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THE INCORPORATION OF ^{3}H-CYTOSINE ARABINOSIDE AND ITS EFFECT ON MURINE LEUKEMIC CELLS (L5178Y)*

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Abstract—Murine leukemic cells, L5178Y, inhibited by a low level of cytosine arabinoside ($3{\cdot}0 \times 10^{-7}$ M) for 6 hr could be rescued by deoxycytidine. Cells exposed to higher concentrations of cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) for short periods of time (2 hr) could not be rescued by deoxycytidine. Acute cell death was the major toxic effect of cytosine arabinoside at the higher concentrations and delay of cell division was of minor importance.

^{3}H-Cytosine arabinoside monophosphate was isolated from DNA and RNA fractions prepared by different methods.

A time-dependent incorporation of cytosine arabinoside was found in the cold acid-soluble, RNA and DNA fractions. The incorporation into RNA could be correlated with irreversible inhibition of cell reproduction.

IT HAD been shown that 1-β-D-arabinofuranosyl cytosine (cytosine arabinoside, ara-C) inhibited the synthesis of DNA in several strains of *Escherichia coli*[1, 2] and in mammalian cells.[3, 4]

In cell culture, cytosine arabinoside was found to be an effective inhibitor of DNA viruses,[5, 6] and in mice and rats an inhibition of a variety of transplantable tumors including Sarcoma 180, Ehrlich carcinoma, leukemias L1210 and L1210/C95 was observed.[7] Cytosine arabinoside was capable of inhibiting the synthesis of SV_{40} viral antigen and infectious virus, but not the production of SV_{40} tumor antigen.[8] Inhibition of marrow regeneration in mice[9] and delay of the primary immune response have been reported.[10] The effect of treatment with cytosine arabinoside on a variety of human neoplasms has been demonstrated. The initial report of possible anticancer activity in humans was that of Talley and Vaitkevicious.[11] Cytosine arabinoside has also been found to be a useful agent for the treatment of viral infections of the eye.[12, 13]

Previous work from this laboratory indicated that the primary site of action of cytosine arabinoside on the reproduction of murine leukemic L5178Y cells was inhibition of the formation of deoxycytidine diphosphate from cytidine diphosphate, an effect that would cause inhibition of the synthesis of DNA and cellular reproduction.[3] Since the only detectable difference in a mutant resistant to cytosine arabinoside was a deficiency in deoxycytidine kinase activity, it was proposed that a phosphorylated derivative of cytosine arabinoside was the active inhibitor of the enzyme or

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enzymes involved in the conversion of cytidine diphosphate to the corresponding derivative of deoxycytidine. It was concluded that the enzyme which phosphorylated cytosine arabinoside was deoxycytidine kinase.[14, 15]

Cytosine arabinoside is extensively converted by whole cells to cytosine arabinoside triphosphate and it was found that this antimetabolite was incorporated into the RNA and DNA fractions in internucleotide linkage.[14] Subsequently, the incorporation of cytosine arabinoside into RNA and DNA of mouse fibroblasts[16] and human leukemic cells[17] has been reported. It seems possible that this incorporation of the analog into nucleic acids might account for a toxic effect on cells.

The present report provides detailed evidence that ^{3}H-cytosine arabinoside was incorporated into both RNA and DNA fractions. Although the metabolic lesion responsible for cytosine arabinoside-induced acute cell death has not been fully elucidated, the time-dependent logarithmic increment of cell death accompanied by the linear time-dependent incorporation into the cold acid-soluble, DNA and RNA fractions suggests that these phenomena may be related.

MATERIALS AND METHODS

The methods of propagation of murine leukemic cells (L5178Y) have been described.[18] 1-β-D-Arabinofuranosyl cytosine hydrochloride was obtained from Dr. J. H. Hunter at the Upjohn Chemical Co. ^{3}H-Cytosine arabinoside was purchased from Schwarz Biochemicals and was used no longer than 45 days after fractionation by chromatography for 96 hr in solvent 1 (Table 1). Before use, a purity of 98 per cent

TABLE 1. TIME COURSE OF SEPARATION OF PYRIMIDINES AND THEIR NUCLEOSIDES BY SOLVENT 1*

Compound	Migration (cm)		R_f value
	Solvent 1†	Solvent 2†	Solvent 3†
Cytosine	48·0	28·0	0·69
Thymine	over	over	0·77
Uracil	34·0	55·0	0·70
Cytosine arabinoside	33·0	23·0	0·75
Thymine arabinoside	43·0	52·0	0·59
Uracil arabinoside	20·0	43·0	0·79
Cytidine	25·0	17·0	0·43
Uridine	13·0	37·0	0·46
Deoxycytidine	51·0	28·0	0·78
Deoxythymidine	over	over	0·83
Deoxyuridine	28·0	56·0	0·80

* After application of the compounds to Whatman No. 1 paper (57 cm × 46 cm), descending chromatograms were removed after 96 hr of separation in solvent 1, 48 hr in solvent 2, and 18 hr in solvent 3. To obtain maximum migration, solvent 1 and solvent 2 were allowed to drip from the end of the paper.

† Solvent 1 = 86% *n*-butanol:conc. NH_4OH (94·5:5·5); solvent 2 = *n*-butanol:formic acid:water (77:10:13); solvent 3 = 5 M NH_4 acetate (pH 9·5):sat. Na tetraborate: 95% ethanol:0·25 M EDTA (pH 9·5) (5:20:55:005).

or more was confirmed by chromatography for 48 hr in solvent 2, and for 18 hr in solvent 3. The radioactive contaminants of particular concern had R_f values corresponding to uracil arabinoside and to cytosine. Since these materials increased in

quantity on storage (−16° in 50% ethanol) they are assumed to arise from the decomposition of cytosine arabinoside.

I. Chromatography

A. *Paper chromatography*. The paper chromatographic systems used[14] accomplished complete separation of cytosine arabinoside from cytosine, uracil, thymine, cytidine, deoxycytidine, uridine, deoxyuridine, uracil arabinoside, thymidine and thymine arabinoside. The aqueous *n*-butanol NH_4OH solvent (Table 1) has been particularly useful because of its resolving power, its capacity for salts, and the volatility of the butanol and NH_3. The R_f values obtained with other solvents are also presented in Table 1.

B. *Column chromatography*. (1) Ecteola cellulose: The ion-exchange cellulose was washed on a 4·5 cm (i.d.) column with 2 l of 0·1 N NaOH followed by 2 l of 0·1 N HCl (50 g of the ion-exchange cellulose). This cycle was repeated twice and the exchanger, finally in the chloride form, was washed with distilled water until the eluate was free of chloride ions. The fines were removed by decanting the supernatant after allowing the suspension to settle for 20 min. This process was repeated twice. Before use, the ion-exchange cellulose column (1 × 16–18 cm) was equilibrated with 15·0 ml of 0·05 M LiCl in 10^{-3} M Tris, pH 7·4, and then with 28·0 ml of 0·0025 M LiCl in 10^{-3} M Tris, pH 7·4. The final pH of the equilibrating effluent was 6·6 to 6·9. The material to be adsorbed on the column was adjusted to pH 7·8 in a volume of 100 ml or less. The phosphate derivatives were eluted in a stepwise manner as indicated below:

Fraction	Solvent	Fraction volume (ml)
1	0·0025 M LiCl 10^{-3} M Tris, pH 7·4	20·0
2	0·05 M LiCl 10^{-3} M Tris, pH 7·4	10·0
3	0·05 M LiCl 10^{-3} M Tris, pH 7·4	5·0
4	0·05 M LiCl 10^{-3} M Tris, pH 7·4	10·0
5	0·08 M LiCl 10^{-3} M Tris, pH 7·4	3·0
6	0·08 M LiCl 10^{-3} M Tris, pH 7·4	2·0
7	0·08 M LiCl 10^{-3} M Tris, pH 7·4	2·0
8	0·08 M LiCl 10^{-3} M Tris, pH 7·4	10·0
9	0·08 M LiCl 10^{-3} M Tris, pH 7·4	1·0
10	0·08 M LiCl 10^{-3} M Tris, pH 7·4	1·0
11	0·08 M LiCl 10^{-3} M Tris, pH 7·4	1·0
12	1·0 M LiCl	5·0
13	1·0 M LiCl	5·0
14	1·0 M LiCl	5·0

The mono-, di-, and triphosphates of cytidine and cytosine arabinoside were recovered in fractions 3, 8, and 12 respectively. Cytidine and cytosine arabinoside were recovered in fraction 1. The ion-exchange cellulose was washed with 100 ml distilled water and resuspended in water at 4° for reuse.

(2) Dowex 1-X4 formate: Dowex 1-X4, 200–400 mesh, chloride form (12 lb) was washed with 4 l. of 2 M sodium formate in 1 M formic acid until the eluate was free of chloride ions and washed with distilled water (pH 6·5) until the effluent was above pH 5·5. The material to be adsorbed on the column was adjusted to pH 8·2 in a volume of 25 ml or less.

The cytidine-5′-phosphate, cytidine-2′-phosphate, cytidine-3′-phosphate and cytosine arabinoside-5′-phosphate were recovered in fractions 1, 4, 7, and 10 respectively.

Fraction	Solvent	Fraction volume (ml)
1	0·01 M HCOOH	40·0
2	0·01 M HCOOH	5·0
3	0·01 M HCOOH	5·0
4	0·01 M HCOOH	40·0
5	0·01 M HCOOH	5·0
6	0·01 M HCOOH	5·0
7	0·01 M HCOOH	40·0
8	0·01 M HCOOH	5·0
9	0·01 M HCOOH	5·0
10	0·05 M HCOOH	100·0

II. *Chemical synthesis of cytosine arabinoside-5′-phosphate*

Cytosine arabinoside-5′-phosphate was prepared according to the method of Tener[19] for the synthesis of phosphomonoesters with 764 mg 1-β-D-arabinofuranosyl cytosine HCl. After the removal of pyridine, the material was diluted with distilled water and adjusted to pH 7·6 with NaOH. The 5′-phosphate derivative was separated from material corresponding to the 2′-and 3′-monophosphates and was recovered by fractionation on a Dowex 1 formate column (2·9 × 14 cm). The column was washed with distilled water until free of u.v.-absorbing material, then eluted with 0·01 M formic acid. Tubes that contained peaks of u.v. -absorbing material and had the approximate 280 mμ/260 mμ ratio of 2:1 corresponding to cytosine arabinoside-5′-phosphate were combined, diluted 5-fold with distilled water, and the volume was reduced with an evaporator under reduced pressure at 25°. The compound was further purified by paper chromatography in solvent 1. The final product was evaporated to dryness at 0·2 mm Hg vacuum and 25°. The yield was 10·7 mg cytosine arabinoside-5′-phosphate. The u.v. absorption spectrum in 0·01 M HCl in a Beckman model DU spectrophotometer was identical with that of cytosine arabinoside. The ϵ_{281} (E_{max}) calculated as the monoammonium salt was found to be $13{\cdot}2 \times 10^3$, a value which was 98 per cent of the theoretical value based on the ϵ_{281} of cytosine arabinoside. Total phosphorous determination by the method of Dryer, Tommes and Routh[20] gave a value of 26 per cent (theoretical for the monoammonium salt = 28 per cent).

Incubation of the synthetic cytosine arabinoside-5′-phosphate with the specific bull semen 5′-phosphatase under conditions described by Heppel and Hilmore[21] released 120 per cent of the theoretical amount of cytosine arabinoside as determined by paper chromatography in solvent 3. Under comparable conditions cytidine-5′-phosphate released 100 per cent of the theoretical amount of cytidine. Titration according to the method of Dixon and Lipkin[22] was used for the determination of arabinose configuration of the sugar moiety. Consumption of 3 μmole periodate, as measured by changes in absorbancy at 227 mμ, required approximately 24 hr for the arabinose derivative compared to 3 hr for the ribose derivative, cytidine-5′-phosphate.

III. *Preparation and hydrolysis of nucleic acids*

A. *Modified salt extraction.* One liter of a logarithmically growing culture (4×10^5 cells/ml) was harvested by centrifugation at approximately 2000 *g* for 30 min. The cells were resuspended in 10·0 ml of growth medium and this process was repeated twice. The washed cells were distributed equally into five 25·0-ml Erlenmeyer flasks (8×10^7 cells/flask) containing 10·0 ml of growth medium. The flasks were preincubated at 37° for 10 min. ^{3}H-Cytosine arabinoside (2370 μc/μmole, $7{\cdot}5 \times 10^{-7}$ M) was added

and the flasks were incubated at 37° for an additional 20 min. The cells were centrifuged and washed with cold medium prior to the fractionation. The cold acid-soluble fraction was extracted four times with 1·0 ml cold 0·2 N $HClO_4$. The radioactivity of the final cold acid-soluble extraction contained no more than 5 per cent of the radioactivity present in the RNA. RNA was extracted with 1·0 ml of cold 1 N $HClO_4$ for 24 hr and washed twice with 1 N $HClO_4$. The residual RNA fraction was extracted twice with 1·0 ml of 0·2 N NaOH at 80° for 20 min. The differential extraction of RNA from DNA by cold 1 N $HClO_4$ has been reported by Ogur and Rosen.[23] A residual RNA fraction, less soluble in cold 1 N $HClO_4$, was reported by Patterson and Docheran.[24] Stansly and Chu[25] reported that the RNA which was less soluble in cold 1 N $HClO_4$ differed from the cold acid-soluble RNA in the uptake of radioactive phosphorous. It seemed possible that a subfractionation of RNA containing ^{3}H-cytosine arabinoside might also occur. The extraction of RNA by cold 1 N $HClO_4$ was followed by alkaline hydrolysis to remove completely the RNA from the DNA. The radioactivity of the last extraction with NaOH was commonly less than 5 per cent of the radioactivity of the DNA fraction. DNA in the residue was extracted first with 8·0 ml then with 2·0 ml of 10% NaCl at 100° for 60 min. After centrifugation, 2½ vol. of 90% ethanol was added to the NaCl extract and the mixture was kept at −16° for 18 hr. The precipitated DNA was recovered by centrifugation, washed once with 95% ethanol, and dissolved in 5·0 ml distilled water.

B. RNA and DNA were isolated by the isopropanol procedure described by Marmur[26] and dialyzed to insure complete removal of alcohol.

C. The RNA prepared by isopropanol fractionation or by Method III A was hydrolyzed in 0·5 N KOH for 22 hr at 37°. The hydrolysate was fractionated on an Ecteola column (1 × 16–18 cm) in the system described above, which separated cytosine arabinoside, cytosine arabinoside monophosphate, cytosine arabinoside diphosphate, and cytosine arabinoside triphosphate. The radioactivity present in the original effluent from the column was identified as cytosine arabinoside by paper chromatography in solvent systems 1, 2 and 3. The cytosine arabinoside monophosphate fraction from the column was reduced to 1·0 ml and treated with alkaline phosphatase in a total volume of 2·0 ml. The amount of enzyme used (2 mg) released 4 μmole inorganic phosphorous from cytidine-2′ (3′)-phosphate when assayed by the method of Brandenberger and Hanson.[27] The hydrolysis was terminated by heating at 80° for 5 min the supernatant fraction recovered after centrifugation, and the volume was reduced to approximately 0·2 ml with a Buchler evaporator. The radioactive products were identified as cytosine arabinoside by paper chromatography in solvent 1. The alkaline-resistant dinucleotide fraction 8 through 14 was desalted by paper chromatography in solvent 1 and the recovered dinucleotide was enzymatically hydrolyzed at 37° for 18 hr by 1700 units of venom phosphodiesterase[28] (free of 5′-nucleotidase activity[29]) to release free cytosine arabinoside. The reaction was terminated by heating for 5 min at 100°. The sediment containing insignificant radioactivity after centrifugation was discarded. The supernatant fraction was diluted with distilled water and rechromatographed on an Ecteola column with carrier amount of chemically prepared cytosine arabinoside-5′-phosphate (0·7 μmole) and cytosine arabinoside (0·7 μmole). Most of the radioactivity of the effluent was identified as cytosine arabinoside by chromatography in solvent 1.

D. *Enzymatic hydrolysis of RNA.* The RNA fraction from Method III A or from

isopropanol fractionation was incubated at 37° for 16 hr with 5000 units[30] of crystalline ribonuclease (bovine pancreas, Worthington), which primarily yields polynucleotides with terminal 3′-phosphates. This hydrolysate was incubated with venom phosphodiesterase as described in Methods III C. The reaction was terminated by the addition of concentrated $HClO_4$, the hydrolysate was centrifuged, and the spuernatant fraction was neutralized with 8 N KOH. The $KClO_4$ was removed by centrifugation, cytosine arabinoside-5′-phosphate (0·7 μmole) was added to the supernatant, and 0·2 N NaOH was added to give pH 8·2. Portions of this enzymatic digest were fractionated on either a Dowex 1 × 4 formate column or an Ecteola column. The fractions which had a 280 mμ/260 mμ ratio of approximately 2:1 were combined and eluted in a position corresponding to cytosine arabinoside monophosphate. This combined fraction was concentrated to 4 ml at reduced pressure in a Buchler evaporator at 35°. After treatment with alkaline phosphatase as described above, the radioactivity in this fraction was identified as cytosine arabinoside by paper chromatography in solvent 1.

E. *Enzymatic hydrolysis of DNA.* The DNA fractions were treated with DNase I (the endonuclease which hydrolyzes DNA to give polynucleotide-5′-phosphates). The DNase I (crystalline, bovine pancreas, Worthington) was standardized according to the method of Kunitz[31] and 5000 units were incubated at 37° for 16 hr. Alternatively, the DNA fractions were hydrolyzed with DNase II (the endonuclease which acts on interdeoxyribonucleotide linkages to yield polynucleotide-3′-phosphates). The DNase II (bovine spleen, Worthington) was standardized according to the method of Kunitz.[31] Approximately 1000 units were incubated at 37° for 16 hr. The DNA hydrolysate was incubated with venom phosphodiesterase to give nucleoside-5′-phosphates with the conditions described previously. The supernatant fraction was diluted with distilled water, cytosine arabinoside-5′-phosphate (0·7 μmole) and cytosine arabinoside (0·7 μmole) were added as carriers, and the mixture was resolved on an Ecteola column. A variable fraction of radioactivity of the original effluent was recovered and identified as cytosine arabinoside by chromatography in solvent 1. The mononucleotide fraction was concentrated, treated with bull semen 5′-phosphatase as described above, and identified as cytosine arabinoside by chromatography in solvent 1.

IV. Kinetics of cell death

A. *The outgrowth method.* Logarithmically growing L5178Y leukemic cells (approximately 1×10^4 cells/ml) in 16 × 125 mm culture tubes were exposed to various concentrations of cytosine arabinoside for different intervals of time. Control cultures containing no cytosine arabinoside were treated in an identical manner. After each incubation period, treated and control cultures were centrifuged for 5 min at approximately 800 *g*; the supernatant fraction containing the analog was discarded and the cells were resuspended in 5 ml of growth medium at 37° without cytosine arabinoside. This washing procedure was repeated twice. A portion of each culture was diluted to 5·0 ml in 0·9% NaCl and the cell number was determined with a Coulter counter. The washed cells were diluted in growth medium with and without deoxycytidine (5×10^{-5} M) and were maintained in the logarithmic phase of growth by dilution in fresh growth medium at 37° for approximately 200 hr. At this time the growth rate of the cells originally treated with cytosine arabinoside was equal to that of the untreated cells. Deoxycytidine had no effect on the growth rate of control cells. The

total inhibition (cell kill plus delay in cell division) was then determined as the fraction of cells present in the treated population at a single point in time when compared with the untreated cells. As shown in Fig. 1, the number of cells in cultures which had been treated for 6 hr with $3{\cdot}3 \times 10^{-6}$ M cytosine arabinoside was 9·2 per cent of the number of cells from control cultures. A population continuously exposed to deoxycytidine (5×10^{-5} M) after 6 hr of treatment with $3{\cdot}3 \times 10^{-6}$ M cystosine arabinoside increased to a cell number that was 14·5 per cent of the control value.

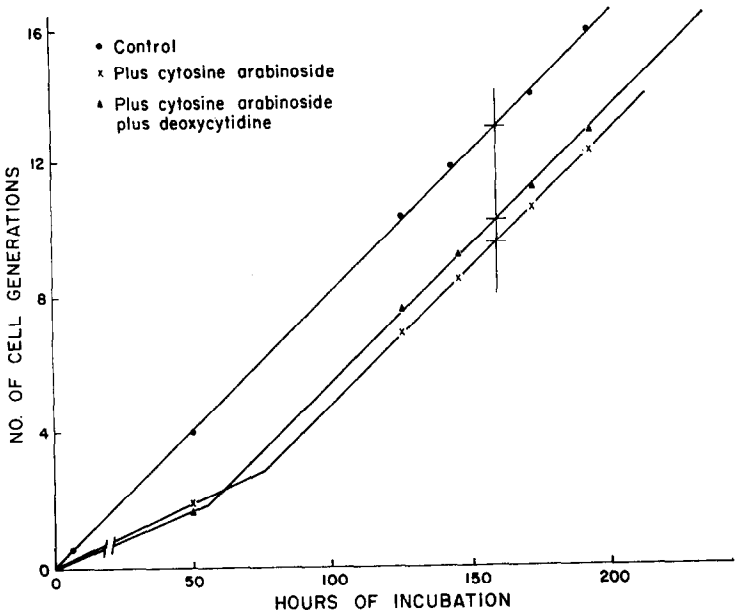

FIG. 1. The outgrowth method; inhibition produced by the incubation of L5178Y cells with $3{\cdot}3 \times 10^{-6}$ M cytosine arabinoside. [L5178Y cells (approximately 1×10^{4}/ml) in the exponential phase of growth were exposed to $3{\cdot}3 \times 10^{-6}$ M cytosine arabinoside for a period of 6 hr. The control and treated cells were centrifuged at approximately 800 *g* for 5 min and resuspended in growth medium (37°) free of drug. This washing procedure was repeated twice. Cells were maintained in the logarithmic phase by dilution in growth medium (37°). A portion of the treated cells (×—×) was grown continuously in the presence of deoxycytidine (5×10^{-5} M). When the growth rates were equivalent, the fraction of cells present in the drug-treated cultures compared to the untreated cultures was determined (●—● = 14·5%; ×—× = 9·2%).

B. *Dilute agar colony method.* A method was developed in this laboratory to clone murine leukemic cells in agar gel suspension. The formation of tridimensional colonies of mammalian cells in an agar overlay has been reported.[32] Three ml of growth medium at 44° containing 0·2% agar (Noble agar) and 15% horse serum was added to culture tubes with and without 0·25 μmole deoxycytidine per tube. The tubes were removed to room temperature and 60 leukemic cells in 2 ml of growth medium containing 15% horse serum was added to each tube to give a final concentration of 0·12% agar. After gently mixing the cells and agar, the tubes were placed upright in an ice bath for 2–4 min, removed from the ice bath and placed in racks which were incubated at 37°. To minimize breakdown of the agar gel, the tubes were placed on foam rubber mats 1–2 in thick, which insulated the tubes from vibration during

the incubation. Cells treated with cytosine arabinoside were treated in the same manner as the control cells and were diluted to provide approximately 60 colony-forming cells per tube. The spherical colonies (approximately 0·1 mm in dia.) were counted 6–10 days later with an AO Spencer darkfield colony counter.

V. *Time course of ^{3}H-cytosine arabinoside uptake*

Leukemic cells (approximately 3×10^5 cells/ml, 20 ml) were exposed to ^{3}H-cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M, 106 μc/μmole) during logarithmic growth in culture for periods ranging from 30–240 min. At the end of the incubation the cells were centrifuged at approximately 800 *g* for 5 min, the supernatant medium was discarded, and the cells were resuspended in 10·0 ml of growth medium at 37° without cytosine arabinoside. This washing procedure was repeated twice. A portion of the washed cells was used to determine cell number and cell viability by the agar colony method. A 9·2-ml portion of the cell suspension was extracted four times with 0·5 ml cold 0·2 N $HClO_4$. The RNA was extracted with 0·5 ml of 0·2 N NaOH at 80° for 20 min and the DNA was precipitated with 0·25 ml cold 1 N $HClO_4$. This extraction was repeated four times. The DNA in the sediment was extracted three times with 0·5 ml of 1 N $HClO_4$ at 80° for 30 min.

Radioactivity in all fractions was determined with a liquid scintillation counter at an efficiency of 16–20 per cent. Quenching was not detected in any of the samples tested.

RESULTS AND DISCUSSION

Cytosine arabinoside, an antimetabolite of deoxycytidine, affects several biochemical events in the cell. Cytosine arabinoside and deoxycytidine can compete with each other for deoxycytidine kinase to form their corresponding phosphorylated derivatives.[14] This competition was also evident in the incorporation of cytosine arabinoside into fractions of whole cells (Table 2). A mutant isolated from L5178Y cells highly

TABLE 2. THE EFFECT OF DEOXYCYTIDINE AND CYTIDINE ON THE INCORPORATION OF ^{3}H-CYTOSINE ARABINOSIDE*

Compounds added	Acid-soluble nucleotides		RNA		DNA	
	(cpm)	(%)	(cpm)	(%)	(cpm)	(%)
^{3}H-cytosine arabinoside	$1{\cdot}45 \times 10^5$	100	1600	100	680	100
^{3}H-cytosine arabinoside + cytidine	$1{\cdot}1 \times 10^5$	75	1030	67	470	70
^{3}H-cytosine arabinoside + deoxycytidine	$0{\cdot}23 \times 10^5$	16	430	27	120	18

* L5178Y cells (3×10^8) in the logarithmic phase of growth were incubated in a volume of 15 ml for 20 min in the presence of ^{3}H-cytosine arabinoside ($3{\cdot}7 \times 10^{-7}$ M, 2370 μc/μmole), cytidine (1×10^{-5} M) and deoxycytidine (1×10^{-5} M) as indicated. After 20 min of incubation, the fractions were prepared as described under Methods and assayed for radioactivity.

resistant to cytosine arabinoside had no detectable deoxycytidine kinase activity, yet grew in culture as rapidly as the parent sensitive line.[33] Thus, deoxycytidine kinase did not appear to be essential for cell reproduction in the absence of cytosine arabinoside, yet it was essential for the conversion of cytosine arabinoside to an active

inhibitor. Furthermore, deoxycytidine triphosphate, which is essential for the synthesis of DNA by mammalian cells, in culture is normally derived from the *de novo* pathway through cytosine ribonucleotide reductase. It has been proposed that the activity of this reductase was inhibited by a phosphoderivative of cytosine arabinoside.[14] Inhibition of cytidine diphosphate reductase may result in cell death, since it can be seen in Fig. 2 that cells exposed to cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M) for 2 hr were

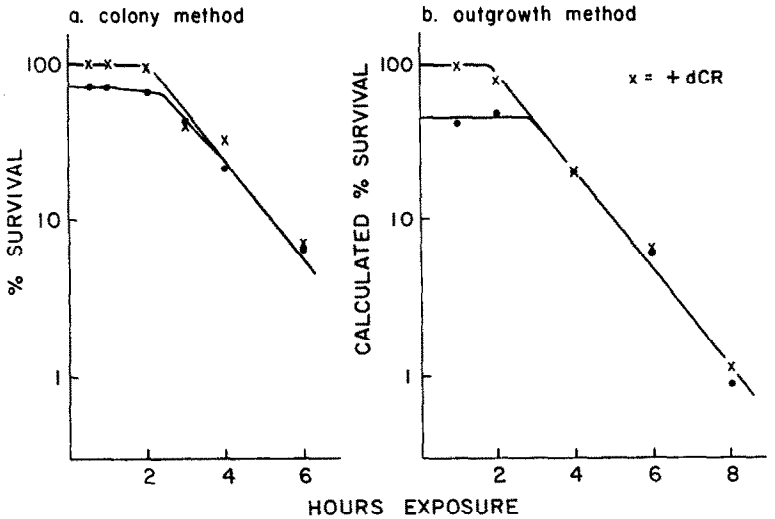

FIG. 2. The kinetics of cell death by the dilute agar colony method and by the outgrowth method. L5178Y cells (approximately 1×10^{4}/ml) in the exponential phase of growth were exposed to $3{\cdot}3 \times 10^{-6}$ M cytosine arabinoside for different periods of time. The cells were removed from the drug and diluted in order to provide 60 colonies per tube (cloning efficiency of noninhibited control cells approximately 75 per cent), which were treated as described in Methods and normalized to a cloning efficiency of 100 per cent. The cells in the outgrowth method were treated as described in Methods.

rescued by subsequent exposure to deoxycytidine. The rescue by deoxycytidine has been attributed to the ability of inhibited cells to form deoxycytidine triphosphate, *via* appropriate kinases from exogenous deoxycytidine,[34] necessary for the synthesis of DNA and cell division. It was of interest that the colony method (Fig. 2a), which measured the ability of the cells to multiply extensively (1 to 10×10^{4} cells per colony), was in close agreement with the outgrowth method (Fig. 2b), which determined cell kill plus delay of cell division, since deoxycytidine could reverse the inhibitory effect of cytosine arabinoside after 2 hr of treatment. It can also be seen from Fig. 3 that cells treated with cytosine arabinoside at $3{\cdot}3 \times 10^{-7}$ M for 6 hr were rescued by deoxycytidine when determined by the outgrowth method. An unknown effect of cytosine arabinoside was implicated, since cells treated for 3–8 hr with $3{\cdot}3 \times 10^{-6}$ M cytosine arabinoside could not be rescued by deoxycytidine although the drug-treated cells effectively converted deoxycytidine to deoxycytidine triphosphate.[34] This cellular injury after acute treatment with the analog was quantitatively similar either in the colony method (Fig. 2a) or in the outgrowth method (Fig. 2b); the rate of cell kill determined by the agar colony method was 52 per cent per hr with a lag period of

2 hr whereas in the outgrowth method the inhibition was 55 per cent per hr with a lag period of 1·8 hr. From these data it is concluded that cell death was the major toxic effect of the analog and that delay of cell division was of minor importance. In addition, exposure to cytosine arabinoside at 1×10^{-6} M or higher for a period of 6 hr was sufficient to produce a similar acute cell death (Fig. 3). From the data of Table 3, it

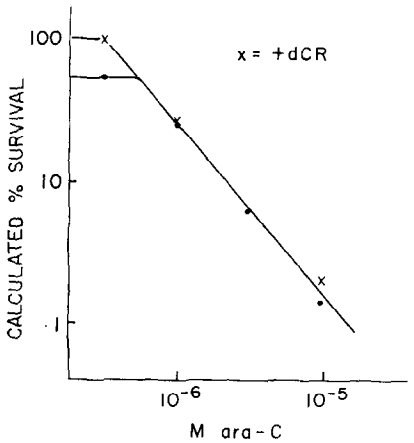

FIG. 3. Inhibition produced by the incubation of L5178Y cells with different levels of cytosine arabinoside for 6 hr. L5178Y cells (approximately 1×10^4/ml) in the exponential phase of growth were exposed to different levels of cytosine arabinoside for 6 hr. The drug was removed and the cells were diluted in growth medium (●—●) and in growth medium containing 5×10^{-5} M deoxycytidine (×—×). The cells were maintained in the logarithmic phase of growth by dilution in growth medium (37°) and the extent of inhibition was determined as described in Methods, IV A (the outgrowth method).

can also be seen that this mechanism of kill was maximal at 1×10^{-5} M cytosine arabinoside after only 2 hr of treatment. It seems possible that this acute cell death, caused by high levels of cytosine arabinoside for short periods of time, results from an incorporation of cytosine arabinoside into the nucleic acids.[14, 16]

TABLE 3. ACUTE CELL DEATH PRODUCED BY EXPOSURE OF CELLS TO CYTOSINE ARABINOSIDE FOR 2 HR*

	0	1×10^{-6}M	3×10^{-6}M	1×10^{-5}M	3×10^{-5}M	1×10^{-4}M	3×10^{-4}M
Total cells inoculated	150	200	180	240	300	720	1250
Total colonies recovered	104	121	114	33	36	80	144
% Recovery	69	61	63	14	12	11	11
% Survivors (normalized)	100	87·8	92·7	20	17	16	16

* L5178Y cells (1×10^4 cells/ml) in the exponential phase of growth were exposed to different concentrations of cytosine arabinoside. After 2 hr of incubation at 37°, the drug-treated and control cells were centrifuged for 5 min at approximately 800 *g* and resuspended in growth medium (37°) without drug. This procedure of centrifugation and resuspension which terminated treatment with drug was repeated twice. After determination of cell concentration, cell viability was determined by the dilute agar colony method.

The results in Table 4 suggest that cells incubated with ^{3}H-cytosine arabinoside have incorporated a portion of the analog in terminal portions of the nucleic acid, since a a part of the radioactivity from RNA was found in the effluent fraction of an Ecteola column, and behaved subsequently as cytosine arabinoside. Although the effluent radioactivity in this experiment was not quantified (Table 4, column 4), it had been

TABLE 4. ENZYMATIC HYDROLYSIS OF ^{3}H-CYTOSINE ARABINOSIDE RNA*

Fraction	Method of preparation	Total radioactivity in RNA (%)	Total radioactivity recovered as cytosine arabinoside (%)
Effluent	salt	18	+
	isopropanol		
Nucleotide	salt	37	12
	isopropanol	30	16

* Approximately 8×10^7 L5178Y cells were incubated at 37° for 20 min with ^{3}H-cytosine arabinoside (2370 μc/μmole, $7{\cdot}5 \times 10^{-7}$ M) and the RNA was prepared by the modified salt extraction method (total cpm, 7640) or the isopropanol method of Marmur[23] (total cpm, 2360), degraded enzymatically (Methods) and chromatographed on an Ecteola column. The monophosphate fraction, after hydrolysis with alkaline phosphatase, behaved like cytosine arabinoside when chromatographed in solvent 1.

found that the radioactivity was not released from the RNA fraction by dialysis. An RNA fraction (1200 cpm) prepared according to the method of Marmur was dialyzed at 4° against 10 vol. of water for 24 hr and 1100 cpm was recovered as the nondialyzable fraction. Of the radioactivity in the mononucleotide fraction from RNA, prepared either by the modified salt extraction method or by the isopropanol fractionation method, 12 and 16 per cent of the radioactivity of the starting material (or approximately 40 per cent of the radioactivity in the mononucleotide fraction) were recovered as cytosine arabinoside after treatment with alkaline phosphatase and chromatography in solvent 1. On the paper chromatogram a radioactive material was found intermediate between cytosine arabinoside monophosphate and cytosine arabinoside. This intermediate material was resistant to alkaline phosphatase. The data in Table 5, which were obtained after alkaline hydrolysis of the RNA fraction, also illustrated that a fraction of the total radioactivity not retained on the Ecteola column chromatographed as cytosine arabinoside. It was also found that at the higher level of analog and with a longer period of incubation (6 hr), the fraction of the total radioactivity which behaved as terminal ^{3}H-cytosine arabinoside increased sharply (Table 5). It is possible that an increase of cytosine arabinoside in the terminal positions of RNA could produce acute cell death.

The major radioactive fraction from RNA of the 20-min incubations chromatographed as a dinucleotide. Inversion of the 2′-hydroxyl group of cytosine arabinoside in RNA presumably had conferred a resistance to hydrolysis by alkali and resulted in a dinucleotide which eluted from the column more slowly than the monophosphoderivatives that constituted the bulk of the material from the RNA fraction as determined by absorbancy at 260 mμ. A slow release of radioactive mononucleotide and effluent radioactivity from the dinucleotide fraction was accomplished by a second treatment with alkali. Thus, at least a part of the radioactivity of the effluent and mononucleotide fractions of the first alkaline hydrolysate might have originated from and internal cytosine arabinoside. When the dinucleotide fraction was treated with

venom phosphodiesterase, free cytosine arabinoside was released in good yield (Table 5, column 4) indicating that the majority of radioactivity was present as dinucleotide. From these studies with RNA prepared by different methods, it was concluded that cytosine arabinoside was incorporated internally in phosphodiester linkage[14] and that terminal incorporation into RNA might also occur. The ratio of

TABLE 5. BASE HYDROLYSIS OF ^{3}H-CYTOSINE ARABINOSIDE RNA*

Fraction	Expt. No.	Method of preparation	Total radioactivity of RNA (%)	Total radioactivity recovered as ^{3}H-cytosine arabinoside (%)
Effluent	1	isopropanol	5	4
	2	isopropanol	6	6
	3	salt	70	35
Mononucleotide	1	isopropanol	4	3
	2	isopropanol	7	3
	3	salt	11	
Dinucleotide + oligonucleotide	1	isopropanol	91	79
	2	isopropanol	87	81
	3	salt	18	

* Approximately 4×10^8 L5178Y cells were incubated for 20 min (Expt. 1 and 2) in the presence of ^{3}H-cytosine arabinoside (2370 μc/μmole, $3{\cdot}7 \times 10^{-7}$ M). In experiment 3, approximately 2×10^8 cells were incubated for 6 hr with $3{\cdot}3 \times 10^{-6}$ M ^{3}H-cytosine arabinoside (215 μc/μmole). The RNA was obtained by base hydrolysis. The total RNA radioactivity of the different experiments were: expt. 1, 7630 cpm; expt. 2, 5280 cpm; expt. 3, 7300 cpm. The hydrolysate was chromatographed on an Ecteola column (Methods) and the recovery of radioactivity is recorded in column 3. The effluent was chromatographed in solvent 1 for 96 hr. The nucleotide fraction was treated with alkaline phosphatase prior to chromatography in solvent 1. The dinucleotide plus oligonucleotide fraction was hydrolyzed with venom phosphodiesterase; the radioactivity was recovered in the effluent fraction from an Ecteola column and identified by chromatography in solvent 1.

cytidine to cytosine arabinoside was approximately 1×10^6:1 for the short-term incubations and 4×10^4:1 for the long-term incubations. For the DNA, enzymatic methods were used to release cytosine arabinoside as a nucleotide. The data in Table 6 indicate that the major fraction of radioactivity which behaved as a mononucleotide on Ecteola columns chromatographed as cytosine arabinoside in solvent 1 after treatment with appropriate phosphatases.

In order to determine whether an incorporation of cytosine arabinoside could be correlated with irreversible inhibition of cell reproduction, a time-course study was carried out. The data of Fig. 4 indicate that the incorporation of cytosine arabinoside into the cold acid-soluble fraction (primarily cytosine arabinoside-5′-triphosphate, secondarily cytosine arabinoside-5′-diphosphate and a small amount of cytosine arabinoside-5′-monophosphate[14]) was slower in the first 2-hr interval than in the interval from 2 to 4 hr. It is possible that cytosine arabinoside increased the activity of deoxycytidine kinase[35] or that deoxycytidine kinase activity is dependent upon the stage of the cell cycle. The mechanism of acute cell death in the 2- to 4-hr interval might be attributed to an acid-soluble phosphorylated derivative of cytosine arabinoside, since it has been found that adenine arabinoside triphosphate was a noncompetitive inhibitor of mammalian DNA polymerase[35] and it has been suggested that cytosine arabinoside triphosphate inhibited mammalian DNA polymerase.[36] The molarity of cytosine arabinoside triphosphate after 2 hr of exposure to the analog in

this study was estimated to be 1×10^{-6} M, whereas the level of deoxycytidine triphosphate in uninhibited cells was estimated to be approximately $1{\cdot}6 \times 10^{-5}$ M. Cytosine arabinoside triphosphate might have produced a significant inhibition of DNA polymerase, since the deoxycytidine triphosphate pool may have been depleted in the analog-treated cells. Phosphorylated derivatives of cytosine arabinoside in the

TABLE 6. INCORPORATION OF ^{3}H-CYTOSINE ARABINOSIDE INTO DNA

Fraction	Expt. No.	Total radioactivity (%)	Total radioactivity recovered as cytosine arabinoside (%)	Unknown
Effluent	1†	35	7	24
	2†	0		
	3†	0		
	4‡	47	35	
Nucleotide	1†	57	45	4
	2†	120	84	
	3†	82	63	
	4‡	48		
Oligonucleotide	1†	19		
	2†	0		
	3†	17		
	4‡	18		

* The DNA of experiments 1 and 2 was prepared by incubation of approximately 4×10^8 cells for 20 min with ^{3}H-cytosine arabinoside ($3{\cdot}7 \times 10^{-7}$ M, 2370 μc/μmole) and the DNA prepared according to the isopropanol method of Marmur[18] (820 and 1650 total cpm, respectively). The DNA of experiment 3 was prepared from similar cells, except that the DNA was prepared by the modified salt extraction method (484 total cpm). The DNA of experiment 4 was prepared by incubation of approximately 2×10^8 cells for 360 min with ^{3}H-cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M, 215 μc/μmole) and alkaline hydrolysis of the RNA prior to DNA extraction (5900 cpm), according to the modified salt extraction method (Methods). After treatment first with DNase I and then with venom diesterase, the DNA hydrolysate of experiments 1, 2, 3 and 4 was fractionated on an Ecteola column, and the mononucleotide treated with bull semen 5′-phosphatase and recovered as cytosine arabinoside by chromatography in solvent 1 (96 hr).

† 20-min incubations.

‡ 360-min incubations.

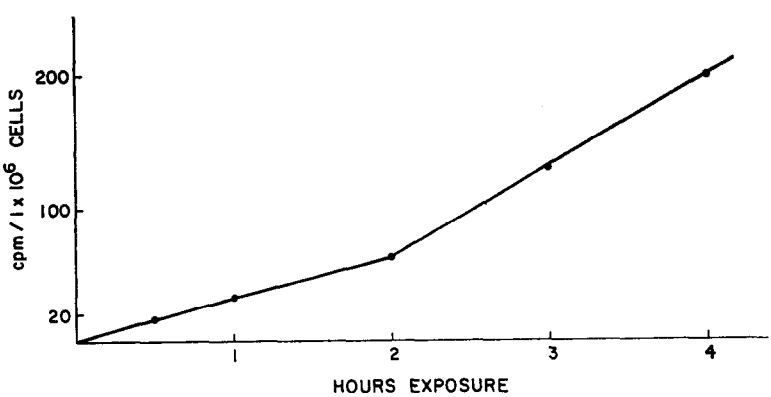

FIG. 4. Time course of ^{3}H-cytosine arabinoside uptake into the cold acid-soluble fraction. L5178Y leukemic cells (approximately 3×10^5/ml) in the exponential phase of growth were exposed to ^{3}H-cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M, 106 μc/μmole). The cold acid-soluble fraction was prepared (Methods) from approximately 6×10^6 cells at each of the periods of time indicated. The radioactivity was determined by liquid scintillation counting.

cold acid-soluble fraction disappeared quickly from the cells when the extracellular analog was removed[32] however, indicating that if this fraction were to produce acute cell death, the continued presence of the anlog was not essential to the process. From the data of Fig. 5 it was evident that the rate of incorporation into DNA was linear in

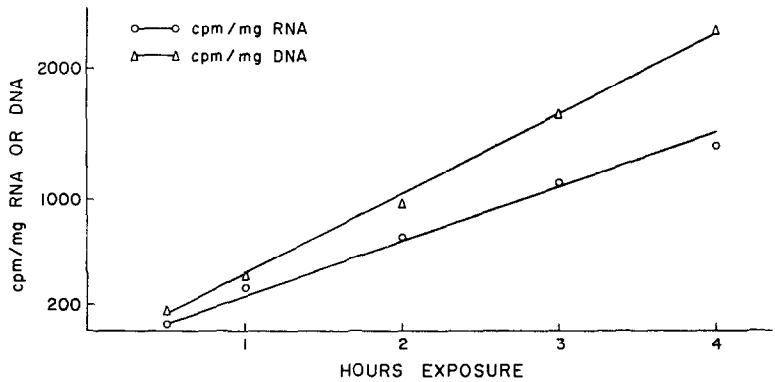

FIG. 5. Time course of ^{3}H-cytosine arabinoside uptake into the RNA and DNA. L5178Y cells (approximately 3×10^5/ml) in the exponential phase of growth were exposed to ^{3}H-cytosine arabinoside ($3{\cdot}3 \times 10^{-6}$ M, 106 μc/μmole). The RNA and DNA were prepared (Methods) from approximately 6×10^6 cells at each of the periods of time indicated. The radioactivity was determined by liquid scintillation counting and the concentration of nucleic acids was determined by the absorbancy at 260 and 280 mμ.

the time interval of 30–240 min, while the rate of incorporation into RNA was linear from 30–180 min. Since neither incorporation could be extrapolated to time zero, it was suggested that a slower rate of initial incorporation had occurred. If the acute cell death produced after 2 hr of incubation with cytosine arabinoside was due to incorporation into RNA, an estimated ratio of cytidine to cytosine arabinoside of $2{\cdot}7 \times 10^4$:1 would be sufficient to obtain this effect, and if the unknown lethal mechanism was due to incorporation into DNA, an estimated ratio of deoxycytidine to cytosine arabinoside of $2{\cdot}0 \times 10^4$:1 would be sufficient. Conversely, it was calculated that a cytidine to cytosine arabinoside ratio of $4{\cdot}7 \times 10^4$:1 and a deoxycytidine to cytosine arabinoside ratio of $3{\cdot}3 \times 10^4$:1 did not prevent the cells from being rescued by subsequent exposure to deoxycytidine.

Similar time-course studies with a higher level ($3{\cdot}3 \times 10^{-5}$ M) of the analog demonstrate that the incorporation of cytosine arabinoside into DNA rapidly reached a plateau at a ratio of deoxycytidine to cytosine arabinoside of $3{\cdot}5 \times 10^4$:1, and that deoxycytidine did not rescue the cells. The rate of cell death at the higher level of cytosine arabinoside was very similar to that described in Fig. 2a. Since at $3{\cdot}3 \times 10^{-5}$ M cytosine arabinoside cell death increased with time while incorporation into the DNA was arrested, these phenomena would not appear to be closely related. Accumulation of cold acid-soluble derivatives is not a favored mechanism of cell death due to the short half-life of this fraction. It seems possible that acute cell death might result from an incorporation of cytosine arabinoside into terminal positions of RNA. The incorporation into RNA was also linear through 180 min of incubation at $3{\cdot}3 \times 10^{-6}$ M or $3{\cdot}3 \times 10^{-5}$ M analog. It seems likely that acute cell death was due to incorporation into the RNA fraction, since these two phenomena were closely related.

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