3}H-TdR, ^{3}H-CR, and ^{3}H-CdR into DNA. While the incorporation of both ^{3}H-TdR and ^{3}H-CR was reduced equally by about 50% at a concentration of Ara-C of 0.05 μg/ml, the same concentration of the drug *increased* the incorporation of ^{3}H-CdR by about 50%. A lower inhibition by Ara-C of CdR than of TdR or CR incorporation was to be expected, since CdR is known to reverse partially the inhibitory effect of Ara-C on DNA synthesis.

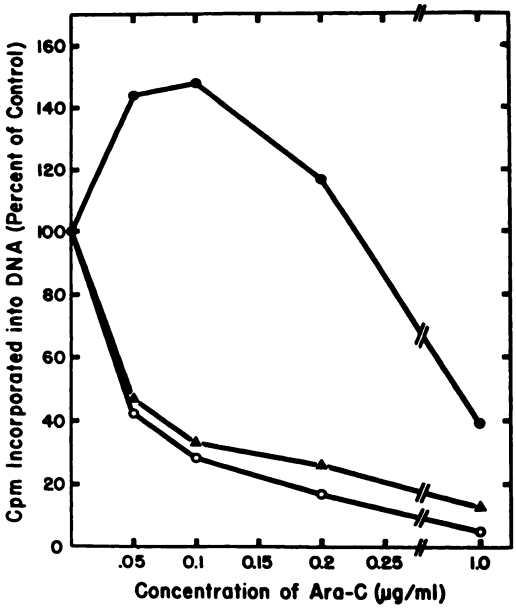

FIG. 1. *Incorporation of tritiated nucleosides into the DNA of RK cells treated with Ara-C*

Actively growing cells were washed twice with Earle's saline and incubated in EDS 1 hr prior to the start of the experiment. The cells were incubated with various concentrations of Ara-C for 3 hours, and the labeled nucleosides were than added: ^{3}H-CR (▲) (1 μC/ml; specific activity, 1.3 C/mmole), or ^{3}H-CdR (●) (1 μC/ml; specific activity, 625 μC/mmole), or ^{14}C-TdR (○) (0.04 μC/ml; specific activity, 31 μC/mmole). Three hours later the cultures were harvested and the amount of radioactivity present in the DNA was determined, as described in Materials and Methods.

Furthermore, if the effects of the drug on DNA synthesis were due to the inhibition of the reduction of CDP to dCDP, as postulated by Chu and Fischer (1), an increased incorporation of CdR into DNA would be obtained in Ara-C-treated cells, since the size of the endogenous pool of dCTP would be decreased. The specific activity of ^{3}H-CdR incorporated into DNA would consequently be higher. However, the increased incorporation of ^{3}H-CdR into DNA might be the result also of a stimulation in the presence of Ara-C of the phosphorylation of ^{3}H-CdR supplied externally. This possibility was tested in the following experiments.

Effect of Ara-C on the Phosphorylation of CdR in Vivo and on the Level of Activity of CdR Kinase

The amount of ^{3}H-CdR phosphorylated by cultures incubated with various concentrations of Ara-C is given in Fig. 2. It is clear that in the presence of Ara-C not only is there an increased amount of ^{3}H-CdR incorporated into DNA, but there is also a considerable stimulation of the phosphorylation of ^{3}H-CdR. These results were somewhat surprising, since Ara-C and CdR are probably phosphorylated by the same enzymes (6) and one would expect, therefore,

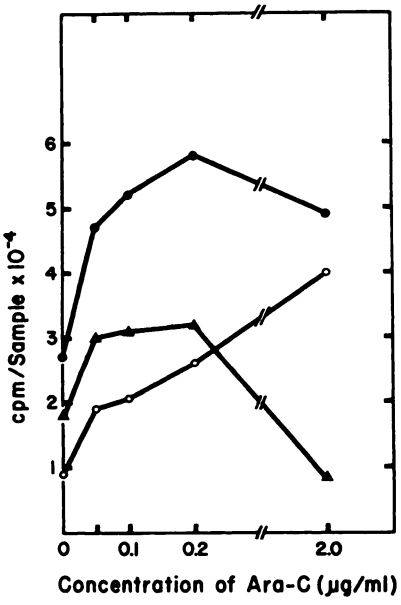

FIG. 2. *Phosphorylation of ^{3}H-CdR and its incorporation into DNA in RK cells treated with Ara-C*

The medium of actively growing cells was changed to EDS 1 hr prior to the start of the experiment. The cells were incubated in EDS containing various concentrations of Ara-C. Three hours later ^{3}H-CdR (5 µC/ml; specific activity, 625 µC/mmole) was added to the cultures which were incubated further for 3 hr. The cells were then harvested and the amount of phosphorylated ^{3}H-CdR present in the acid-soluble pool, as well as in DNA, was determined, as described in Materials and Methods. Total ^{3}H-CdR phosphorylated (^{3}H-CdR in pool and in DNA) (●); phosphorylated ^{3}H-CdR in the pool (○); ^{3}H-CdR incorporated into DNA (▲).

TABLE 1

CdR kinase activity in extracts of Ara-C-treated and untreated cells

Actively growing RK cells were incubated for 4 hr in EDS containing various concentrations of Ara-C. The cells were harvested and cell extracts were prepared. The phosphorylation of ^{3}H-CdR, as well as the dephosphorylation of ^{3}H-dCMP, by the cell extracts was tested as described in Materials and Methods. The results are the average of duplicate samples. The maximum variation between the individual samples was 10%.

Concentration of Ara-C (µg/ml)	^{3}H-CdR phosphorylated (µµmoles/sample)	^{3}H-dCMP dephosphorylated (µµmoles/sample)
0	1.14	1.04
0.1	1.60	—
1	1.84	—
10	2.08	0.98
Ara-C (10 µg/ml) + puromycin (20 µg/ml)	1.07	

a decrease rather than a stimulation of the rate of phosphorylation of CdR in the presence of Ara-C, unless Ara-C induces an increase in the level of activity of CdR kinase.

The activity of CdR kinase present in extracts of cells that had been incubated with various concentrations of Ara-C was determined. Since the activity of the enzyme was tested in crude extracts, the rate of dephosphorylation of dCMP was also determined in order to be sure that any increase observed in the level of phosphorylation of CdR by the extracts was not due to a decrease in the activity of phosphatases. Table 1 shows that exposure of the cultures to Ara-C increased the level of activity of CdR kinase and that this increase occurred only in the cultures in which protein synthesis was allowed to take place. Incubation of the cells with Ara-C had no effect, on the other hand, on the level of activity of dCMP kinase.

The effect of time of incubation of the cultures with Ara-C on the level of activity of CdR kinase, as well as on the amount of phosphorylated Ara-C present in the intra-

TABLE 2

Effect of time of incubation of cultures with Ara-C on the level of activity of CdR kinase and on the amount of Ara-C phosphorylated

Actively growing RK cells were incubated in EDS with unlabeled Ara-C (0.2 μg/ml) or ^{3}H-Ara-C (0.2 μg/ml; specific activity, 1 μC/0.2 μg). At various times thereafter, cultures were harvested and assayed for CdR kinase activity, as described in the legend to Table 1, and for the amount of ^{3}H-Ara-C phosphorylated, as described in Materials and Methods.

Time of incubation with Ara-C (hr)	CdR kinase activity (μμmoles phosphorylated/ sample)	Ara-C phosphorylated (μg/sample × 10^2)
0	0.59	—
2	0.82	1.82
4	0.96	3.08
6	1.15	3.79
8	1.11	4.07

cellular pool of the cultures, was tested. Table 2 shows that the activity of CdR kinase increased for the first 6 hr after the addition of Ara-C to the culture. Thereafter, there was no further increase in the activity of the enzyme and only a small increase in the amount of phosphorylated Ara-C present in the intracellular pool.

Since incubation of cells with Ara-C leads to an increase in the level of activity of CdR kinase, the increased incorporation of ^{3}H-CdR into the DNA of Ara-C-treated cultures is, therefore, not necessarily an indication of a decrease in the endogenous pool of dCTP in the cells; it may be the reflection only of an increase in the amount of phosphorylated derivatives arising from ^{3}H-CdR supplied externally.

Effect of Ara-C on the Reduction of CDP to dCDP in Vivo

In an experiment similar to the one designed by Chu and Fischer (1), the effect of Ara-C on the ability of RK cells to reduce CDP to dCDP *in vivo* was tested. The results of these experiments are summarized in Table 3. While the amount of phosphorylated CdR derived from CR present in the intracellular acid-soluble pool did not decrease in the Ara-C-treated RK cells, the amount of incorporation of the CdR derivatives into DNA was severely diminished. (Similar results were obtained by adding Ara-C and CR simultaneously instead of preincubating the cells with Ara-C.) Thus, it is clear that the inhibition of DNA synthesis by Ara-C cannot be due to the exhaustion of CdR derivatives from the system, since DNA synthesis was inhibited, even though these compounds were present in adequate amounts in the acid-soluble pool.

In the particular experiment summarized in Table 3, the total amount of CDP reduced (dCMP in the acid-soluble pool and dCMP incorporated into DNA) was diminished by incubation of the cells with Ara-C. This may be due to the lack of withdrawal of dCDP from the pool as a result of the inhibition of DNA synthesis in the drug-treated cells and to a consequent negative feedback exerted on the further reduction of CDP to dCDP. In some ex-

TABLE 3

Effect of Ara-C on the synthesis of ^{3}H-dCMP from ^{3}H-CR

Actively growing RK cultures were incubated for 3 hr in EDS containing various concentrations of Ara-C. ^{3}H-CR (5 μC/ml; specific activity, 15 C/mmole) was added, and the cells were incubated for 3 more hours at 37°. The cells were harvested and the amount of ^{3}H-CMP and ^{3}H-dCMP (and the di- and triphosphates) in the intracellular acid-soluble pool, as well as the amount of ^{3}H-dCMP incorporated into DNA, was determined as described in Materials and Methods. The results are the average of duplicate samples. The maximum variation between the individual samples was 16%.

Concentration of Ara-C (μg/ml)	^{3}H-CMP in acid-soluble pool	^{3}H-dCMP in acid-soluble pool	^{3}H-dCMP in DNA	Total ^{3}H-CDP reduced
0	74[a]	1.4	8.4	9.8
0.1	80	1.8	6.0	7.8
0.2	67	1.7	3.8	5.5
1.0	66	2.0	1.4	3.4
5.0	85	1.7	0.3	2.0

[a] Cpm/sample × 10^{-4}.

periments, however, we have observed that the size of the acid-soluble pool of ^{3}H-dCMP derived from ^{3}H-CR was increased considerably in the Ara-C treated cells. We have as yet been unable to determine why after exposure of the cells to Ara-C the size of this pool remained unchanged in some experiments while it increased in others.

The conflict between our findings and those of Chu and Fischer (1) could be attributed to differences in the cell lines used. However, although this possibility has not been excluded, it seemed rather unlikely and we preferred to look elsewhere for an explanation. One of the obvious differences between the two sets of experiments lay in the types of medium employed for cell cultivation: Our experiments were performed in a chemically defined medium containing a relatively low concentration (3%) of dialyzed serum and thus devoid of nucleosides; Chu and Fischer used a medium containing 10% *nondialyzed* horse serum, which presumably therefore contained nucleosides. Since Ara-C induces an increase in the level of activity of CdR kinase, as well as of TdR kinase (19), the size of the pool of dCTP and dTTP will be increased when the cells are incubated in the presence of the corresponding nucleosides and Ara-C, and an inhibition of the reduction of CDP to dCDP may result (20, 21).

TABLE 4
Effect of CdR on the reduction of CDP in Ara-C-treated cells

Actively growing RK cells were incubated for 3 hr either with Ara-C, CdR, or both. ^{3}H-CR (5 μC/ml; specific activity, 15 C/mmole) was then added, and the cells were incubated for 3 more hours at 37°. The cells were harvested and the amount of ^{3}H-dCMP (and its di- and triphosphates) in the acid-soluble pool, as well as the amount of ^{3}H-dCMP incorporated into DNA, was determined as described in Materials and Methods. The results are the average of duplicate samples; the maximum variation between the individual samples was 18%.

CdR (μg/ml)	Ara-C (μg/ml)	^{3}H-dCMP in acid-soluble pool (cpm/sample $\times 10^3$)	^{3}H-dCMP in DNA (cpm/sample $\times 10^3$)
0	0	9.1	60.5
0	1	9.9	6.2
0.05	0	8.2	43.8
0.05	1	3.5	4.9

We tested whether the inhibitory effect of Ara-C on the reduction of CMP to dCMP is affected by the presence of CdR in the medium and found that this was indeed the case (Table 4). Whereas Ara-C did not affect the amount of ^{3}H-dCMP derived from ^{3}H-CR that accumulated in the acid-soluble pool of cells incubated in medium without CdR, Ara-C caused a decrease in the amount of ^{3}H-dCMP produced in the cells incubated with CdR. Thus, although Ara-C has no direct effect on the reduction of CMP to dCMP in RK cells, it may, depending upon the experimental conditions, affect indirectly the rate at which this reaction takes place. Whether this holds true also for the L5178Y cells used by Chu and Fischer remains to be established.

Effect of CdR on the Ability of RK Cells to Phosphorylate Ara-C

It is well established that in a variety of systems the inhibitory effect of Ara-C on DNA synthesis is reversed competitively by CdR. This observation has been taken as an indication that the drug affects the endogenous synthesis of dCDP. However, CdR inhibits the phosphorylation of Ara-C (6), and the reversal by CdR of the inhibitory effect of Ara-C may be due primarily to its interference with the phosphorylation of the drug. Figure 3 shows that in RK cells CdR prevents the accumulation of phosphorylated derivatives of Ara-C in the acid-soluble pool and that the degree of inhibition of the phosphorylation of Ara-C by various concentrations of CdR is paralleled by the degree of reversal of the inhibition of DNA synthesis. Thus, CdR probably reverses the inhibition of DNA

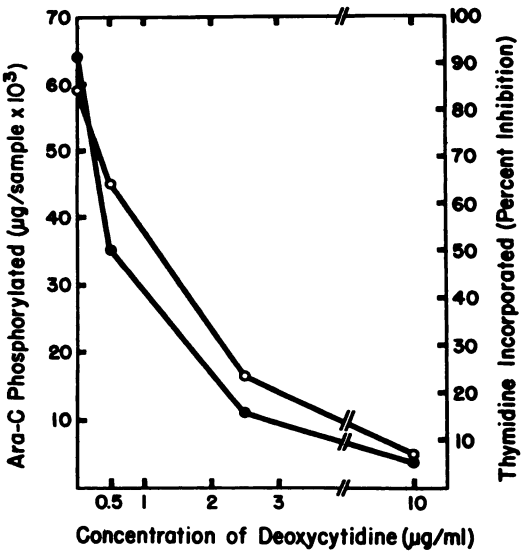

FIG. 3. *Inhibition by CdR of the phosphorylation of Ara-C*

The medium of actively growing RK cells was changed to EDS 1 hr prior to the start of the experiment. (A) Part of the cultures was incubated with ^{3}H-Ara-C (0.5 µg/ml; specific activity, 1 µC/0.5 µg) and various concentrations of CdR. Three hours thereafter, the cultures were harvested and the amount of phosphorylated ^{3}H-Ara-C present in the acid-soluble pool was determined, as described in Materials and Methods. (B) Part of the cultures was incubated with Ara-C (0.5 µg/ml) and various concentrations of CdR. Three hours thereafter ^{14}C-TdR (0.04 µC/ml; specific activity, 31 µC/mmole) was added. One hour later the cells were harvested and the amount of radioactivity in the DNA was determined. ^{3}H-Ara-C phosphorylated (●); ^{14}C-TdR incorporated (○).

synthesis by Ara-C primarily because it prevents the formation of phosphorylated Ara-C, the actual inhibitor of DNA synthesis.

DISCUSSION

The experiments presented in this paper demonstrate the following: (1) In RK cells treated with Ara-C the amount of CdR (supplied externally) which becomes phosphorylated and which accumulates in the intracellular acid soluble pool is increased. The increase in the rate of phosphorylation of CdR by Ara-C-treated cultures is correlated with an increase in the level of activity of CdR kinase in these cultures. (2) Although Ara-C has no effect on the rate of reduction of CDP to dCDP in RK cells incubated in medium free of CdR, this reaction is partially inhibited when the cultures are incubated with CdR *plus* Ara-C. (3) CdR reverses the inhibitory effect of Ara-C on DNA synthesis by preventing the phosphorylation of the drug.

Our experiments show that the inhibition of DNA synthesis in RK cells treated with Ara-C is not due to a lack of formation of dCTP resulting from an inhibition of the reduction of CDP to dCDP; these experiments confirm *in vivo* the findings reported by Moore and Cohen (5), who showed that Ara-CTP does not greatly affect the enzymes obtained from Novikoff and Ehrlich tumor cells catalyzing this reaction.

Ara-C will inhibit the reduction of CDP when the cells are incubated with low concentrations of CdR. This inhibition is probably related to the increased activity of CdR kinase in Ara-C-treated cells, the accumulation of dCTP in the acid-soluble pool, and a consequent negative feedback inhibition of the reduction of CDP to dCDP (20, 21). Since TdR kinase is also increased by incubation of the cells with Ara-C (19) and since dTTP is an inhibitor of the reduction of CDP to dCDP (20, 21), it seems reasonable to assume that low concentrations of TdR supplied externally will also tend to inhibit this reaction in the Ara-C-treated RK cells.

The increase in the level of CdR kinase, as well as the increase in the amount of CdR phosphorylated in cultures treated with Ara-C, cannot be ascribed to a partial synchronization of cell division by Ara-C (4) and to a resulting increase in the number of cells containing the enzyme in an active form, because the experiments were carried out during only a relatively small part of the cell growth cycle (which is about 30 hr, under the conditions used). Furthermore, the fact that the decrease in the activity of CDP reductase was affected by CdR to a greater extent in Ara-C treated cells than in untreated cells would also

indicate that the level of activity of CdR kinase in these cells was increased.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Institutes of Health (AI-03362) and from the National Science Foundation (GB-4995), and by a U.S. Public Health Research Career Program Award (5-K3-AI-19,335) from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.* **11,** 423 (1962).
2. S. Silagi, *Cancer Res.* **25,** 1446 (1965).
3. A. Doering, J. Keller and S. S. Cohen, *Cancer Res.* **26,** 2444 (1966).
4. J. K. Kim and M. L. Eidinoff, *Cancer Res.* **25,** 698 (1965).
5. E. C. Moore and S. S. Cohen, *J. Biol. Chem.* **242,** 2116 (1967).
6. M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.* **14,** 333 (1965).
7. W. A. Creasey, R. J. Papâc, M. E. Markiw, P. Calabresi and A. Welch, *Biochem. Pharmacol.* **15,** 1417 (1966).
8. S. S. Cohen, *Progr. Nucleic Acid Res.* **5,** 1 (1966).
9. H. E. Kaufman and E. D. Maloney, *Arch. Ophthalmol.* **69,** 626 (1963).
10. G. E. Underwood, C. A. Wisner and S. D. Weed, *Arch. Ophthalmol.* **72,** 505 (1964).
11. A. S. Kaplan, *Virology* **4,** 435 (1957).
12. H. Eagle, *Science* **130,** 432 (1959).
13. T. Kamiya, T. Ben-Porat and A. S. Kaplan, *Virology* **26,** 577 (1965).
14. C. Hamada, T. Kamiya and A. S. Kaplan, *Virology* **28,** 271 (1966).
15. P. Reichard, *J. Biol. Chem.* **236,** 1150 (1961).
16. T. Ben-Porat and A. S. Kaplan, *Virology* **16,** 261 (1962).
17. C. A. Decker, *J. Am. Chem. Soc.* **87,** 4027 (1965).
18. G. Schmidt and S. J. Tannhauser, *J. Biol. Chem.* **161,** 83 (1945).
19. S. Kit, A. de Torres and D. R. Dubbs, *Cancer Res.* **26,** 1859 (1966).
20. A. Holmgren, P. Reichard and L. Thelander, *Proc. Natl. Acad. Sci. U.S.* **59,** 830 (1965).
21. E. C. Moore and R. B. Hurlbert, *J. Biol. Chem.* **241,** 4802 (1966).