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The Effect of 1-β-D-Arabinofuranosylcytosine on Growth, Viability, and DNA Synthesis of Mouse L-cells[1]

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SUMMARY

An examination of the effects of 1-β-D-arabinosylcytosine (ara-C) on DNA synthesis of mouse L-cells has been made, and these effects have been compared with effects on viability. Measurements on the incorporation of radioactive precursors into acid-insoluble material showed that ara-C strongly inhibited DNA synthesis while having relatively little effect on RNA or protein synthesis. It was found that 3.6×10^{-7} M ara-C inhibited cell division for at least 24 hr, except for the single division of cells initially in G_2. Over this time, however, there was no decrease in cell viability, even though DNA synthesis was reduced by more than 97% over the first 14 hr of exposure to ara-C. Results of studies on growth and cell viability suggest that at higher concentrations (7.2×10^{-6} M or greater) ara-C rapidly killed S phase cells but temporarily blocked the progression of cells from G_1 into S, and thus partially protected against its own toxicity. The observed effects are discussed in relation to the current models for the mechanism of action of ara-C. Our observations do not appear to be consistent with a model in which ara-C acts by blocking the reduction of CDP nor with one in which ara-C acts by incorporation into DNA. Rather, our results agree with a model in which inhibition of DNA synthesis is the result of inhibition of DNA polymerase.

INTRODUCTION

ara-C[3] has been shown to inhibit multiplication and DNA synthesis in cultured mammalian cells (7, 24), as well as multiplication of some DNA viruses (2) and malignant tumors (13, 28). At concentrations above 2 mM, ara-C has also been reported to inhibit division and DNA synthesis in *Escherichia coli* (17). A review of the data on arabinosylnucleosides up to 1965 has been given by Cohen (5).

At least 3 possible mechanisms for the action of ara-C have been proposed. Chu and Fischer (3) have suggested that ara-C inhibits the reduction of CDP to dCDP, blocking the production of dCTP and thereby preventing DNA synthesis. As evidence, they reported that the ara-C-induced inhibition of growth could be reversed by deoxycytidine and that ara-C decreased the conversion of uridine-^{3}H to deoxycytidine nucleotides but not to cytidine nucleotides. However, Moore and Cohen (20) have shown that the 5′-diphosphate of ara-C and ara-CTP inhibited ribonucleotide reduction in a partially purified enzyme preparation from rat tumor no more effectively than did dCTP. This would suggest that unless the intracellular concentrations of the 5′-diphosphate of ara-C and ara-CTP are exceedingly high in ara-C-treated cells reduction is probably not significantly inhibited by ara-C. More convincing evidence that inhibition of DNA synthesis is not due to inhibition of the reduction step comes from the work of Kaplan *et al.* (14). They have observed in rabbit kidney cells that reduction of CDP was not affected by ara-C unless small amounts of deoxycytidine were present in the medium. They suggested that the inhibition of CDP reduction was due to an increase in the dCTP pool resulting in feedback inhibition of CDP reductase and attributed the inhibition observed by Chu and Fischer to the use of undialyzed serum, possibly containing deoxycytidine.

Incorporation of ara-C into the DNA of mammalian cells has been reported (4, 6, 24) and it has been suggested (24) that this incorporation might be the cause of inhibition of DNA synthesis and cell death. The observation that ara-C induced rapid, irreversible loss of viability was interpreted as evidence for this model. Recently, the incorporation of ara-C into DNA in an *in vitro* DNA-synthesizing system has been reported (19). It was found that most of the incorporated ara-C was confined to the 3′-hydroxyl terminus of the DNA chain, suggesting that ara-C incorporated into the end of the growing DNA molecule might be preventing further addition of nucleotides. These observations will be examined in more detail later.

Finally, Kimball and Wilson (16) have suggested that inhibition of DNA synthesis might be the result of inhibition at the DNA polymerase level. They reported that ara-C inhibited DNA polymerase activity in crude extracts of Ehrlich ascites cells and that the inhibition could be prevented by dCTP. Furth and Cohen (10) have found that ara-CTP inhibited calf thymus DNA polymerase and that the inhibition was competitive with dCTP.

Implicit in most studies carried out thus far with ara-C has been the assumption that the inhibition of DNA synthesis induced by the drug is the cause of cell death (*i.e.*, loss of cell

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[3] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CTP, 5′-triphosphate of ara-C; FUdR, 5-fluorodeoxyuridine.

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proliferative capacity). This assumption seems quite reasonable in view of the specific effect of ara-C on DNA synthesis and on the survival of S phase cells (Ref. 15 and Charts 3 and 5). In order to examine the relationship between inhibition of DNA synthesis and loss of cell viability in more detail, we have undertaken a study of the effects of ara-C on mouse L-cells. It was found that DNA synthesis could be inhibited by more than 97% for nearly 14 hr without significant effect on cell viability, suggesting that the relationship between inhibition of DNA synthesis and viability may not be simple. Furthermore, since DNA synthesis must resume for the cells to form colonies, inhibition of DNA synthesis cannot be the result of the incorporated irreversible blocking by the ara-C molecules of further elongation of the growing DNA chains. Inhibition of deoxycytidine-^{3}H incorporation should not occur if inhibition of DNA synthesis were due only to a block in the reduction of CDP to dCDP. Yet we have found that ara-C had no significant effect on the conversion of deoxycytidine-^{3}H to intracellular nucleotides, while severely inhibiting its incorporation into DNA.

MATERIALS AND METHODS

Materials. All radioactive compounds were obtained from the Radiochemical Centre, Amersham, England. Thymidine-^{3}H was labeled in the methyl group with a specific activity of 17.4 Ci/mmole or nominally in the 6-position, with a specific activity of 5.0 Ci/mmole. Deoxycytidine-^{3}H and uridine-^{3}H were labeled in the 5-position, with specific activities of 15.5 and 22.7 Ci/mmole, respectively. Leucine-^{3}H was labeled in the 4- and 5-positions, with a specific activity of 1.0 Ci/mmole. NCS solubilizer was obtained from Amersham/Searle Corp., Des Plaines, Ill. Nucleotides used as chromatography markers were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. ara-C hydrochloride was purchased from the Sigma Chemical Co., St. Louis, Mo. All other nucleosides were purchased from General Biochemicals, Chagrin Falls, Ohio. Stock solutions of nucleosides at 100 times the final exposure concentration were made in Connaught Medical Research Laboratories 1066 medium (21) lacking nucleosides and coenzymes and were neutralized with KOH. Solutions were stored at 4° and used within 1 week of preparation. No alteration could be detected in solutions of ara-C stored at 4° for several months when tested for ability to inhibit growth of L-cells.

Cell Growth and Viability. Mouse L-cells, strain L60T (27), were grown at 37° as suspension cultures in spinner flasks or in plastic tubes in a rotating wheel. Unless otherwise indicated, the growth medium was Connaught Medical Research Laboratories 1066 lacking all nucleosides and coenzymes and supplemented with 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.) which had been extensively dialyzed. Cell stocks were diluted every 1 or 2 days with fresh medium to maintain the cultures in exponential growth. Under these conditions, the population doubling time was approximately 16 hr. Total cell number was measured by counting on a Model F Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cell viability was determined by the plating technique of Puck, Marcus, and Cieciura (23) with CMRL 1066 supplemented with 10% undialyzed fetal bovine serum as plating medium. In some experiments the plating medium lacked nucleosides and coenzymes and was supplemented with 10% dialyzed serum. Dishes were incubated for 12 to 14 days at 37° in a humidity-controlled atmosphere containing 5% carbon dioxide. The plating efficiency of untreated controls was 70 to 90%, and no difference was observed whether the cells were plated in medium containing nucleosides and undialyzed serum or in medium lacking nucleosides and supplemented with dialyzed serum.

Experiments to determine the effects of drugs on cell viability were carried out in the following way. Cells were grown in a 1.0-liter spinner flask and distributed into 200-ml spinners 2 to 3 hr before the addition of any chemicals. During this 2- to 3-hr interval, aliquots were removed for counting and plating to determine growth and plating efficiency prior to the addition of drugs. At Time 0 drugs were added, and determinations of growth and viability were continued for the required length of time. In most experiments, aliquots of the cell suspension were centrifuged once and resuspended in fresh growth medium before dilution and plating.

Determination of DNA Synthesis. The rate of DNA synthesis was determined by measuring the incorporation of thymidine-^{3}H or deoxycytidine-^{3}H into acid-insoluble material during a 15-min or a 30-min pulse at 37°. RNA and protein synthesis were measured by the incorporation of uridine-^{3}H and leucine-^{3}H, respectively, into acid-insoluble material during a 15-min pulse. When acid-insoluble material from cells labeled with DNA or RNA precursors was treated with 0.5 N NaOH at 37° for 24 hr to hydrolyze the RNA, it was found that less than 0.1% of the thymidine-^{3}H counts were made acid soluble, and 5% of the deoxycytidine-^{3}H counts and 92% of the uridine-^{3}H counts were rendered acid soluble. Therefore, the incorporation of thymidine and deoxycytidine was predominantly into DNA, and incorporation of uridine was predominantly into RNA. The depletion of radioactive precursors from the medium due to uptake into cells was negligible in all experiments reported in this paper.

Determination of Nucleotide Synthesis from Exogenous Nucleosides. For determination of the amount of nucleotides synthesized from exogenous nucleosides, aliquots of cell suspensions (10^6 cells) treated with thymidine-^{3}H or deoxycytidine-^{3}H were centrifuged, washed twice with ice-cold phosphate-buffered saline (9), and extracted 3 times with 2 ml of 0.2 N ice-cold perchloric acid. The acid-insoluble fraction was dissolved in 1.0 ml of NCS solubilizer and counted in 10 ml of toluene containing 0.05 g/liter of POPOP and 6.0 g/liter of PPO in a liquid scintillation counter. The acid-soluble extract was neutralized by addition of 2.0 N KOH, and the $KClO_4$ precipitate was removed by centrifugation. After descending chromatography of aliquots of the supernatant on Whatman No. 3MM paper with 1-butanol:H_2O, (86:14) (18), no detectable radioactive nucleosides were found, indicating that the 2 phosphate-buffered saline washes effectively removed nucleosides from the cells. Radioactive nucleotides were separated and identified with descending chromatography on diethylaminoethyl cellulose paper (DE-81) with a solvent consisting of concentrated NH_4OH:formic acid:H_2O

(1:20:79). Chromatography of extracts from labeled cells was also carried out on a Whatman No. 3MM paper with 95% ethanol:1 M ammonium acetate (75:30), pH 7.5, or 95% ethanol:1 M ammonium acetate saturated with sodium tetraborate (75:30) (26). For determination of the position of the radioactivity, the developed chromatograms were cut into 0.5-inch strips along the path of the spots, eluted with 0.1 ml of H_2O, 0.2 ml of ethanol, and 1.0 ml of NCS solubilizer, and counted on a liquid scintillation counter. Nonradioactive markers were located as ultraviolet-absorbing spots. Only thymidine-^{3}H nucleotides were detected in extracts from thymidine-^{3}H-labeled cells, and only deoxycytidine-^{3}H nucleotides were found in extracts from deoxycytidine-^{3}H-labeled cells.

RESULTS

Growth Studies. For determination of the effect of ara-C on the growth of L-cells, ara-C in concentrations ranging from 3.0×10^{-9} M to 3.6×10^{-4} M was added to exponentially growing cultures, and the total cell number was measured as a function of time thereafter. In cultures containing ara-C at 3.0×10^{-9} and 3.0×10^{-8} M (Chart 1), the cell number continued to increase at the same rate as the control for at least 15 days. After a lag of approximately 1 day, which is not apparent on the chart, cells grown in 3.0×10^{-7} M ara-C grew exponentially with a doubling time of 42 hr for 33 days, after which the doubling time decreased to 31 hr. The doubling time then remained constant up to at least 125 days.

At higher ara-C concentrations (Chart 2), a more detailed examination of growth curves over shorter time intervals indicated that the cell number increased normally for 3 to 4 hr after addition of the drug; then, no further increase was apparent for times up to 24 hr. Since the length of G_2 phase for our cell line is 3.5 hr, it appears that division of G_2 cells was uninhibited, but the progression to mitosis of cells that were in S phase at the time of ara-C addition was blocked. After 5 to 10 hr in the presence of high concentrations of ara-C, the total cell number started to decrease slightly, presumably due to cell lysis.

Viability Studies. The effect on cell viability of exposure to various concentrations of ara-C for various lengths of time is shown in Chart 3. At Time 0, ara-C was added at the concentrations shown, and at various times thereafter aliquots of the cell suspension were removed, washed once, diluted, and plated. Viability is expressed as percentage of survival (ratio of the plating efficiency of the treated cells to the plating efficiency immediately before addition of ara-C). A concentration of 3.6×10^{-7} M ara-C had no effect on cell viability for nearly twice the doubling time of the cells, although cell multiplication was completely inhibited during the interval from 4 to 28 hr. At 3.6×10^{-6} M ara-C, there was a slow decrease in survival for approximately 16 hr then a more rapid drop to 3% survival at 30 hr. In some experiments at this concentration, however, the survival was not significantly affected during the first 16 hr of exposure, but dropped rapidly thereafter, as in Chart 3. At 3.6×10^{-5} M and 3.6×10^{-4} M ara-C, there was an almost immediate drop in survival to between 30 and 40%, values which correspond approximately to the fraction of cells in exponentially growing cultures which are outside the DNA synthetic phase.

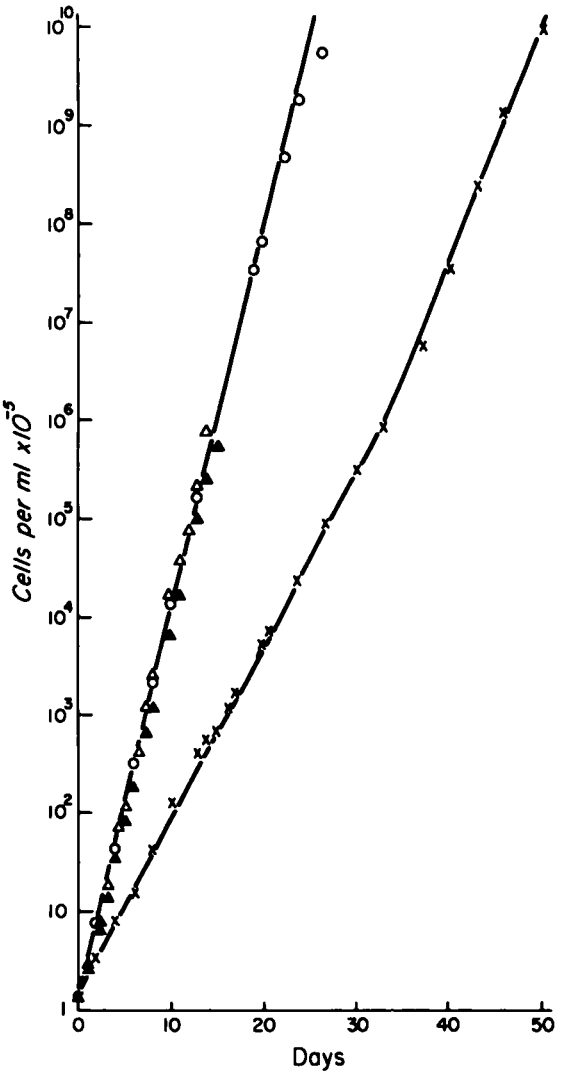

Chart 1. The effect of ara-C on L-cell multiplication over several weeks. ara-C was added to L-cell cultures at Day 0 and cultures were diluted every 2 days with fresh medium containing appropriate concentrations of ara-C. ○, control; △, 3.0×10^{-9} M ara-C; ▲, 3.0×10^{-8} M ara-C; X, 3.0×10^{-7} M ara-C. All values along the ordinate must be multiplied by 10^5 to attain the correct values.

The survival curve obtained at an ara-C concentration of 3.6×10^{-4} M corresponded almost exactly to that obtained when L-cells were exposed to high-specific-activity thymidine-^{3}H (data not shown, but see Chart 5). The plateau from 2 to 4 hr is the result of the division of G_2 cells offsetting the killing of G_1 cells as they enter S. However, the relatively long plateau lasting up to 10 hr when cells were treated with 3.6×10^{-5} M ara-C suggested that G_1 cells were prevented from entering S phase and were temporarily protected from the toxic effect of the drug. Thus ara-C seemed to have the ability, under certain circumstances, to protect, at least

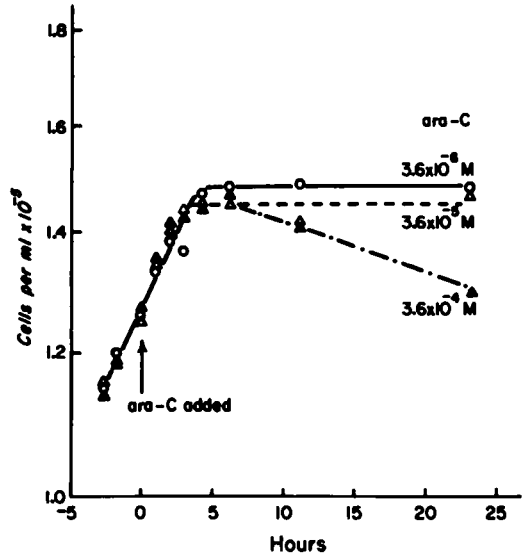

Chart 2. The effect of ara-C on the division of L-cells. ara-C at the concentrations shown was added at Time 0 to exponentially growing cultures of L-cells. All values along the ordinate must be multiplied by 10^5 to attain the correct values.

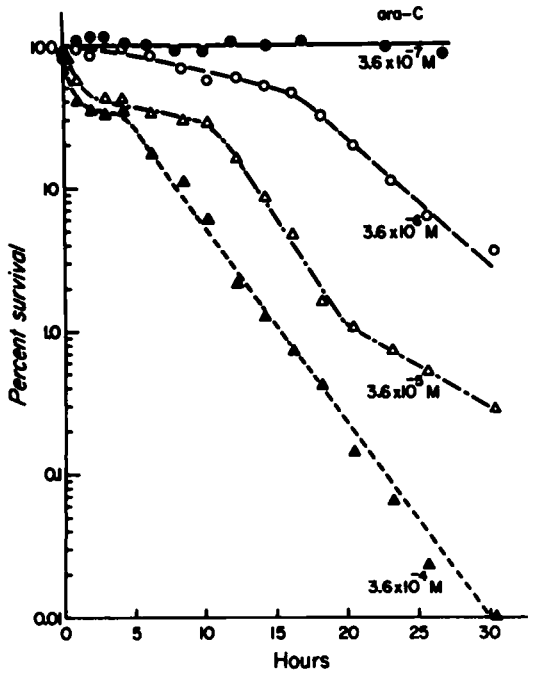

Chart 3. Survival of cells as a function of time after addition of ara-C. Identical cultures were exposed to ara-C at the concentrations indicated, and at various times aliquots were removed, washed once, diluted and plated in 1066 medium containing nucleosides, and supplemented with 10% fetal bovine serum.

partially, against its own toxicity. The rapid drop in cell survival observed at ara-C concentrations of 3.6×10^{-5} M or greater has been examined in more detail using a microliter syringe for removal of 2-μl aliquots of the cell suspension which were plated directly into Petri dishes for colony assay. The results of this experiment are shown in Chart 4. Although the plating medium contained deoxycytidine, cell viability was rapidly reduced by ara-C. Since the viable cell number was the same before and immediately after drug addition, it is clear that the small amount of ara-C carried over into the plating medium with the cells did not affect the plating efficiency.

The results of Charts 3 and 4, combined with the observation that ara-C had no affect on the progression of G_2 cells through mitosis (Chart 2), suggested that in L-cells, as in other systems (15), ara-C might be acting primarily on S phase cells. Further evidence for this was provided by the results of an experiment in which the effects of ara-C and thymidine-^{3}H alone were compared with the effect of a combined treatment. These results are given in Chart 5. When thymidine-^{3}H (*Curve D*) or ara-C (*Curve A*) were added to separate cultures, the initial drop in survival was to approximately the same level. From *Curve B*, it can be seen that when cells were first treated for 2 hr with ara-C the addition of thymidine-^{3}H at that time appeared to have little effect, presumably because ara-C inhibits DNA synthesis. *Curve C* shows that in a culture pretreated with thymidine-^{3}H for 2 hr the addition of ara-C produced no further killing. In fact, a comparison of *Curves C* and *D* indicates that the addition of ara-C at least temporarily protected against the killing due to thymidine-^{3}H. The fact that the addition of ara-C to thymidine-^{3}H-treated cells produced no further killing suggests that the initial killing induced by ara-C is limited to S phase cells. Furthermore, since the survival of cells treated with only thymidine-^{3}H decreased rapidly after 2 hr due to the entry of G_1 cells into S phase, while the addition of ara-C at 2 hr extended the shoulder in the survival curve for at least an additional 2 to 3 hr, it appears that ara-C temporarily blocked the progression of G_1 cells into S phase.

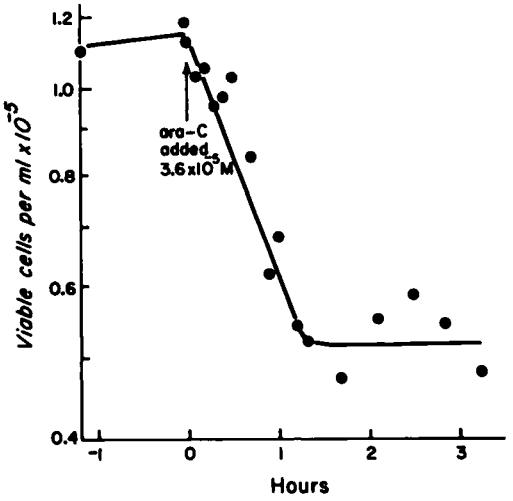

Chart 4. The effect of short exposures to ara-C on cell viability. ara-C was added at Time 0 and 2-μl aliquots were dispensed directly into dishes containing 1066 medium plus nucleosides and 10% fetal bovine serum. All values along the ordinate must be multiplied by 10^5 to attain the correct values.

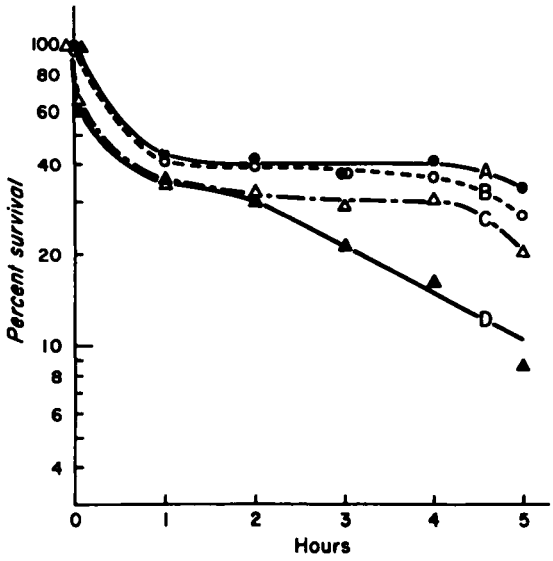

Chart 5. Comparison of the survival of cells exposed separately to 3.0×10^{-5} M ara-C or 2 μCi/ml thymidine-^{3}H (specific activity, 17.4 Ci/mmole) with the survival of cells treated with ara-C and thymidine-^{3}H 2 hr apart. *A*, ara-C added at Time 0; *B*, ara-C added at Time 0, then thymidine-^{3}H added at 2 hr; *C*, thymidine-^{3}H added at Time 0, then ara-C added at 2 hr; *D*, thymidine-^{3}H added at Time 0. At the times indicated, viability was assayed as in Chart 3.

Chu and Fischer (4) have reported that ara-C-treated L5178Y leukemic cells could be rescued[4] by deoxycytidine, and Young and Fischer (29) have made similar observations with Chinese hamster cells. From the results illustrated in Charts 3 and 4, it appeared that whether L-cells were washed before plating or not rapid killing of S phase cells could not be reversed by the deoxycytidine (3.8×10^{-5} M) present in the plating medium. Additional attempts were made to rescue ara-C-treated L-cells by exposure to concentrations of deoxycytidine in excess of that normally found in the plating medium. Cells were treated with ara-C at 3×10^{-5} M for various times, then washed once, diluted, and plated in either CMRL 1066 lacking nucleosides and coenzymes and supplemented with dialyzed serum or the same medium containing deoxycytidine at 3mM. The results are shown in Table 1 and indicate no significant difference in the plating efficiency whether deoxycytidine was present or absent in the plating medium.

The results of an additional attempt to observe any rescue phenomenon are shown in Chart 6. It was found that if deoxycytidine at approximately 100 times the concentration of ara-C was added at the same time as ara-C no killing occurred, and if deoxycytidine was added 8 hr after ara-C, additional killing was prevented, although ara-C was still

Table 1

The effect of posttreatment with deoxycytidine on the viability of ara-C-treated L-cells

L-cells were exposed to 3.0×10^{-5} M ara-C for various times, washed once, diluted, and plated in medium supplemented with 10% dialyzed serum and lacking all nucleosides or containing deoxycytidine at 3.0×10^{-3} M.

Time in ara-C (hr)	Plating efficiency in medium (%)	
	– deoxycytidine	+ deoxycytidine
0	76	81
0.5	72	75
1.0	70	66
2.0	42	54
3.0	34	37

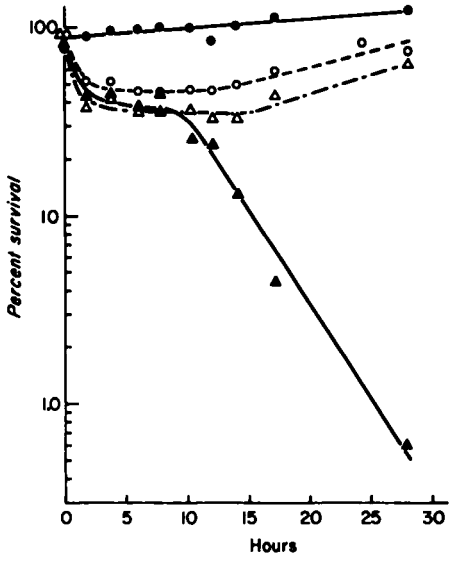

Chart 6. The effect of viability of deoxycytidine added to L-cell cultures exposed to ara-C. ●, 3.6×10^{-5} M ara-C and 3.6×10^{-3} M deoxycytidine, both added at Time 0; ○, 7.2×10^{-6} M ara-C added at Time 0 followed by the addition of 7.2×10^{-4} M deoxycytidine at 8 hr; △, 3.6×10^{-5} M ara-C added at Time 0, followed by 3.6×10^{-3} M deoxycytidine at 8 hr; ▲, 3.6×10^{-5} M ara-C added at Time 0. At the times indicated, viable cell number was assayed by the method described in Chart 3.

present in the medium. The increase in viable cell number starting at 16 hr (8 hr after addition of deoxycytidine) coincided with an increase in the total cell number and was due to the division of cells which were piled up in G_1 at the time deoxycytidine was added. It is apparent from this experiment that deoxycytidine did not reverse any ara-C-induced loss of viability which had occurred up to the time of addition of deoxycytidine, but rather only protected against further killing. When this experiment was repeated at an ara-C concentration of 1.8×10^{-5} M with 1.8×10^{-3} M deoxycytidine added 2 hr or 6 hr after ara-C, similar results were obtained.

Thus all attempts to rescue cells either by washing and plating them into dishes containing deoxycytidine or by

[4]By "rescue," we mean a posttreatment which increases the observed fraction of viable cells; by "protection," we mean a pre- or concurrent treatment which completely or partially prevents a decrease in the fraction of viable cells.

adding high concentrations of deoxycytidine to the medium were unsuccessful in our system.

Inhibition of DNA Synthesis. In order to compare the effect of ara-C on viability as illustrated in Chart 3 with its effect on DNA synthesis, cells were exposed to several concentrations of ara-C and pulsed with thymidine-^{3}H for 15 min at various times after drug addition. As Chart 7 shows, the effect on DNA synthesis was pronounced and rapid. Even the relatively low concentration of 3.6×10^{-7} M ara-C inhibited DNA synthesis by 97% within 2 hr, although it had been found that viability was not appreciably affected for nearly 30 hr or almost twice the generation time. At this concentration, RNA and protein synthesis, as measured by incorporation of uridine-^{3}H and leucine-^{3}H, were not significantly affected (<5% inhibition). These results suggest that it is possible specifically to inhibit DNA synthesis almost totally for more than a generation time without effecting cell viability. At higher concentrations of ara-C, DNA synthesis was further inhibited, and Chart 3 indicates that cell killing does occur, but no direct correlation between killing and inhibition of DNA synthesis has been established.

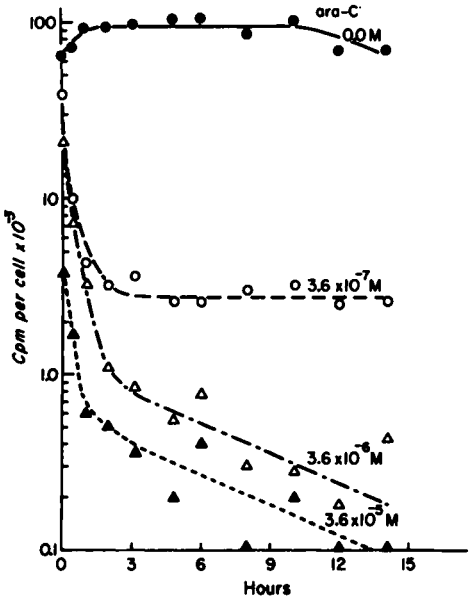

Chart 7. The effect of continuous exposure to ara-C on thymidine-^{3}H incorporation into acid-insoluble material. L-cells were exposed to ara-C at several concentrations and at the times indicated pulsed for 15 min with 1 μCi/ml thymidine-^{3}H (specific activity, 5.0 Ci/mmole). The points are plotted at the beginning of the pulse. All values along the ordinate must be multiplied by 10^3 to attain the correct values.

Since we had shown that deoxycytidine could protect against the lethal effects of ara-C, it was of interest to determine whether a similar type of protection could be observed for the inhibition of DNA synthesis. In an experiment in which the uptake of thymidine-^{3}H was measured in the presence of various mixtures for ara-C and deoxycytidine during a 30-min pulse, it was found that deoxycytidine could protect against the inhibition of DNA synthesis induced by ara-C and that approximately a 20-fold excess of deoxycytidine was sufficient to completely overcome the ara-C-induced inhibition (F. L. Graham and G. F. Whitmore, unpublished data).

Synthesis of Nucleotides from Exogenous Nucleosides. The observation (Charts 3 and 7) that ara-C can apparently inhibit DNA synthesis by more than 97% for approximately 1 generation time without a concomitant reduction in survival makes it doubly important to show that the failure to incorporate thymidine-^{3}H is not simply due to a reduced production of TTP-^{3}H. Therefore, for measurement of the conversion of thymidine-^{3}H to intracellular nucleotides, cells treated with 3.0×10^{-5} M ara-C were exposed to thymidine-^{3}H for 15 min, an acid-soluble extract was made, and aliquots were chromatographed on diethylaminoethyl cellulose paper (see "Materials and Methods"). The acid-insoluble fraction was dissolved in 1.0 ml of NCS solubilizer and counted on a liquid scintillation counter to determine the incorporation into DNA. The conversion of thymidine-^{3}H into intracellular nucleotides and into acid-insoluble material is shown in Table 2. Up to 4 hr after addition of ara-C, there was little change in the amount of TTP-^{3}H accumulated in the intracellular pool during the 15-min pulse, although the amount of radioactivity incorporated into acid-insoluble material had decreased to 0.26% of the control. At 11 hr, incorporation into DNA was down to 0.05% of the control value, while the TTP-^{3}H concentration was reduced to only approximately 30% of the control. Hence it appears highly unlikely that the inhibition of incorporation of thymidine-^{3}H into acid-insoluble material observed in ara-C-treated cells is due to a block in production of TTP-^{3}H.

In a study similar to the above but with deoxycytidine-^{3}H, it was found that after a 2-hr exposure to ara-C (3.0×10^{-5} M) the pool of dCTP-^{3}H was approximately 3 times that of the control, while the rate of incorporation into acid-insoluble material was reduced to 3% of the control. This increased content of dCTP-^{3}H could be due to the increased kinase activity reported by Kaplan *et al.*(14) or to a reduced utilization of the triphosphate. This experiment indicated that cells treated with ara-C can convert at least small amounts of deoxycytidine-^{3}H to dCTP-^{3}H. Nevertheless, the finding that all attempts to use deoxycytidine to rescue cells from the ara-C-induced loss of viability were unsuccessful posed the question of whether such cells can convert appreciable amounts of exogenous deoxycytidine to the nucleotide form. Since a failure to synthesize sufficient dCTP might account for the inability of deoxycytidine to rescue ara-C-killed cells, an attempt was made to measure the rate of conversion of exogenous deoxycytidine-^{3}H to dCTP-^{3}H at concentrations of deoxycytidine similar to those used in the rescue experiments. For this purpose, cells were pretreated with ara-C for various times, and deoxycytidine-^{3}H at 10^4 cpm/mμmole, 3.0×10^{-3} M, was added to the medium. The cells were then incubated in the presence of both ara-C and deoxycytidine for an additional 4 hr, at which time acid-insoluble extracts were made, and

Table 2

The conversion of thymidine-^3H to intracellular nucleotides in ara-C-treated L-cells

L-cells were pulsed for 15 min with 2 μCi/ml thymidine-^3H (specific activity, 17.4 Ci/mmole) at various times after addition of 3.0×10^{-5} M ara-C. Radioactivity in the acid-insoluble fraction (DNA) and in acid-soluble nucleotides was determined as described in "Materials and Methods."

Conditions	Incorporation (cpm/10^3 cells)				
	TMP-^3H	TDP-^3H	TTP-^3H	DNA	DNA (% control)
Control (no ara-C)	2.10	8.61	62.2	244.0	100
ara-C for:					
0 hr	2.89	14.00	57.3	47.7	19.5
1.0 hr	1.87	7.09	66.1	2.2	0.91
2.0 hr	0.95	4.55	57.2	1.16	0.48
4.0 hr	0.89	2.07	53.0	0.64	0.26
11.0 hr	3.05	6.38	17.8	0.12	0.05

Table 3

The ability of ara-C-treated L-cells to synthesize deoxycytidine nucleotides from exogenous deoxycytidine

L-cells were treated with ara-C at 3.0×10^{-5} M; at the times shown, 3×10^{-3} M deoxycytidine at 10^4 cpm/mμmole was added. After 4 hr, acid-soluble nucleotides and acid-insoluble incorporation were determined as described in "Materials and Methods."

	Nucleotide (mμmoles/10^8 cells)				
	dCMP	dCDP	dCTP	DNA	DNA (% control)
Control (no ara-C)	445	18.6	86.4	234	100
Time in ara-C before addition of deoxycytidine					
0	523	15.6	88.7	209	89
2	611	12.3	115.5	57	24
4	421	9.3	67.7	39	17

aliquots were chromatographed as before. The results of this experiment are given in Table 3. It was found that both in the presence and absence of ara-C the total amount of deoxycytidine nucleotide derived from exogenous deoxycytidine was sufficient to support DNA synthesis for several hr, even if this was the only source of DNA cytosine. Thus it appears that the inability of deoxycytidine to rescue cells exposed to ara-C was not due to an inability of the cells to convert deoxycytidine to intracellular nucleotides.

DISCUSSION

Silagi (24) has reported that low concentrations of ara-C irreversibly stop DNA synthesis in L-cells. However, the results of our studies on mouse L-cells show that 3.6×10^{-7} M ara-C can inhibit DNA synthesis by more than 97%, while at the same time reducing RNA and protein synthesis no more than 5%. Under these conditions the viability of the cells remains essentially unchanged for nearly 2 generation times, although during this time the cells increase in size until they are more than double the volume of control cells (F. L. Graham and G. F. Whitmore, unpublished data). Thus, cells can undergo unbalanced growth for periods greater than 1 generation time without necessarily losing viability. At concentrations of 7.2×10^{-6} M or greater, the irreversible loss of viability after short exposures is limited to cells in the DNA-synthetic period, and treatments of 1 to 2 hr are sufficient to kill all of the S phase cells.

Although the specific effects of ara-C on both DNA synthesis and the viability of S phase cells might suggest that cell death is due to the inhibition of DNA synthesis, no direct correlation between these two effects can be made from our results. For this reason we will discuss the action of ara-C under 2 separate headings: inhibition of DNA synthesis and induction of cell death. By this separation, however, we do not wish to suggest that these two effects are necessarily the results of different mechanisms; what we wish to emphasize is that an explanation of how ara-C inhibits DNA synthesis does not immediately show how cell death results from such an inhibition, especially if it is only temporary.

Inhibition of DNA Synthesis. The three current models for the ara-C-induced inhibition of DNA synthesis have already been mentioned in the "Introduction." The results obtained by Moore and Cohen (20) and Kaplan *et al.*(14) indicate that ara-C probably does not inhibit DNA synthesis by

blocking the reduction of CDP. Further evidence against this model is provided by our data in Table 3, which show that ara-C-treated L-cells could synthesize enough deoxycytidine nucleotides from exogenous deoxycytidine to support DNA synthesis for several hr; yet DNA synthesis was inhibited by 83%.

Incorporation of ara-C into both RNA and DNA has been observed (4, 6, 24). However, it seems unlikely that incorporation into RNA could account for the inhibition of DNA synthesis. On the other hand, the suggestion that ara-C inhibits DNA synthesis by being incorporated at the end of the growing DNA chain, thus blocking its elongation (19), is difficult to reconcile with our observation that DNA synthesis can be severely inhibited with no effect on cell viability (Charts 3 and 7). If incorporation is the cause of inhibition, then it should also be lethal, since DNA synthesis must resume in order for the cells to divide and form colonies. Doering *et al.*(7) reached the same conclusion by showing that inhibition of DNA synthesis in asynchronous L-cells could be reversed by washing the cells free of ara-C. However, the interpretation of their results is complicated by the fact that ara-C would cause the accumulation of cells in front of S phase and, upon removal of the drug, these cells would progress into S and presumably synthesize DNA at a rapid rate. In their experiments, this could not be distinguished from reinitiation of DNA synthesis in cells which were already in S phase at the time of addition of ara-C.

Recently, Momparler (19) has shown in an *in vitro* DNA-synthesizing system that the ^{3}H-labeled 5′-monophosphate of ara-C is incorporated into DNA with 90% of the label confined to the 3′-hydroxyl terminus of the DNA strand and that a doubling of the amount of denatured DNA in the reaction mixture results in a doubling of the amount of the ^{3}H-labeled 5′-monophosphate of ara-C incorporated. While these results suggest that ara-C incorporation could indeed block DNA synthesis, our data on L-cells are not in agreement with this hypothesis. Evidence will be presented in the next paper (11) that, although ara-C incorporation into DNA occurs in intact cells, the incorporated ara-C is not confined to the 3′-hydroxyl position and thus does not appear to block extension of DNA strands. The observations described in the present paper are instead consistent with a model for ara-C action in which DNA polymerase is inhibited by ara-CTP (10, 16). Further analysis of this model is presented in the next paper (11).

Induction of Cell Death. It appears that ara-C incorporation into DNA cannot easily account for inhibition of DNA synthesis. However, rapid and irreversible killing of ara-C-treated cells has been interpreted as evidence that the drug might be lethal when incorporated into DNA (24). Certainly, in our system we were unable to rescue cells treated with ara-C at concentrations of 7.2×10^{-6} M or greater (Chart 6), even after exposures of less than 2 hr. We have observed a similarity in survival curves from FUdR and thymidine-^{3}H treatment of L-cells (F. L. Graham and G. F. Whitmore, unpublished data) which suggests that FUdR may also be lethal specifically for S phase cells. Hydroxyurea, another inhibitor of DNA synthesis, has also been shown to be toxic for S phase cells after short exposures (1, 25). However, there is no evidence that FUdR is incorporated into DNA (12), and in the case of hydroxyurea such a mechanism of action seems even more unlikely. The observation that 2 or perhaps 3 completely different inhibitors of DNA synthesis are able to kill S phase cells after short exposures suggests that the specific inhibition of DNA synthesis for even a brief period might be lethal, no matter how this inhibition is induced. However, the data of Charts 3 and 7 show that 97% inhibition of DNA synthesis is not lethal over more than a generation time (3.6×10^{-7} M ara-C), while 3.6×10^{-5} M ara-C, which inhibits DNA synthesis by more than 97%, is lethal within 1 to 2 hr. Therefore, if we postulate that cell death is due to a massive inhibition of DNA synthesis, then we must also postulate a mechanism whereby a relatively small amount of DNA synthesis, as little as 3% of the normal rate, can prevent loss of viability.

The kinetics of killing illustrated in Chart 3 are difficult to explain in terms of mechanisms at the subcellular level. At 3.6×10^{-5} M ara-C kills S phase cells within 1 to 2 hr; there then follows a plateau lasting several hr which apparently is due to a block in the entry of G_1 cells into S. Of possible relevance are certain observations of Lark and Lark (17) on the effects of ara-C on *E. coli.* They observed that when cells treated with ara-C were washed free of the drug premature initiation of DNA synthesis did not occur as it does in cells exposed to a thymineless condition. It may be that ara-C prevents initiation of DNA synthesis, and thus in mammalian cells blocks the entry of G_1 cells into S phase. This would explain the extended plateau in the survival curves of L-cells treated with 3.6×10^{-5} M ara-C. However, it is not possible to explain on this basis why, at higher concentrations (3.6×10^{-4} M ara-C, Chart 3), ara-C no longer appears to block the flow of G_1 cells into S.

Chu and Fischer (4) have suggested that a correlation exists between incorporation of ara-C into the RNA of L5178Y cells and irreversible loss of cell viability. It is known that RNA synthesis is not limited to the S phase of the cycle (8, 22), and, therefore, it might be expected that cells in other phases of the cell cycle would also be sensitive to the drug. Since our data, as well as those of Karon and Shirakawa (15), show that the drug is specifically toxic for S phase cells, it would seem that lethality is probably not the result of ara-C incorporation into RNA.

From the results of our studies on L-cells and from the observations reported by other investigators, it appears that any model for ara-C killing of mammalian cells should account for the following facts. It is possible to inhibit DNA synthesis and cell division for several hr without affecting cell viability. At severe levels of DNA synthesis, inhibition (*i.e.*, at high ara-C concentrations), S phase cells are specifically killed within 1 to 2 hr. The effect of ara-C on S phase cells may be similar to the effect of at least 2 other inhibitors of DNA synthesis, FUdR and hydroxyurea. It is possible that rapid killing of S phase cells is a feature of all inhibitors of DNA synthesis and may be induced by a single mechanism.

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REFERENCES

1. Bacchetti, S., and Whitmore, G. F. The Action of Hydroxyurea on Mouse L-Cells. Cell Tissue Kinet., *2:* 193–211, 1969.
2. Buthala, D. A. Cell Culture Studies on Antiviral Agents: 1. Action of Cytosine Arabinoside and Some Comparison with 5-Iodo-2-deoxyuridine. Proc. Soc. Exptl. Biol. Med., *115:* 69–77, 1964.
3. Chu, M. Y., and Fischer, G. A. A Proposed Mechanism of Action of 1-β-D-Arabinofuranosyl-Cytosine as an Inhibitor of the Growth of Leukemic Cells. Biochem. Pharmacol., *11:* 423–430, 1962.
4. Chu, M. Y., and Fischer, G. A. The Incorporation of ^{3}H-Cytosine Arabinoside and Its Effect on Murine Leukemic Cells (L5178Y). Biochem. Pharmacol., *17:* 753–767, 1968.
5. Cohen, S. S. Introduction to the Biochemistry of D-Arabinosyl Nucleosides. Progr. Nucleic Acid Res., *5:* 1–88, 1966.
6. Creasey, W. A., Papac, R. J., Markiw, M. E., Calabresi, P., and Welch, A. D. Biochemical and Pharmacological Studies with 1-β-D-Arabinofuranosylcytosine in Man. Biochem. Pharmacol., *15:* 1417–1428, 1966.
7. Doering, A., Keller, J., and Cohen, S. S. Some Effects of D-Arabinosyl Nucleosides on Polymer Synthesis in Mouse Fibroblasts. Cancer Res., *26:* 2444–2450, 1966.
8. Doida, Y., and Okada, S. Determination of Switching-off and Switching-on Time of Overall Nuclear RNA Synthesis. Nature, *216:* 272–273, 1967.
9. Dulbecco, R., and Vogt, M. Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exptl. Med., *99:* 167–182, 1954.
10. Furth, J. J., and Cohen, S. S. Inhibition of Mammalian DNA Polymerase by the 5′-Triphosphate of 1-β-D-Arabinofuranosylcytosine and the 5′-Triphosphate of 9-β-D-Arabinofuranosyladenine. Cancer Res., *28:* 2061–2067, 1968.
11. Graham, F. L., and Whitmore, G. F. Studies in Mouse L-cells on the Incorporation of 1-β-D-Arabinofuranosylcytosine into DNA and on Inhibition of DNA Polymerase by 1-β-D-Arabinofuranosylcytosine-5′-Triphosphate. Cancer Res., *30:* 2636–2644, 1970.
12. Heidelberger, C. Fluorinated Pyrimidines, Progr. Nucleic Acid Res., *4:* 1–50, 1965.
13. Howard, J. B., Cevik, N., and Murphy, M. L. Cytosine Arabinoside (NSC-63878) in Acute Leukemia in Children. Cancer Chemotherapy Rept., *50:* 287–291, 1966.
14. Kaplan, A. S., Brown, M., and Ben-Porat, T. Effect of 1-β-D-Arabinofuranosylcytosine on DNA Synthesis. I. In Normal Rabbit Kidney Cell Cultures. Mol. Pharmacol., *4:* 131–138, 1968.
15. Karon, M., and Shirakawa, S. The Locus of Action of 1-β-D-Arabinofuranosylcytosine in the Cell Cycle. Cancer Res., *29:* 687–696, 1969.
16. Kimball, A. P., and Wilson, M. J. Inhibition of DNA Polymerase by β-D-Arabinosylcytosine and Reversal of Inhibition by Deoxycytidine-5′-triphosphate. Proc. Soc. Exptl. Biol. Med., *127:* 429–432, 1968.
17. Lark, K. G., and Lark, C. L. Evidence for Two Distinct Aspects of the Mechanism Regulating Chromosome Replication in *Escherichia coli.* J. Mol. Biol., *10:* 120–136, 1964.
18. Markham, R., and Smith, J. D. Chromatographic Studies on Nucleic Acids. I. Technique for the Identification and Estimation of Purine and Pyrimidine Bases, Nucleosides and Related Substances. Biochem. J., *45:* 294–298, 1949.
19. Momparler, R. L. Effect of Cytosine Arabinoside 5′-Triphosphate on Mammalian DNA Polymerase. Biochem. Biophys. Res. Commun., *34:* 465–471, 1969.
20. Moore, E. C., and Cohen, S. S. Effect of Arabinonucleotides on Ribonucleotide Reduction by an Enzyme System from Rat Tumor. J. Biol. Chem., *242:* 2116–2118, 1967.
21. Parker, R. C. Methods of Tissue Culture, Ed. 3. pp. 77–79. New York: Paul B. Hoeber, Inc., 1961.
22. Pfieffer, S. E., and Tolmach, L. J. RNA Synthesis in Synchronously Growing Populations of HeLa S3 Cells. I. Rate of Total RNA Synthesis and Its Relationship to DNA Synthesis. J. Cellular Comp. Physiol., *71:* 77–94, 1968.
23. Puck, T. T., Marcus, P. I., and Cieciura, S. J. Clonal Growth of Mammalian Cells *in Vitro*: Growth Characteristics of Colonies from Single HeLa Cells with or without a "Feeder" Layer. J. Exptl. Med., *103:* 273–284, 1956.
24. Silagi, S. Metabolism of 1-β-D-Arabinofuranosylcytosine in L-cells. Cancer Res., *25:* 1446–1453, 1965.
25. Sinclair, W. K. Hydroxyurea: Effects on Chinese Hamster Cells Grown in Culture. Cancer Res., *27:* 297–308, 1967.
26. Thomson, R. Y. Purines, Pyrimidines and Their Derivatives. *In:* I. Smith (ed.), Chromatographic and Electrophoretic Techniques, Vol. 1, Chromatography, p. 234–245. New York: Interscience Publishers, Inc., 1960.
27. Till, J. E., Whitmore, G. F., and Gulyas, S. Deoxyribonucleic Acid Synthesis in Individual L-Strain Mouse Cells. II. Effects of Thymidine Starvation. Biochim. Biophys. Acta., *72:* 277–289, 1963.
28. Wodinsky, I., and Kensler, C. J. Activity of Cytosine Arabinoside (NSC-63878) in a Spectrum of Rodent Tumours. Cancer Chemotherapy Rept., *47:* 65–68, 1965.
29. Young, R. S. K., and Fischer, G. A. The Action of Arabinosylcytosine on Synchronously Growing Populations of Mammalian Cells. Biochem. Biophys. Res. Commun., *32:* 23–29, 1969.