Biochemical Pharmacology, Vol. 17, pp. 741–751. Pergamon Press. 1968. Printed in Great Britain

EFFECTS OF CYTOSINE ARABINOSIDE ON THE CELL VIABILITY AND UPTAKE OF DEOXYPYRIMIDINE NUCLEOSIDES IN L5178Y CELLS*

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(*Received* 31 *August* 1967; *accepted* 16 *October* 1967)

Abstract—Cytosine arabinoside inhibited the incorporation of ^{3}H-deoxythymidine into DNA. L5178Y leukemic cells prelabeled with ^{3}H-deoxythymidine and treated with high levels of cytosine arabinoside were also shown to lose radioactivity from the DNA at a logarithmic rate.

Pretreatment of cells with high levels of cytosine arabinoside markedly increased the incorporation of ^{3}H-deoxycytidine into the cold acid-soluble and DNA fractions, but the treated cells could not be rescued by deoxycytidine.

Cells pretreated with methotrexate incorporated less ^{3}H-cytosine arabinoside into the DNA and RNA and were sensitized to the acute killing effect of high levels of cytosine arabinoside. Short periods of pretreatment with methotrexate killed leukemic cells, although the cloning medium contained hypoxanthine, deoxythymidine and serine.

1-β-D-ARABINOFURANOSYL cytosine (cytosine arabinoside) has been demonstrated to be an inhibitor of several strains of *Escherichia coli*,[1, 2] mammalian cells in culture,[3, 4] DNA viruses,[5, 6] and a variety of transplantable tumors in mice and in rats.[7] Cytosine arabinoside inhibited the synthesis of SV_{40} antigen and infectious virus,[8] the regeneration of bone marrow in mice,[9] and the primary immune response.[10] In humans, cytosine arabinoside has been studied for the treatment of neoplasms[11] and for the treatment of virus infections of the eye.[12, 13]

It has been proposed that cytosine arabinoside inhibits the formation of deoxycytidine diphosphate from cytidine diphosphate.[3, 14, 15] From the studies of mutants resistant to cytosine arabinoside, it was concluded that the enzyme which phosphorylates cytosine arabinoside is deoxycytidine kinase.[16, 17]

It has been suggested that the incorporation of ^{3}H-cytosine arabinoside into the DNA or RNA fractions of mammalian cells[16, 18–20] might result in cell death. The present report concerns the effects of cytosine arabinoside on the incorporation of ^{3}H-deoxycytidine and ^{3}H-deoxythymidine by murine leukemic cells. The modification of the incorporation of cytosine arabinoside by methetrexate has also been investigated.

MATERIALS AND METHODS

The methods of propagation of murine leukemic cells (L5178Y) have been described.[21] Cells used in all experiments were in the exponential phase of growth and

* This work was supported by Grants T-17 and T-23 from the American Cancer Society.

were maintained at 37° in growth medium during the experiment. 1-β-D-Arabinofuranosyl cytosine hydrochloride was obtained from Dr. J. H. Hunter of the Research Laboratories of the Upjohn Company. ^{3}H-cytosine arabinoside, labeled in the 5 and 6 positions of the pyrimidine ring, purified by chromatography as described previously,[20] and ^{3}H-deoxythymidine, methyl labeled, were purchased from Schwarz Biochemicals. ^{3}H-Deoxycytidine, labeled in the pyrimidine ring and in the sugar moiety, was purchased from Tracer Labs. Deoxycytidine hydrochloride and deoxythymidine were purchased from Schwarz Biochemicals; hypoxanthine was purchased from Nutritional Biochemicals Corp.; and methotrexate was purified according to a method previously described.[22]

I. *Cells prelabeled with ^{3}H-deoxythymidine*

L5178Y cells (30 ml, $1{\cdot}7 \times 10^5$/ml) were grown in medium containing ^{3}H-deoxythymidine (2530 μc/μmole, $1{\cdot}31 \times 10^{-7}$ M) for 20 hr. After incubation, the cells were centrifuged, washed twice with growth medium at 37°, and diluted in 90 ml of growth medium to obtain $2{\cdot}2 \times 10^5$ cells per ml. A portion of the cells (45 ml) was incubated for 6 hr with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M). The treated and control cells were harvested at 37° by centrifugation and resuspended in 10·0 ml of growth medium free of cytosine arabinoside. This washing procedure was repeated twice and the cells were diluted in growth medium. At intervals of 0, 6, 18, 43 and 66 hr, a portion of each cell incubation was transferred to a Hopkin's tube containing a column of 0·05 ml cold 0·25 M sucrose solution. The Hopkin's tubes were centrifuged at approximately 200 *g* for 5 min, the medium was removed, the tubes were rinsed twice with cold medium, the column of sucrose was aspirated, and the remaining fluid was removed with absorbent paper. The packed cells were extracted four times with cold 0·2 N $HClO_4$ and the RNA was hydrolyzed and removed by three successive extractions with 0·2 N NaOH for 20 min at 80°. The DNA readioactivity was recovered by extraction at 80° for 20 min with 1 N $HClO_4$. This extraction was repeated twice. The content of RNA and DNA was determined by the absorbancy at 260 mμ and 280 mμ in the extracts. This method was found to be in close agreement with the orcinol and diphenylamine methods for the determination of RNA and DNA.[23]

Radioactivity was determined in a liquid scintillation counter with an efficiency of 16–20 per cent. Quenching by the radioactive samples was not detected.

II. *^{3}H-Deoxythymidine incorporation during 6 hr of exposure to cytosine arabinoside*

Logarithmically growing cells (20·0 ml, $3{\cdot}7 \times 10^5$/ml) were distributed into 4 culture tubes. Cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) and deoxycytidine (5×10^{-5} M) were added as shown in Table 1. ^{3}H-Deoxythymidine (2530 μc/μmole, $1{\cdot}3 \times 10^{-7}$ M) was added and the cultures were incubated for 6 hr. At the end of the incubation a portion from each culture was transferred to a Hopkin's tube containing a column of sucrose solution and the cold acid-soluble, RNA and DNA fractions prepared as described in Methods, section I.

III. *^{3}H-Deoxythymidine incorporation after exposure to cytosine arabinoside*

L5178Y cells (10 ml, approximately $3{\cdot}0 \times 10^5$ cells/ml) were exposed to cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) for 6 hr; the cells were centrifuged and resuspended in

TABLE 1. ^{3}H-DEOXYTHYMIDINE INCORPORATION DURING EXPOSURE TO CYTOSINE ARABINOSIDE*

Cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M)	Deoxycytidine (5×10^{-5} M)	DNA (cpm/cell $\times 10^3$)
+	—	8·2
+	+	10·0
—	—	14·9
—	+	14·1

* L5178Y cells (10 ml, $3{\cdot}7 \times 10^5$/ml) in the exponential phase of growth were incubated for 6 hr with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M), ^{3}H-deoxythymidine ($1{\cdot}3 \times 10^{-7}$ M, 2530 μc/μmole) and deoxycytidine (5×10^{-5} M) as indicated.

5·0 ml of growth medium free of cytosine arabinoside. This washing procedure was repeated twice. Control cells were prepared in an identical manner except that cytosine arabinoside was omitted. Treated and control cells were diluted to 1×10^5 cells per ml in growth medium. ^{3}H-Deoxythymidine (2530 μc/μmole, $2{\cdot}0 \times 10^{-8}$ M) and deoxycytidine (5×10^{-5} M) were added as shown in Table 2. After 19 hr of incubation, a portion from each culture was transferred to a Hopkin's tube containing a column of sucrose and the cold acid-soluble RNA and DNA fractions prepared as described in Methods, section I.

IV. *Sensitization of cells to cytosine arabinoside by methotrexate*

Methotrexate ($1{\cdot}6 \times 10^{-7}$ M), hypoxanthine (2×10^{-5} M) and serine ($1{\cdot}4 \times 10^{-3}$ M) were added to a 50-ml culture (2×10^4 cells/ml). After 4 hr of incubation, an aliquot (25 ml) was centrifuged, washed, and resuspended in 10 ml of growth medium to remove methotrexate. This washing procedure was repeated twice. The washed cells were divided into two portions; one was incubated with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) and deoxythymidine (2×10^{-6} M), and the other with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) alone. The remaining aliquot (25 ml) containing methotrexate was divided into two portions; one was incubated with cytosine arabinoside alone, and the other with cytosine arabinoside plus deoxythymidine as shown in Table 7. The cells were collected by centrifugation and resuspended in 10 ml of growth medium. This washing procedure was repeated twice and the cell viability was determined by the dilute agar colony method[20] in cloning medium containing hypoxanthine (2×10^{-5} M), deoxythymidine ($2{\cdot}0 \times 10^{-6}$ M), serine ($1{\cdot}4 \times 10^{-3}$ M) and deoxycytidine (5×10^{-5} M).

V. *Incorporation of ^{3}H-cytosine arabinoside after pretreatment of cells with methotrexate*

Four 200-ml incubation flasks containing $7{\cdot}3 \times 10^4$ cells/ml were prepared. At the beginning of the experiment, methotrexate ($1{\cdot}6 \times 10^{-7}$ M), hypoxanthine (2×10^{-5} M) and serine ($1{\cdot}4 \times 10^{-3}$ M) were added to flasks 3 and 4. After 4 hr of incubation, ^{3}H-cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M, 106 μc/μmole) was added to flasks 1, 2 and 3, and deoxythymidine (2×10^{-6} M) to flask 4. The untreated cells of flask 4 were used as controls in the viability study. After an additional 4-hr incubation with ^{3}H-cytosine arabinoside, cell viability was determined by the dilute agar colony

method in a growth medium containing hypoxanthine (2×10^{-5} M), deoxythymidine ($2{\cdot}0 \times 10^{-6}$ M), serine ($1{\cdot}4 \times 10^{-3}$ M) and deoxycytidine (5×10^{-5} M). The remaining cells of each incubation flask were then centrifuged at approximately 2000 *g* for 45 min, washed, and resuspended in 9·0 ml of growth medium. A portion of the washed cells (3·0 ml) was diluted in 200 ml of growth medium and incubated at 37° for 18 hr. The remaining washed cells (6·0 ml) and the cells incubated for 18 hr were fractionated for cold acid-soluble, RNA and DNA as described in Methods, section I (Tables 5 and 6).

VI. *Effect of cytosine arabinoside and methotrexate on the uptake of* ^{3}*H-deoxycytidine*

Cells in the logarithmic phase of growth (1×10^6 cells/50 ml) were incubated with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) for 6 hr, centrifuged, washed twice, and resuspended in 10 ml of growth medium. The washed cells were diluted in growth medium containing ^{3}H-deoxycytidine (5×10^{-5} M of 100 μc/μmole or 1×10^{-5} M of 10 μc/μmole). Control cells were prepared in an identical manner except that cytosine arabinoside was omitted. After 2 hr of incubation for flasks containing 5×10^{-5} M deoxycytidine (100 μc/μmole) and 24 hr for flasks containing 1×10^{-5} M deoxycytidine (10 μc/μmole), the cells were centrifuged, washed twice with cold medium, and the cold acid-soluble, RNA and DNA fractions prepared as described in Methods, section I (Table 3).

A portion of cells in the exponential phase of growth (3×10^5/ml) were incubated with methotrexate ($1{\cdot}6 \times 10^{-7}$ M), hypoxanthine (2×10^{-5} M) and serine ($1{\cdot}4 \times 10^{-3}$ M). After 4 hr of incubation, ^{3}H-deoxycytidine (75·8 μc/μmole, $3{\cdot}3 \times 10^{-6}$ M), cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M), methotrexate ($1{\cdot}6 \times 10^{-7}$ M) and deoxythymidine (2×10^{-6} M) were added to cells as shown in Table 7. After an additional 4 hr of incubation (total incubation time, 8 hr), the cells were centrifuged, washed twice, and resuspended in 10 ml of growth medium. A portion from each incubation was removed to determine cell viability, and from the remaining cells the cold acid-soluble, RNA and DNA fractions were prepared as described in Methods, section I (Table 4).

RESULTS AND DISCUSSION

L5178Y cells, prelabeled with ^{3}H-deoxythymidine and subsequently treated with cytosine arabinoside for 6 hr, lost DNA radioactivity logarithmically from the culture during 66 hr of study (Fig. 1). Control cells without exposure to cytosine arabinoside retained radioactivity quantitatively after a small initial loss. The generation time of cytosine arabinoside-treated cells (23 hr) was much slower than that of the untreated cells (10 hr). The prolonged generation time was the result of cell death, since it has been shown that acute cell death and not delay of cell division was the major toxic effect of high levels of cytosine arabinoside.[20] The large increase in cell number of the drug-treated population (Fig. 1) demonstrates that some inhibited cells were capable of a limited number of divisions prior to cell death. The rate of ^{3}H-deoxythymidine loss from DNA was approximately 3·0 per cent per hr as compared to a cell death rate of approximately 2·2 per cent per hr in the time interval of 18–66 hr. The small difference in the rates might not be significant. The constant fractional percentage kill of the cell population could not be attributed to reutilization of cytosine arabinoside released after cell death, since the analog would have been

diluted in the growth medium to an extremely low level. The finding that radioactivity from ^{3}H-cytosine arabinoside in the cold acid-soluble fraction fell to very low levels after incubation of treated cells at 37° for 18 hr in growth medium free of cytosine arabinoside suggests that the continued presence of this fraction was not essential to

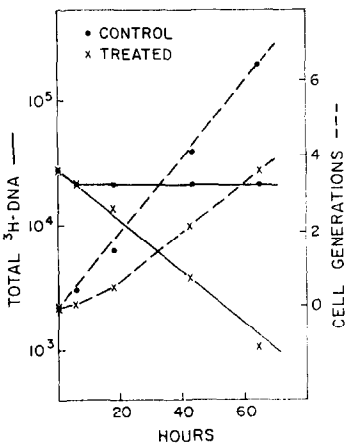

FIG. 1. L5178Y cells ($2{\cdot}2 \times 10^5$ cells/ml) prelabeled with ^{3}H-deoxythymidine for 19 hr were incubated for 6 hr with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M), washed free of the analog, and incubated at 37° in growth medium. At the times indicated, the DNA fraction was prepared as described in Methods, section I. Cell numbers were determined with a Coulter counter.

cell death. It is probable that the levels of inhibitory derivatives of cytosine arabinoside were not uniform within individual cells of the nonsynchronized population, even individual cells at the same stage of the cell cycle would not contain identical inhibitory amounts of cytosine arabinoside. Thus, a cellular heterogeneity in content of inhibitory cytosine arabinoside would contribute to a constant fractional kill of the cell population.

The effects of cytosine arabinoside on the incorporation of ^{3}H-deoxythymidine were also investigated. It has been reported that cytosine arabinoside ($1{\cdot}2 \times 10^{-6}$ M) inhibited the conversion of ^{3}H-uridine into cold acid-soluble ^{3}H-deoxycytidine nucleotides,[3] and it was proposed that the site of this inhibition was cytidine diphosphate reductase. In these studies, deoxycytidine (5×10^{-5} M) could partially prevent the inhibition of the incorporation of radioactive derivatives of uridine into the DNA.[1] The data of Table 1 demonstrate that the presence of cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) inhibited (45 per cent) the incorporation of ^{3}H-deoxythymidine into the DNA, and that the inhibition was partially prevented by deoxycytidine (5×10^{-5} M). The inhibition by cytosine arabinoside of the incorporation of ^{3}H-deoxythymidine into DNA was presumably due to a deficiency of deoxycytidine triphosphate. The protective effect of deoxycytidine is attributed: (1) to inhibition of the phosphorylation of cytosine arabinoside by competition for deoxycytidine kinase, and (2) to provision of deoxycytidine triphosphate to the cells. An additional effect of deoxycytidine, after deamination, was to dilute the pool of radioactive deoxythymidylic acid. The data of Table 2 demonstrate that cells pretreated with cytosine arabinoside for 6 hr and washed free of the analog incorporated ^{3}H-deoxythymidine as efficiently as the untreated cells.

A similar observation with L-cells has been reported.[24] In populations of L5178Y cells pretreated with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) for 6 hr, the DNA content per cell ($1{\cdot}2 \times 10^{-8}$ mg/cell) remained constant during a 24-hr period. The radioactivity of the DNA of the treated cells might be due in part to an increase of thymidine kinase activity, as has been reported.[25] Although the pretreated cells incorporated ^{3}H-deoxythymidine as well as the controls, it should be emphasized that approximately

TABLE 2. ^{3}H-DEOXYTHYMIDINE INCORPORATION IN CELLS PRETREATED WITH CYTOSINE ARABINOSIDE*

Cytosine arabinoside pretreatment ($3{\cdot}3 \times 10^{-6}$ M)	Deoxycytidine (5×10^{-5} M)	DNA (cpm/cell $\times 10^3$)
—	—	$14{\cdot}6 \pm 0{\cdot}8$
+	—	$16{\cdot}1 \pm 1{\cdot}1$
+	+	$10{\cdot}2 \pm 0{\cdot}8$
—	+	$11{\cdot}1 \pm 0{\cdot}7$

* L5178Y cells (10 ml, $3{\cdot}0 \times 10^6$ cells) in the exponential phase of growth were incubated with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) for 6 hr. Treated and control cells were diluted to approximately 1×10^5/ml in growth medium containing deoxycytidine (5×10^{-5} M) and incubated for 19 hr with ^{3}H-deoxythymidine ($2{\cdot}0 \times 10^{-8}$ M, 2530 μc/μmole). A portion of each culture was transferred to a Hopkin's tube containing a column of sucrose and the DNA extracted. The average results of 3 experiments are presented.

95 per cent of the treated cells were non-viable and rapidly lost DNA, but were capable of several divisions prior to cell death. In a situation which might be analogous, it has been reported that 50 per cent of the P815Y cell population treated with 5-iodo-deoxyuridine (resulting in 99·7 per cent nonviable cells) were capable of a normal rate of incorporation of deoxythymidine.[26]

It has been found that cells treated with a high level of cytosine arabinoside for short periods of time could not be rescued by deoxycytidine.[20] The data of Table 3 demonstrate that the cells treated with these high levels of analog incorporated much more ^{3}H-deoxycytidine into the cold acid-soluble and the DNA fractions than did

TABLE 3. PHOSPHORYLATION OF ^{3}H-DEOXYCYTIDINE IN CELLS AFTER EXPOSURE TO CYTOSINE ARABINOSIDE*

	(cpm/cell $\times 10^3$)			
	2 hr		24 hr	
Treatment	dCMP†	DNA	dCMP†	DNA
No cytosine arabinoside	1·6	1·2	0·62	1·2
With cytosine arabinoside	7·8	6·8	1·7	4·0

* L5178Y cells (1×10^6 cells/50 ml), after pretreatment with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) for 4 hr, were exposed to ^{3}H-deoxycytidine either 5×10^{-5} M (100 μc/μmole) or 1×10^{-5} M (10 μc/μmole) and incubated at 37°. After 2 hr (100 μc/μmole) and 24 hr (10 μc/μmole) of incubation, the cold acid-soluble, RNA and DNA fractions were prepared.

† dCMP = the combined cold acid-soluble phosphate derivatives of deoxycytidine.

the untreated cells. This increase could be attributed to several possible phenomena: 1) a depletion of cellular deoxycytidine triphosphate, which has been found to be an efficient inhibitor of the deoxycytidine kinase of L5178Y cells;[27] 2) an increase of deoxycytidine kinase activity may be related, because partially purified deoxycytidine kinase from L5178Y cells has been shown to be activated by cytosine arabinoside.[28] The pool size of the radioactive ^{3}H-deoxycytidine phosphates of cells treated with cytosine arabinoside was estimated to be 0·21 mμmole/10^6 cells, whereas in other studies[17] the pool size of non-radioactive deoxycytidine phosphates in normal L5178Y cells was 0·020 mμmole/10^6 cells. It is apparent, therefore, that the majority of the treated cells which could not be rescued by deoxycytidine[22] had sufficient deoxycytidine triphosphate for DNA synthesis.

If cell death were due to incorporation of cytosine arabinoside into the DNA, an interruption of DNA synthesis might prevent cell death. From the data of Table 4, it is evident that the incorporation of ^{3}H-deoxycytidine into cellular DNA was decreased

TABLE 4. INCORPORATION OF ^{3}H-DEOXYCYTIDINE IN CELLS PRETREATED WITH METHOTREXATE*

	No methotrexate pretreatment				Methotrexate pretreatment			
	Group No.							
Compound added	1	2	3	4	5	6	7	8
Deoxythymidine	0	+	0	+	0	+	0	+
Cytosine arabinoside	0	0	+	+	0	0	+	+
DNA (cpm/mg)	56·8	39·0	109	149	27·2	128	24·3	132

* L5178Y cells (3×10^5/ml) in the exponential phase of growth were incubated with methotrexate ($1{\cdot}6 \times 10^{-7}$ M) for 4 hr in a medium containing hypoxanthine (2×10^{-5} M) and serine ($1{\cdot}4 \times 10^{-3}$ M). After 4 hr of incubation, ^{3}H-deoxycytidine ($3{\cdot}3 \times 10^{-6}$ M, 75·8 μc/μmole) was added to all cultures. Cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) and deoxythymidine (4×10^{-6} M) were added and the incubation was continued for an additional 4 hr. The cells were washed and the DNA fraction was prepared.

by pretreatment with methotrexate in a medium containing hypoxanthine and serine and that the incorporation of ^{3}H-deoxycytidine was increased 5-fold with the addition of deoxythymidine to the depleted cells. In cells not pretreated with methotrexate, nonradioactive deoxythymidine diluted ^{3}H-deoxythymidine derived from ^{3}H-deoxycytidine (Table 4, group 2). When cytosine arabinoside and deoxythymidine were present at the same time (Table 4, groups 4 and 8), the radioactivity in DNA increased sharply. This increase might result from a depletion of nonradioactive deoxycytidine phosphates by cytosine arabinoside and an increased activity of deoxycytidine kinase as discussed above. When ^{3}H-deoxycytidine, the favored substrate of deoxycytidine kinase, and cytosine arabinoside were of equal molarity, it was expected that significant amounts of ^{3}H-deoxycytidine would be incorporated into methotrexate-treated cells (Table 4, groups 3, 4, 7 and 8). It is possible that the increased radioactivity seen in the presence of cytosine arabinoside alone (group 3) is due to activation of deoxycytidine kinase by cytosine arabinoside.[20]

Since pretreatment with methotrexate inhibited the incorporation of ^{3}H-deoxycytidine into DNA, the effect of pretreatment upon the incorporation of cytosine arabino-

side into the DNA and RNA was studied. The results (Table 5) demonstrate that cells deficient in deoxythymidine triphosphate incorporated less ^{3}H-cytosine arabinoside into DNA and RNA and that the addition of deoxythymidine increased the incorporation of the analog into DNA and RNA to levels above that of the untreated cells. The increased radioactivity of the DNA in the presence of deoxythymidine presumably

TABLE 5. INCORPORATION OF ^{3}H-CYTOSINE ARABINOSIDE IN CELLS PRETREATED WITH METHOTREXATE*

Incubation time	Compound added	Flask No. 1	2	3
	Methotrexate pretreatment	0	+	+
	^{3}H-cytosine arabinoside	+	+	+
	Deoxythymidine	0	0	+
4 hr	RNA (cpm/mg)	566	483	659
	DNA (cpm/mg)	527	297	670
18 hr	RNA (cpm/mg)	242	302	390
	DNA (cpm/mg)	405	560	790

* Approximately $1{\cdot}5 \times 10^7$ L5178Y cells ($7{\cdot}3 \times 10^4$/ml) per flask were preincubated for 4 hr with methotrexate ($1{\cdot}6 \times 10^{-7}$ M) in medium containing hypoxanthine ($2{\cdot}0 \times 10^{-5}$ M) and serine ($1{\cdot}4 \times 10^{-3}$ M). ^{3}H-cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M, 106 μc/μmole) and deoxythymidine (2×10^{-6} M) incubation was continued for 4 hr. A portion of the cells was incubated for 18 hr in medium free of cytosine arabinoside. The RNA and DNA fractions were prepared as described in Methods, section I.

reflected a partial degree of synchrony in the methotrexate-treated cell population. After washing the cells to remove free ^{3}H-cytosine arabinoside, it was found that the radioactivity of the cold acid-soluble fraction was similar in treated and control cell populations (430 cpm/10^6 cells for controls, 470 cpm/10^6 cells for methotrexate-treated cells, and 480 cpm/10^6 cells for cells treated with methotrexate and deoxythymidine). The extent of incorporation of ^{3}H-cytosine arabinoside into the DNA seemed to be dependent on the presence of deoxythymidine triphosphate.

The increase in radioactivity in the DNA fraction of cells incubated for an additional 18 hr presumably resulted from additional incorporation of a portion of the very high levels of cytosine arabinoside triphosphate present after 6 hr of incubation with the analog. It is apparent that incorporation of the analog into DNA of the cell population occurred while DNA loss also occurred (Fig. 1). A loss of radioactivity from the RNA fraction in the 18-hr incubation would result from the shorter half-life of RNA when compared to DNA.

Since the levels of cytosine arabinoside in the nucleic acids were modified by treatment with methotrexate, the effects on cell viability were examined. The data in Tables 6 and 7 demonstrate that cytosine arabinoside killed a larger fraction of cells when pretreated with methotrexate and that the addition of deoxythymidine did not have a significant effect. Although the population treated with methotrexate and cytosine arabinoside increased in number during 18 hr of incubation in medium free of antimetabolites, the viable fraction remained constant (Table 6). Cells pretreated with methotrexate and subsequently treated with cytosine arabinoside apparently undergo division prior to cell death, as do cells treated with cytosine arabinoside alone. Cells treated with methotrexate were killed slowly (Fig. 2), although hypoxanthine, serine

TABLE 6. SENSITIZATION OF CELLS TO CYTOSINE ARABINOSIDE BY PRETREATMENT WITH METHOTREXATE*

Incubation time	Compound added	Flask No. 1	2	3	4
	Methotrexate pretreatment	0	+	+	0
	^{3}H-cytosine arabinoside	+	+	+	0
	Deoxythymidine	0	0	+	0
4 hr	Normalized (%) survivors	23	5·5	5·4	100
18 hr	Normalized (%) survivors	21	4·6	6·3	100

* L5178Y cells from the experiment reported in Table 5 were diluted just before extraction of the cold acid-soluble, RNA and DNA fractions and the viability was determined by the dilute agar colony method.[22] A minimum of 200 colonies was counted for each set. The cloning efficiency of drug-free controls was approximately 75 per cent. All values were normalized to 100 per cent.

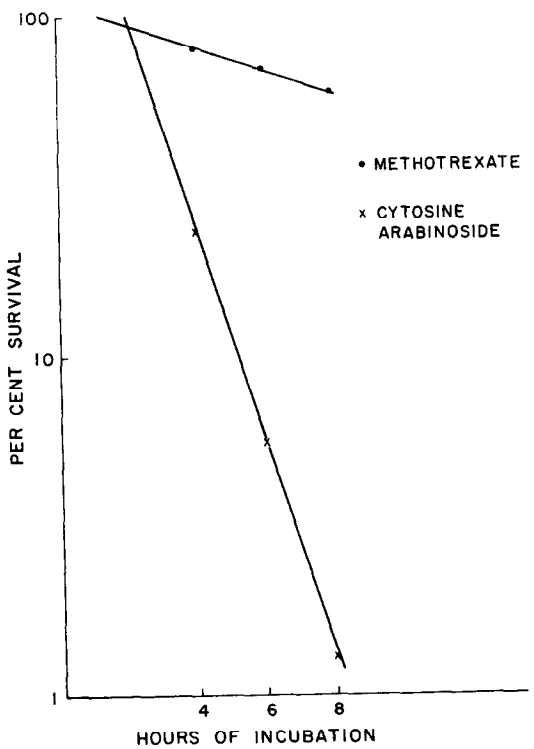

FIG. 2. Kinetics of cell death by methotrexate and cytosine arabinoside. L5178Y cells (approximately 1×10^4 cells/ml) in the exponential phase of growth were exposed to methotrexate ($1{\cdot}6 \times 10^{-7}$ M) in medium containing hypoxanthine (2×10^{-5} M) and serine ($1{\cdot}4 \times 10^{-3}$ M), or to cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) for different periods of time. The analogs were removed and cell viability was determined by the dilute agar colony method.[20] The cloning medium contained deoxythymidine (2×10^{-6} M), hypoxanthine (2×10^{-5} M), serine ($1{\cdot}4 \times 10^{-3}$ M) and deoxycytidine (5×10^{-5} M). A minimum of 200 colonies was counted for each determination. The cloning efficiency of the drug-free control cells was approximately 75 per cent. All values were normalized to 100 per cent.

TABLE 7. SENSITIZATION OF CELLS TO CYTOSINE ARABINOSIDE BY METHOTREXATE*

Incubation time	No methotrexate pretreatment			Methotrexate pretreatment					
	No cytosine arabinoside	+ Cytosine arabinoside	+ Methotrexate	+ Cytosine arabinoside, − methotrexate		+ Cytosine arabinoside, + methotrexate		− Cytosine arabinoside, + methotrexate	
				− Deoxythymidine	+ Deoxythymidine	− Deoxythymidine	+ Deoxythymidine	− Deoxythymidine	+ Deoxythymidine
2 hr	100	94	94	14	10	7·0	9·0	56	78
4 hr	100	20	81	4·3	2·5	4·0	4·0	52	75

* L5178Y cells (2×10^4/ml) in the exponential phase of growth were pretreated with methotrexate ($1{\cdot}6 \times 10^{-7}$ M) for 4 hr in a medium containing hypoxanthine (2×10^{-5} M) and serine ($1{\cdot}4 \times 10^{-3}$ M). Cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M), deoxythymidine (2×10^{-6} M) and methotrexate ($1{\cdot}6 \times 10^{-7}$ M) were added as indicated and the incubation was continued for an additional 2-hr and 4-hr periods. Cell viability was determined by the dilute agar colony method[22] in a medium containing hypoxanthine (2×10^{-5} M), serine ($1{\cdot}4 \times 10^{-3}$ M), deoxythymidine (2×10^{-6} M) and deoxycytidine (5×10^{-5} M). A minimum of 200 colonies was counted for each group. The cloning efficiency of the untreated control cells was approximately 75 per cent. All values were normalized to 100 per cent.

and deoxythymidine were present in the cloning medium and the fractional cell kill was calculated to be 7 per cent per hr with a lag period of 1·2 hr. The fractional cell kill with cytosine arabinoside treatment ($3{\cdot}3 \times 10^{-6}$ M) was calculated to be 55 per cent per hr (Fig. 2) with a lag period of 2 hr. Pretreatment with methotrexate increased the killing effect of cytosine arabinoside (Tables 6 and 7). Until the mechanism of methotrexate sensitization to cytosine arabinoside is understood, it may be difficult to relate these observations to the possible toxic effects of incorporation of cytosine arabinoside into the RNA and DNA of mammalian cells. In the system used, methotrexate would have caused an accumulation of cells early in the DNA synthetic phase. It is possible that cytosine arabinoside incorporated into the DNA or RNA of these partially synchronized cells produced a greater degree of cell death than when the analog was incorporated in other stages of DNA synthesis.

Acknowledgements—The proficient technical assistance of Mrs. Shue-Ying Liu, Mrs. Barbara Morcaldi and Miss Barbara Moroson is gratefully acknowledged.

* Note added in proof: It has recently been found (Glenn A. Fischer and Carl von Essen) that L5178Y cells treated for 2 hr with cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) have an increased sensitivity to irradiation from a ^{137}Cs source.

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