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COMPARATIVE STUDIES OF LEUKEMIC CELLS SENSITIVE AND RESISTANT TO CYTOSINE ARABINOSIDE*

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Abstract—A clone of L5178Y cells, resistant to growth inhibition caused by 1-β-D-arabinofuranosylcytosine (cytosine arabinoside), has been isolated. Suspensions of the drug-resistant cells had an impaired capacity to form phosphorylated derivatives of cytosine arabinoside. The enzymatic capacity of extracts of the resistant cells was correspondingly defective in the conversion of cytosine arabinoside and deoxycytidine to the corresponding 5′-phosphate esters. It is proposed that a phosphorylated form of cytosine arabinoside is the active inhibitor of an enzyme involved in the conversion of cytidine (as the 5′-diphosphate) to the corresponding derivative of deoxycytidine.

OUR previous studies demonstrated that the primary growth inhibitory effect of 1-β-D-arabinofuranosylcytosine (cytosine arabinoside) upon leukemia cells (L5178Y) in culture is to prevent the conversion of cytidine into phosphorylated derivatives of deoxycytidine (and hence, of DNA), without greatly affecting the conversion of uridine into phosphorylated derivatives of uridine, cytidine, or RNA.[1] This evidence has been substantiated by whole-cell studies with ^{3}H-uridine as the substrate, in the presence and absence of cytosine arabinoside, in which the various pyrimidine nucleoside mono-, di- and triphosphates were recovered. Although the principal radioactivity occurred in known phosphoderivatives of uridine, it was found that inhibition by cytosine arabinoside appeared to affect primarily the incorporation of ^{3}H-uridine into deoxycytidine-5′-diphosphate without greatly affecting the formation of cytidine-5′-diphosphate.[2]

MATERIALS AND METHODS

Cell reproduction experiments were done under conditions described previously.[3] The L5178Y line resistant to cytosine arabinoside, *ca*^{k}, was derived by the incubation of 1×10^8 L5178Y cells (parent strain) at 37° with a level of cytosine arabinoside (1×10^{-6} M) sufficient completely to inhibit the reproduction of the sensitive cells. The medium containing the inhibitor was renewed every 5 days. After incubation for 12 days, reproduction of the resistant cells present in the initial population had provided about 10^6 cells. At this time the medium was replaced by one containing none of the deoxycytidine analog, single cells were isolated,[3] and the progeny were recovered as a clone; this procedure provided the line, *ca*^{k}, used in these studies. This line has quantitatively retained its characteristic resistance during continuous reproduction

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in culture for a period of 36 months in medium free from inhibitor, and the modal chromosome number (40) has not differed from that of the sensitive L5178Y cells.[4] The concentration of inhibitor required to decrease the number of cell generations, per unit time, to about one half that obtained in the drug-free controls (Gd50), was $4{\cdot}7 \times 10^{-6}$ M, an increase by a factor of 36 over that required to produce a comparable inhibition of the original parent L5178Y clone (Fig. 1).

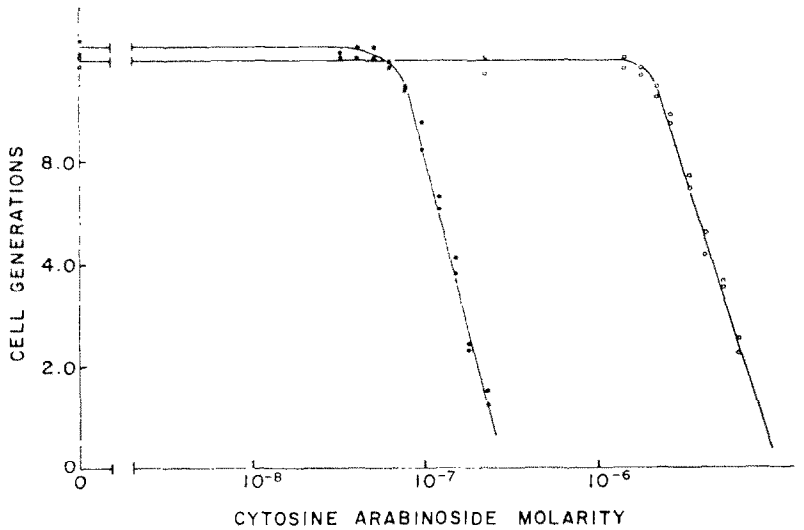

FIG. 1. Degree of resistance of *ca*^{k} cells to cytosine arabinoside. Sensitive (●) and resistant (○) cells at an initial concentration of 2×10^3 per ml were incubated at approximately 37° with levels of cytosine arabinoside (in duplicate) for a period of 96 hr. The level of analog that would have provided only one-half the generations undergone by the control (Gd_{50}) was determined. The results presented were obtained in one of several similar experiments.

For studies of the entry of cytosine arabinoside into sensitive and resistant cells and its subsequent phosphorylation, a previously described method was used.[5] In these experiments, 1×10^8 cells per ml in growth medium containing ^{3}H-cytosine arabinoside ($7{\cdot}5 \times 10^{-6}$ M, 542 μc/μmole), were incubated at 37°; aliquots of 0·8 ml were removed at 0, 5, 12·5, and 20 min, respectively, and centrifuged at 0° in Wintrobe hematocrit tubes for 10 min at approximately 800 *g*. After determination of the packed-cell volume, the cells were extracted with cold 0·2 N $HClO_4$ to obtain the acid-soluble fraction, which was neutralized with KOH; the supernatant fraction was analyzed on Ecteola columns for free analog and for phosphorylated derivatives, according to the method described below for the study of deoxycytidine kinase activity. From these determinations, the ratio in the cells of ^{3}H-cytosine arabinoside and the ratio of its phosphorylated derivatives to the concentration of the analog in the medium were calculated. Of the packed cell volume, 20% consisted of extracellular fluid, and the cytosine arabinoside in the medium was not significantly altered during the period of incubation. Consequently, from the ^{3}H-cytosine arabinoside of the packed cells, 20% was subtracted and the remainder (80%) represented the intracellular levels of free ^{3}H-cytosine arabinoside.

In other whole-cell incubations in which the DNA and RNA fractions were recovered, $3{\cdot}2 \times 10^8$ cells of sensitive and of resistant lines were simultaneously incubated

at 37° for 20 min in 20 ml of growth medium containing ^{3}H-cytosine arabinoside ($3{\cdot}7 \times 10^{-7}$ M; 2,370 μc/μmole). To stop the reaction, tubes were transferred to an ice-bath and centrifuged at 0° for 10 min at about 800 *g* in 12-ml conical centrifuge tubes. The supernatant fraction was withdrawn with a pipet, and most of the residual supernatant fluid was removed by wiping the inner surfaces of the inverted tubes with absorbent paper. The cells were then fractionated for (a) cold acid-soluble fraction, (b) RNA, and (c) DNA, as in the previous work.[1] The total radioactivity of each fraction was determined with a liquid scintillation counter. The mono-, di- and triphosphate derivatives of cytosine arabinoside were separated on cellulose anion-exchange paper AE 30,[6] and on Ecteola columns (Table 2).

For the preparation of deoxycytidine kinase, cells from culture were harvested during their logarithmic phase of growth with a continuous flow centrifuge (Servall model RC-2) at 0° and 13,000 *g*. The cells were washed twice with ice-cold serum-free medium, the suspension was frozen rapidly and thawed three times, in a dry ice–ethanol bath; they were then centrifuged at 0° for 1 hr at 30,000 *g*. The supernatant fluid thus obtained was stored at −4°. The complete enzyme incubation mixture in 0·2-ml final volume contained ATP, $2{\cdot}25 \times 10^{-3}$ M; $MgCl_2$, $3{\cdot}5 \times 10^{-3}$ M; ^{3}H-deoxycytidine, 0·0015 μmoles; protein, 0·2 mg; Tris buffer, 10·0 μmoles (pH 7·95).

The reaction was terminated by heating for 2 min in a boiling-water bath and, after centrifugation, a 0·1-ml aliquot was diluted to 4·5 ml (pH 7·5). Subsequently, 1·2 μmoles of cytidine-5′-monophosphate and 1·2 μmoles of cytosine arabinoside in a volume of 0·5 ml were added, and the phosphorylated derivatives were adsorbed on Ecteola hand columns (1 × 10 cm) in the chloride form.[7] Columns were prepared from aliquots of an Ecteola suspension in water, which had been prepared previously by the elution of uv.-absorbing material with 12 liters of 0·1 N NaOH, followed by 12 liters of 0·1 N HCl and 12 liters of distilled water. Before use the hand columns were charged with 0·003 N HCl until the pH of the effluent reached 2·6–2·7. The phosphorylated compounds were absorbed from a 4·0 ml aliquot (pH 7·5) of the diluted reaction mixture. The unreacted substrate was recovered in 15 ml of water wash and in the first 5-ml fraction containing 0·01 N HCl as the developer. The phosphate esters were recovered with 0·2 N HCl, and their radioactivity was quantified in a liquid scintillation counter; their cytosine content was determined by the absorption at 280 mμ.

Radioactive ^{3}H-deoxycytidine and ^{3}H-cytosine arabinoside, labeled primarily in the pyrimidine ring, were obtained from Schwarz Bio-Research Inc. The ^{3}H-deoxycytidine was purified on Dowex-50 H^+ form resin (200 × 400 mesh), followed by chromatography in a system previously described, in which deoxycytidine was seperated from cytidine with 1·0 N HCl as the developer.[1] The HCl in the fraction recovered was partially removed by storage for 48 hr in a vacuum desiccator containing dry NaOH. Subsequently, the volume was reduced by distillation at approximately 40° in a Buchler flash evaporator, and an equal volume of water was added to dilute the remaining HCl. This process was repeated nine times. The remaining acid was neutralized with NaOH and the purity confirmed by paper chromatography and by the spectrum in the u.v. region (specific activity determined in the liquid scintillation counter: 328 μc/μmole). The purity of the ^{3}H-cytosine arabinoside was confirmed by its homogeneity on chromatography (Whatman 1 paper and three solvent systems). Two solvent

systems (1 and 2)* are able to separate cytosine arabinoside from cytidine, while the third system, no. 3,* in addition separates deoxycytidine, cytosine arabinoside, uracil arabinoside,† cytidine, and cytosine. The radioactive material was stored at pH 7·0, −4°, and a concentration of 40 μc/ml. After each successive interval of 4 months, a small amount of decomposition product was removed by rechromatography in solvent 3 and elution from paper.

RESULTS AND DISCUSSION

Cytosine arabinoside inhibits the growth of certain transplantable neoplasms of mice[8] and is currently being studied as a possible tumor inhibitory substance in man[9,10]. A comparative biochemical study of murine leukemic cells sensitive and resistant to cytosine arabinoside was undertaken not only to provide information that might be relevant to an understanding of the disposition and effectiveness of this agent in man but also to develop a better understanding of the metabolism and mode of action of this inhibitor of the formation of deoxycytidine-5′-diphosphate.

It would be predicted that a mutant drug-resistant cell would differ from sensitive cell in biochemical reactions unrelated to the property of resistance when: (1) heterologous cell lines (e.g. P815Y mastocytoma cells and cells of the Mecca lymphosarcoma[4]), or (2) noncloned lines, or (3) mutants that differ from the parent-sensitive clone in chromosome number[11] are compared. The method of selection of the ca^k mutant clone, however, was designed to recover the progeny of one cell in which a mutagenic event had conferred resistance (a "single-step" mutant). Furthermore, the chromosomes of line ca^k could not be distinguished from those of the sensitive L5178Y cells.[4] It has also been found that the sensitivity of ca^k cells is unaffected with respect to a number of antimetabolites[4] (e.g. methotrexate, pyrimethamine, azauridine, 5-fluorodeoxyuridine and 5-iododeoxyuridine), which appear to inhibit the reproduction of L5178Y cells at sites other than those involving the enzymatic formation of deoxycytidine-5′-diphosphate. These data are in agreement with earlier findings concerning mutation to resistance to methotrexate in L5178Y cells, from which it had been concluded that a single mutational event that confers resistance could affect but one biochemical property of the mutant cell.[4, 12–14] Thus it is possible that ca^k cells differ from L5178Y-sensitive cells in but one biochemical aspect and that this alteration is the molecular basis of resistance to cytosine arabinoside.

The intracellular concentrations of free cytosine arabinoside were very similar in sensitive and resistant cell populations (Table 1), a circumstance indicating that the extent of cellular uptake of the drug is not related to the degree of resistance in this line. Furthermore, resistance could not be attributed to deamination of cytosine arabinoside to uracil arabinoside, or to cleavage to cytosine, since descending chromotography of aliquots of media or of cold-acid-soluble fractions in solvent 3 for 72 hr demonstrated that 80 to 100% of the radioactivity traveled with known cytosine arabinoside. Since the radioactivity of the fraction from Ecteola columns that would contain phosphorylated derivatives of cytosine arabinoside was considerably reduced in resistant cell preparations (Table 1), similar fractions were prepared from larger

* Solvent 1: 20 ml ammonium acetate, pH 9·5; 80 ml saturated sodium tetraborate; 220 ml 95% ethanol; 1 ml 0·25 M EDTA. Solvent 2: 400 ml isobutyric acid; 208 ml water; 0·4 ml concentrated ammonium hydroxide. Solvent 3: 94·6 ml 86% *n*-butanol; 5·4 ml concentrated ammonium hydroxide.

† The uracil arabinoside was generously provided by Dr. Jack Fox of the Sloan-Kettering Institute, Rye, N.Y.

quantities of sensitive and resistant cells to characterize the phosphate esters present and to isolate the nucleic acid fractions.

In order to characterize the phosphorylated derivatives of cytosine arabinoside, the 5′-phosphate ester was synthesized[2] and used as a known reference compound. The reference material used for the diphosphate fractions was deoxycytidine-5′-diphosphate; for triphosphate fractions deoxycytidine-5′-triphosphate was used as

TABLE 1. RATIO OF INTRACELLULAR FREE ^{3}H-CYTOSINE ARABINOSIDE AND ITS PHOSPHATE ESTERS TO EXTRACELLULAR FREE ^{3}H-CYTOSINE ARABINOSIDE

	Sensitive		Resistant	
	^{3}H-CA cell pack	^{3}H-CAP cell pack	^{3}H-CA cell pack	^{3}H-CAP cell pack
Incubation (min)	^{3}H-CA medium	^{3}H-CA medium	^{3}H-CA medium	^{3}H-CA medium
0	0·28	0·25	0·40	0·13
5	0·39	0·63	0·46	0·14
12·5	0·60	0·82	0·62	0·28
20	0·78	1·0	0·78	0·28

Sensitive and resistant cells at a concentration of 1×10^8 per ml were incubated at 37° with ^{3}H-cytosine arabinoside ($7{\cdot}5 \times 10^{-6}$ M, 542 μc/μmole) and aliquots were removed for each time interval by centrifugation. From the cold-acid-soluble fraction, free cytosine arabinoside was separated from the phosphorylated derivatives on Ecteola hand columns and the recoveries of radioactivity and of added carriers were determined. After correction for extracellular fluid (containing free cytosine arabinoside) of the packed-cell volume, intracellular concentrations of free cytosine arabinoside and its phosphorylated derivatives were calculated.

the carrier. The cold-acid-soluble fractions of sensitive and resistant cells were resolved into mono-, di- and triphosphate fractions by chromatography on Ecteola columns developed with a gradient of LiCl in 1×10^{-3} M Tris buffer, pH 7·4 (Table 2).

TABLE 2. TOTAL RADIOACTIVITY OF SENSITIVE AND RESISTANT CELLS

Cell line	CAP	CADP (cpm $\times 10^{-3}$)	CATP	RNA	DNA
Sensitive	7·5	34	103	4·3	0·52
Resistant	4·9	17	39	2·1	0·17

Sensitive and resistant cells ($3{\cdot}2 \times 10^8$ each) were incubated for 20 min in the presence of ^{3}H-cytosine arabinoside (2,370 μc/μmole), $3{\cdot}7 \times 10^{-7}$ M; the reaction was terminated by chilling. After centrifugation, 1·0 μmole of each carrier was added, the cell fractions were isolated and the recovery of radioactivity and nonradioactive carriers was determined. Abbreviations: cytosine arabinoside, CA; cytosine arabinoside-5′-phosphate, CAP; cytidine-5′-diphosphate, CADP; cytidine-5′-triphosphate, CATP.

The behavior of the radioactivity recovered in the monophosphate peak was identical with that of 1-β-D-arabinofuranosylcytosine-5′-phosphate in several solvent systems and, after treatment with bull semen 5′-phosphatase, 90% or more was converted to free cytosine arabinoside. The cytosine arabinoside was recovered in solvent systems 1 and 3. The triphosphate fraction stored at 4° (pH 7·0) for four weeks was

in part converted to material with the chromatographic behavior of a diphosphate and to a lesser extent to material resembling the 5′-monophosphate. Under the same conditions of storage, the diphosphate fraction was in part converted to material with the chromatographic behavior of the 5′-monophosphate. After treatment of these monophosphate fractions with 5′-phosphatase, the radioactivity was essentially all recovered with the carrier cytosine arabinoside after chromatography in solvent 3. It was concluded that the sensitive cells (and to a lesser degree the resistant cells) convert cytosine arabinoside to the 5′-monophosphate ester, and apparently to the corresponding cytosine arabinoside di- and triphosphoderivatives as well.

The radioactivity in RNA and DNA fractions of sensitive cells has been recovered to the extent of approximately 50 to 90% as cytosine arabinoside after the sequential treatments with (1) RNase or DNase, (2) venom phosphodiesterase, (3) chromatography on a neutral Ecteola column and recovery of the phosphoderivatives, (4) treatment with alkaline phosphatase or bull semen 5′-phosphatase, and (5) recovery of cytosine arabinoside in solvent 3. From these results it has been concluded that this analog can be incorporated into phosphonucleotide linkage in both RNA and DNA fractions of the cell. The lower radioactivity of DNA and RNA fractions of resistant cells incubated with ^{3}H-cytosine arabinoside may be attributed to the lower levels of the cytosine arabinoside-5′-triphosphate found in these cells (Table 2).

In order to determine whether the lower level of phosphorylated derivatives of cytosine arabinoside found in resistant cells might result from a kinase deficiency, comparative enzyme studies of sensitive and resistant cells were undertaken with cytosine arabinoside and deoxycytidine as substrates. For the enzymatic phosphorylation of deoxycytidine, a broad range of pH (7·0–9·0) resulted in 64 to 67% conversion of substrate to phosphorylated derivatives (the mono-, di- and triphosphates). A concentration of ATP of $3{\cdot}25 \times 10^{-3}$ M was optimal for phosphorylation when the

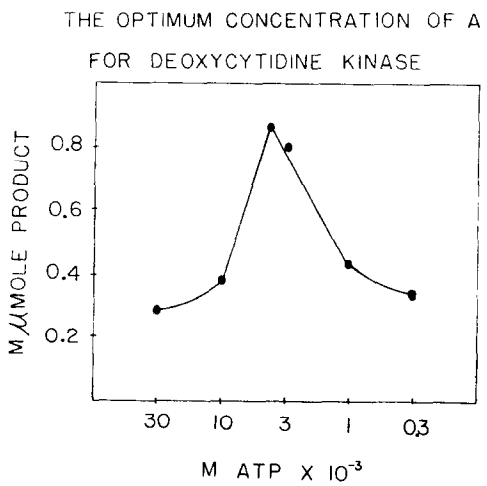

FIG. 2. The optimum concentration of ATP for deoxycytidine kinase. An enzyme preparation of L5178Y cells (0·2 mg protein) was incubated with $7{\cdot}5 \times 10^{-6}$ M radioactive deoxycytidine (328 μc/μmole), Tris buffer 5×10^{-2} (pH 7·95), and $MgCl_2$ $3{\cdot}0 \times 10^{-3}$ M, in a final volume of 0·2 ml at 37° for 20 min. After heating, the phosphorylated products were recovered with Ecteola hand columns and the radioactivity (and the nonradioactive carrier cytidine-5′-monophosphate) quantitated.

concentration of $MgCl_2$ was 3×10^{-3} M (Fig. 2); at an ATP concentration of $2{\cdot}25 \times 10^{-3}$ M, the optimal concentration of $MgCl_2$ was $3{\cdot}5 \times 10^{-3}$ M (Fig. 3). The phosphorylating system was not saturated with a substrate molarity of $4{\cdot}12 \times 10^{-6}$ M, and the phosphorylation was linearly related to time during the first 20 min of incubation (Fig. 4). Under these conditions, at equal concentrations of protein, the phosphorylation of deoxycytidine was only 57% that of the sensitive cells. This enzymatic

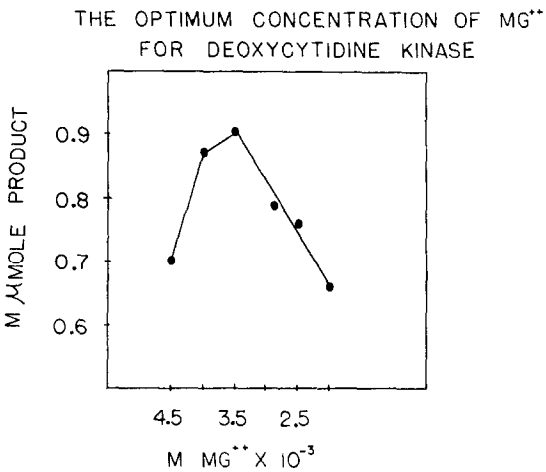

FIG. 3. The optimum concentration of Mg^{2+} for deoxycytidine kinase. An enzyme preparation of L5178Y cells (0·2 mg protein) was incubated with $7{\cdot}5 \times 10^{-6}$ M radioactive deoxycytidine (328 μc/μmole), Tris buffer 5×10^{-2} M (pH 7·95) and ATP $2{\cdot}25 \times 10^{-3}$ M, in a final volume of 0·2 ml incubated at 37° for 20 min. After heating, the phosphorylated products were recovered with Ecteola hand columns and the nonradioactive carrier cytidine-5′-phosphate and radioactivity quantitated.

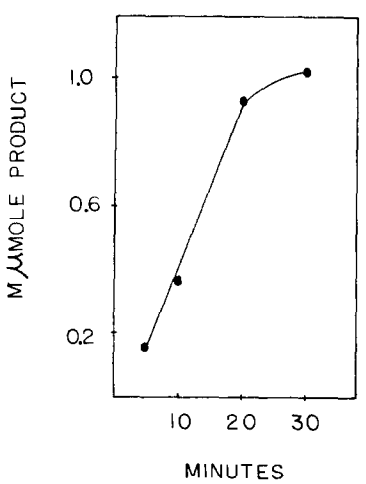

FIG. 4. The linear time course of deoxycytidine kinase activity. An enzyme preparation of L5178Y cells (3·0 mg protein) was incubated at 37° with $7{\cdot}5 \times 10^{-6}$ M radioactive deoxycytidine (328 μc/μmole), Tris buffer 5×10^{-2} M, ATP $2{\cdot}25 \times 10^{-3}$ M and $MgCl_2$ $3{\cdot}5 \times 10^{-3}$ M, and incubated at 37° (volume 3·0 ml). Aliquots of 0·2 ml were removed at points in time and the phosphorylated products recovered with Ecteola hand columns.

deficiency might indicate that deoxycytidine kinase of sensitive cells is capable of the phosphorylation of cytosine arabinoside. Thus molar excess of nonradioactive cytosine arabinoside ($2{\cdot}28 \times 10^{-5}$ M) diminished by 33% the phosphorylation of ^{3}H-deoxycytidine ($7{\cdot}14 \times 10^{-6}$ M) in enzyme preparations of sensitive cells, an effect possibly attributable to a common site for phosphorylation, presumably deoxycytidine kinase. Deoxycytidine at $7{\cdot}5 \times 10^{-6}$ M thus prevented by 60% the phosphorylation of an excess of ^{3}H-cytosine arabinoside ($7{\cdot}5 \times 10^{-5}$ M), indicating further that the affinity of deoxycytidine kinase for cytosine arabinoside is much less than that for deoxycytidine. The inability of deoxycytidine, even at $2{\cdot}25 \times 10^{-4}$ M, completely to prevent the phosphorylation of ^{3}H-cytosine arabinoside ($7{\cdot}5 \times 10^{-5}$ M) (as well as at the presence of the lower levels of ^{3}H-cytosine arabinoside (Table 3)) in the enzyme preparation from sensitive cells may be due to either the existence of a second enzyme that can phosphorylate cytosine arabinoside or to other effects.

TABLE 3. THE EFFECT OF DEOXYCYTIDINE (dCR) UPON THE PHOSPHORYLATION OF ^{3}H-CYTOSINE ARABINOSIDE (CA)

H^3-CA $7{\cdot}5 \times 10^{-6}$ M / dCR	Sensitive		Resistant	
	cpm-CAP	% Inhibition	cpm-CAP	% Inhibition
0	13.6×10^3		5×10^3	
$7{\cdot}5 \times 10^{-6}$ M	$2{\cdot}9 \times 10^3$	78	$1{\cdot}9 \times 10^3$	61
$2{\cdot}25 \times 10^{-4}$ M	$2{\cdot}5 \times 10^3$	78	$1{\cdot}9 \times 10^3$	61
H^3-CA $7{\cdot}5 \times 10^{-5}$ M / dCR				
0	$67{\cdot}5 \times 10^3$		$32{\cdot}0 \times 10^3$	
$7{\cdot}5 \times 10^{-6}$ M	$27{\cdot}1 \times 10^3$	60	$20{\cdot}5 \times 10^3$	36
$2{\cdot}25 \times 10^{-4}$ M	$18{\cdot}1 \times 10^3$	73	$18{\cdot}8 \times 10^3$	45

Cell-free extracts (4 to 5 mg protein/ml) from sensitive and resistant cell lines were incubated at 37° in a volume of 0·2 ml for 20 min with H^3-CA (542 μc/μmole), $7{\cdot}5 \times 10^{-6}$ M, and $7{\cdot}5 \times 10^{-5}$ M as the substrate, ATP $2{\cdot}25 \times 10^{-3}$ M, $MgCl_2$ $3{\cdot}5 \times 10^{-3}$ M, and Tris buffer 5×10^{-2} M (pH 8). Phosphorylated cytosine arabinoside (CAP) was recovered from Ecteola columns and the per cent conversion determined in the presence of different levels of nonradioactive deoxycytidine.

The enzyme preparation obtained from resistant *ca*^{k} cells, as compared to that from the parent line, was deficient in the capacity to phosphorylate ^{3}H-cytosine arabinoside (Table 3). It was also found that cytosine arabinoside was not deaminated to uracil arabinoside or cleaved to cytosine by the enzyme preparation of either line, to a degree sufficient to affect significantly the levels of these substrates. Also, the deamination of cytosine arabinoside to uracil arabinoside was not detected in whole-cell incubations of either sensitive or resistant cell line. Although uracil arabinoside is not an effective inhibitor of the reproduction of sensitive cells (Gd_{50} = approximately 1×10^{-4} M) deamination of the antimetabolite by *ca*^{k} cells could not be implicated as a factor in the mechanism of resistance.

Deoxycytidine was less effective in preventing phosphorylation of cytosine arabinoside by the resistant enzyme preparation (Table 3), and the total phosphorylation in sensitive and resistant cell preparations, not inhibited by a molar excess of deoxycytidine, was very similar. Thus a defective capacity in resistant cells for conversion of

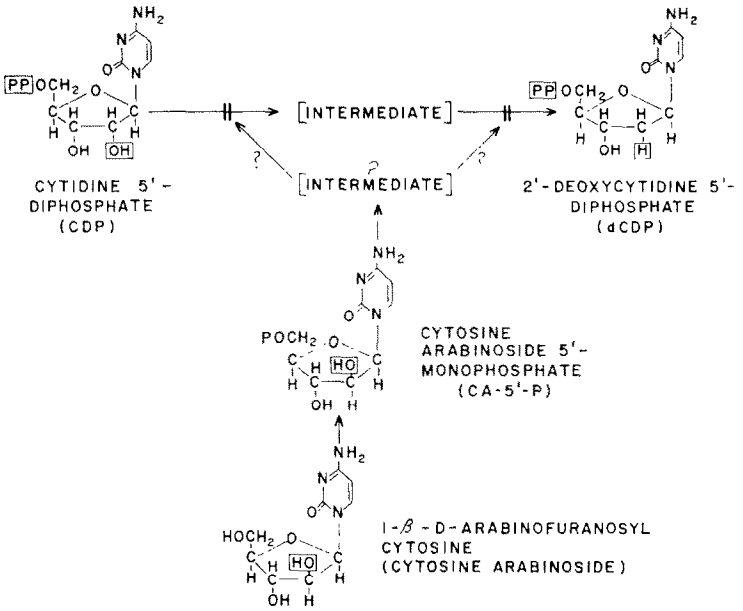

FIG. 5. A proposed mechanism of action of cytosine arabinoside as an inhibitor of the growth of leukemic cells.

cytosine arabinoside to the 5′-phosphate ester, presumed to account for the property of resistance during cellular reproduction, indicates that a phosphorylated derivative of cytosine arabinoside inhibits an enzyme that is involved in the formation of deoxycytidine-5′-diphosphate from a derivative of cytidine (Fig. 5).

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REFERENCES

1. M. Y. CHU and G. A. FISCHER, *Biochem. Pharmacol.* **11**, 423 (1962).
2. M. Y. CHU and G. A. FISCHER. Unpublished work.
3. G. A. FISCHER and A. C. SARTORELLI, *Meth. med. Res.* **10**, 247 (1964).
4. G. A. FISCHER and C. von ESSEN, *Biochem. Pharmacol.* In press.
5. R. E. HANDSCHUMACHER and G. A. FISCHER, *Meth. med. Res.* **10**, 269 (1964).
6. I. W. DELAMORE and W. H. PRUSOFF, *Biochem. Pharmacol.* **8**, 336 (1961).
7. S. M. WEISSMAN, R. M. S. SMELLIE and J. PAUL, *Biochim. biophys. Acta* **45**, 101 (1960).
8. J. S. EVANS, E. A. MUSSER, G. D. MENGEL, K. R. FORSBLAD and J. H. HUNTER, *Proc. Soc. exp. Biol. (N.Y.)* **106**, 350 (1961).
9. R. W. TALLEY and V. K. VAITKEVICIUS, *Blood* **21**, 352 (1963)
10. ARNOLD D. WELCH, *Ann. N.Y. Acad. Sci.* In press.
11. M. VOGT, *Genetics* **44**, 1257 (1959).
12. G. A. FISCHER, *Cancer Res.* **19**, 372 (1959).
13. G. A. FISCHER, *Biochem. Pharmacol.* **7**, 75 (1961).
14. G. A. FISCHER, *Biochem. Pharmacol.* **11**, 1233 (1962).