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INHIBITION OF INTRACELLULAR PYRIMIDINE RIBONUCLEOTIDE REDUCTION BY DEOXYCYTIDINE, ARABINOSYLCYTOSINE AND HYDROXYUREA*

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Abstract—A correlation has been made between the ability of selected agents to inhibit intracellular pyrimidine nucleotide reduction and the ability of these agents to inhibit the growth of L5178Y cells. Both deoxycytidine and its analogue, arabinosylcytosine, appeared to inhibit intracellular cytidine phosphate reduction. The depletion of deoxycytidine phosphate pools may play a role in arabinosylcytosine toxicity by enhancing the action of arabinosylcytosine at one or more of its proposed sites of action. Hydroxyurea inhibited uridine phosphate reduction, and this inhibition correlated well with the growth inhibition caused by this agent. The inability of hydroxyurea to affect cytidine phosphate reduction suggests that there may be a cytidine phosphate reductase present in L5178Y cells which either is insensitive to inhibition by hydroxyurea or is sequestered at an intracellular site which is relatively inaccessible to this drug.

One of the current trends in cancer chemotherapy is to elucidate the mechanisms by which the various anti-cancer drugs inhibit biochemical targets in the cell and then to relate these findings to the death of tumor cells. However, many anti-cancer agents have been found to have multiple biochemical sites of action, most prominently noted at different drug levels or periods of exposure to the cell. Also, a single biochemical target appears to be of variable significance in different cell lines. It has, therefore, been difficult to determine how single anti-cancer agents cause tumor cell death.

Four lines of evidence suggest that one site of action of the deoxycytidine analogue, arabinosylcytosine, is ribonucleoside diphosphate reductase, the enzyme responsible for providing deoxyribonucleotides for DNA synthesis. The incorporation of [^{3}H]-uridine into deoxycytidine phosphate pools decreased in the presence of arabinosylcytosine with no detectable decrease in incorporation into cytidine phosphate pools of L5178Y cells [1]; arabinosylcytosine inhibited conversion of [^{14}C]adenosine-5′-phosphate to [^{14}C]deoxyadenosine-5′-phosphate by a cell-free extract of carcinoma 755 ascites cells [2]; a single-step L5178Y cell mutant resistant to arabinosylcytosine was cross-resistant to thymidine and possessed elevated deoxycytidine phosphate levels, suggesting possible elevated reductase activity [3]; and a mutant hamster cell line resistant to arabinosylcytosine was cross-resistant to hydroxyurea [4]. Contrary to these findings, phosphorylated derivatives of both arabinosylcytosine [5] and deoxycytidine [6] acted neither as an allosteric nor as a competitive inhibitor of cytidine diphosphate reduction by a partially purified Novikoff rat tumor reductase, and pool size studies employing mouse embryo cells indicated that, while arabinosylcytosine initially lowered the deoxycytidine triphosphate pools by 50 per cent, this pool returned to normal gradually and the other deoxyribonucleoside triphosphate pools doubled in size in 30 min [7].

Ribonucleoside diphosphate reductase inhibition has been proposed as the mechanism by which hydroxyurea causes toxicity in mammalian cells on the basis of its ability to inhibit partially purified reductase from Novikoff rat tumor cells [8, 9] and also of its ability to deplete the purine deoxyribonucleoside triphosphate pools of mouse embryo cells [7]. However, hydroxyurea appeared to have little effect on the pyrimidine deoxyribonucleoside triphosphate pools of mouse embryo cells [7], even though it was able to prevent reduction of cytidine diphosphate to deoxycytidine diphosphate by purified Novikoff rat tumor reductase [8, 9].

From these findings it is difficult to draw a conclusion as to whether or not arabinosylcytosine and hydroxyurea cause adverse effects by inhibition of ribonucleoside diphosphate reductase within mammalian cells. Consequently, we have investigated the ability of deoxycytidine, arabinosylcytosine and hydroxyurea to inhibit pyrimidine ribonucleotide reduction within L5178Y murine leukemia cells and have attempted to correlate this inhibition with the inhibition of L5178Y cell growth caused by these agents.

METHODS

Deoxycytidine (CdR), cytidine-5′-phosphate, deoxycytidine-5′-phosphate, uridine-5′-phosphate, deoxyuridine-5′-phosphate and thymidine-5′-phosphate were

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obtained from CalBiochem, La Jolla, Calif. Arabinosylcytosine (Ara-C) was obtained from Upjohn Co., Kalamazoo, Mich., and hydroxyurea (HU) was obtained from K & K Laboratories, Plainview, N. Y. Uridine-6-^{3}H (104 mCi/mg) (^{3}H-UR) was supplied by New England Nuclear Corp., Boston, Mass., and diethylaminoethyl (DEAE) thin-layer cellulose plastic backed sheets were supplied by Brinkmann Instruments, Westbury, N. Y. Fischer's medium and horse serum were obtained from GIBCO, Grand Island, N. Y., and Reeve Angel glass-fiber filters were obtained from Fisher Scientific Co., Medford, Mass.

Growth inhibition studies. Approximately 2×10^3 L5178Y cells were incubated at 37° in replicate 5-ml aliquots of Fischer's medium containing 10% horse serum (FMS) and increasing concentrations of the agent being studied. After 96 hr, the cell number in each tube was determined with a model B Coulter particle counter, and the number of apparent cell generations (population doublings) which occurred at each concentration of the selected agent was calculated. By dividing the apparent cell generations which occurred in the control and multiplying by 100, the per cent of control cell generations was calculated.

Incorporation of ^{3}H-uridine into intracellular pyrimidine nucleotide pools and into DNA. After incubation with the agent being studied, the cells were incubated in [^{3}H]uridine, washed three times with 5 ml cold Fischer's medium (FM) to remove unphosphorylated derivatives of [^{3}H]uridine and the cell number was determined with a model B Coulter counter. The cell pellet was then suspended in 0·5 ml of 10% trichloroacetic acid (TCA), kept for 15 min at 4° and centrifuged. The cold acid-soluble (CAS) supernatant resulting from this treatment was immersed in a boiling water bath for 30 min to hydrolyze polyphosphates to monophosphates, followed by the removal of TCA by repeated ether extractions of TCA until the pH exceeded 3.

Distribution of radioactivity among the pyrimidine monophosphate pools present in the CAS fraction was determined by modifying two previously described chromatographic procedures [10, 11] and combining them in a two-dimensional chromatographic scheme. From each cold acid-soluble fraction, 20 μl was applied to a thin-layer DEAE cellulose plastic backed sheet to which UMP, CMP, dUMP, dCMP and TMP (8 μg each) had previously been applied as carrier. This sheet was then chromatographed in 0·2 N formic acid–0·02 M NH_4 formate (50:50). This sheet was then dried and chromatographed in 5 M NH_4 acetate (pH 10)–saturated potassium tetraborate–95% ethanol–0·25 M EDTA, pH 10 (5:40:40:0·25). This chromatographic procedure gave a clear separation of the various pyrimidine nucleoside monophosphates with the exception of TMP, which traveled coincidently with dUMP. The various unphosphorylated pyrimidine derivatives traveled ahead of the nucleotides in the second dimension and thus were well separated from the nucleoside monophosphates. Nucleotide spots were detected with a 254-nm u.v. light, and the radioactivity present in these spots was determined by cutting them out and counting them in a model 3375 Packard Tricarb liquid scintillation spectrometer in a POPOP* (0·05 g/l.)–PPO(4 g/l.)–toluene liquid scintillation mixture.

Radioactive incorporation into DNA was determined by modifying a previously described glass-fiber filter method [12]. The pellet remaining after the TCA treatment was suspended in 1 ml of 0·1 N NaOH and placed in a boiling water bath for 10 min to hydrolyze RNA. The tubes were then cooled and 5 ml of 10% TCA was added to precipitate DNA. The contents of each tube were then filtered through Reeve Angle glass-fiber filters to trap the DNA; the filters were then dried and the radioactivity present in the DNA was determined by counting in the liquid scintillation mixture described above.

* POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazobyl)]benzene; PPO = 2,5-diphenyloxazole.

RESULTS

Effect of deoxycytidine on intracellular pyrimidine nucleotide reduction. The effect of deoxycytidine on the incorporation of [^{3}H]uridine into intracellular pyrimidine nucleotide pools is presented in Table 1. Increasing concentrations of deoxycytidine appear to

Table 1. Effect of deoxycytidine on [^{3}H]uridine incorporation into intracellular pyrimidine nucleotide pools*

Deoxycytidine concn	Activity (cpm/10^6 cells)				
	CMP	dCMP	UMP	dUMP TMP	Total CAS
0	17,440	1,210	108,540	1,050	128,240
2×10^{-6} M	21,870	1,240	99,940	690	123,740
4×10^{-6} M	19,130	1,010	126,300	1,320	147,740
8×10^{-6} M	23,990	930	96,990	840	122,750
$1{\cdot}6 \times 10^{-5}$ M	21,550	690	112,800	1,090	136,140
$3{\cdot}2 \times 10^{-5}$ M	21,220	650	110,050	1,100	133,010

* The 5×10^6 to 1×10^7 L5178Y cells were preincubated in 40 ml FMS containing increasing concentrations of deoxycytidine for 70 min at 37°. These cells were then incubated in 2 ml FMS containing the appropriate deoxycytidine concentration and 20 μCi of [^{3}H]uridine for 15 min at 37°. The incubation was terminated by addition of 10 ml cold FM, and radioactive incorporation into intracellular pyrimidine nucleotide pools was determined as described in Methods. Each value represents the arithmetic mean of three experiments.

have little effect on [^{3}H]uridine incorporation into pyrimidine ribonucleotide pools and also had little effect on incorporation into the dUMP–TMP pools, except at the lowest concentration employed. Incorporation of radioactivity into deoxycytidine phosphate pools, however, appears to decrease with increasing deoxycytidine levels.

These incorporation data have been treated to better illustrate the effect of deoxycytidine on intracellular pyrimidine nucleotide reduction. Within the cell there is a pool of ribonucleotides as well as a pool of deoxyribonucleotides, and the ribonucleoside diphosphate reductase enzyme is responsible for converting ribonucleotides to deoxyribonucleotides. The amount of radioactivity which accumulates in the deoxyribonucleotide pool over the 15-min incubation in [^{3}H]-uridine precursor is a function both of the activity of the reductase enzyme and of the amount of radioactive label which is present in the ribonucleotide pool. To minimize any fluctuations in deoxyribonucleotide radioactivity due to fluctuations in radioactive incorporation into ribonucleotide pools, the ratio of accumulated deoxyribonucleotide radioactivity to accumulated ribonucleotide radioactivity has been taken as an indication of intracellular nucleotide reduction. By dividing the ratio occurring in the presence of deoxycytidine by the untreated control ratio and multiplying by 100, the per cent of control ribonucleotide reduction was obtained.

These data are illustrated graphically in Fig. 1. It appears that deoxcytidine effectively inhibits the reduction of cytidine phosphate while having little effect on uridine phosphate reduction, except at the lowest deoxycytidine level employed.

Effect of arabinosylcytosine on intracellular pyrimidine nucleotide reduction, radioactive incorporation into DNA and L5178Y cell growth. The effect of arabinosylcytosine on the incorporation of [^{3}H]uridine into intracellular pyrimidine nucleotide pools and into DNA is depicted in Table 2. Arabinosylcytosine has little effect on the incorporation of ^{3}H-uridine into ribonucleotide pools except at the highest concentration employed, where some inhibition of incorporation is apparent. Arabinosylcytosine also has little effect on the radioactivity appearing in the dUMP–TMP pools, while it inhibits radioactive incorporation both into deoxycytidine phosphate pools and into DNA.

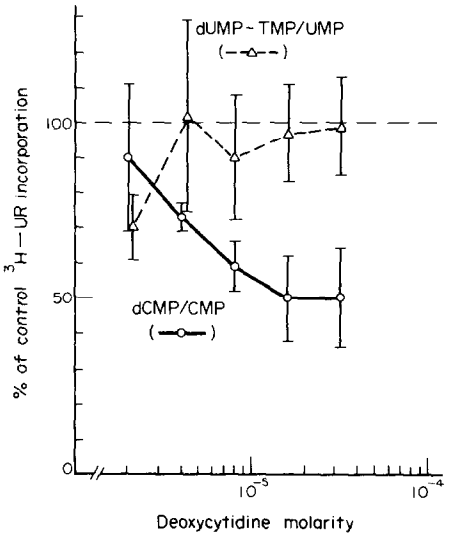

Fig. 1. Effect of deoxycytidine on intracellular pyrimidine nucleotide reduction. The data in Table 1 have been treated as described in the text to better illustrate the effect of deoxycytidine on intracellular pyrimidine nucleotide reduction. Each point represents the arithmetic mean of three experiments and the brackets indicate the standard error of the mean.

These data have been treated to better correlate arabinosylcytosine inhibition of cytidine phosphate reduction and radioactive incorporation into DNA. The data concerning radioactive incorporation into pyrimidine nucleotide pools have been treated as described for deoxycytidine. As is the case with the incorporation of radioactivity into intracellular deoxyribonucleotide pools, radioactive incorporation into DNA is a function of several variables. One of these variables is the uptake of the radioactive precursor into intracellular nucleotide pools, and another variable is the intracellular metabolism of the nucleotide precursor to deoxyribonucleotide triphosphate. To minimize an effect on radioactive incorporation into DNA due to alteration in uptake, the ratio of the

Table 2. Effect of arabinosylcytosine on [^{3}H]uridine incorporation into intracellular pyrimidine nucleotide pools and into DNA*

Ara-C concn	Activity (cpm/10^6 cells)					
	CMP	dCMP	UMP	dUMP–TMP	Total CAS	DNA
0	25,850	1,480	159,070	1,060	187,850	7,680
1×10^{-7} M	22,790	930	160,950	1,090	185,780	5,670
2×10^{-7} M	25,910	660	156,060	960	183,590	3,060
4×10^{-7} M	16,550	310	136,830	660	154,570	1,730

* The 5×10^6 to 1×10^7 L5178Y cells were preincubated in 40 ml FMS containing increasing arabinosylcytosine concentrations for 60 min at 37°. The cells were then incubated in 2 ml FMS containing the appropriate arabinosylcytosine concentration and 20 μCi of [^{3}H]uridine for 15 min at 37°. The incubation was terminated by addition of 10 ml cold FM, and radioactive incorporation into intracellular pyrimidine nucleotide pools and into DNA was determined as described in Methods. Each value represents the arithmetic mean of three experiments.

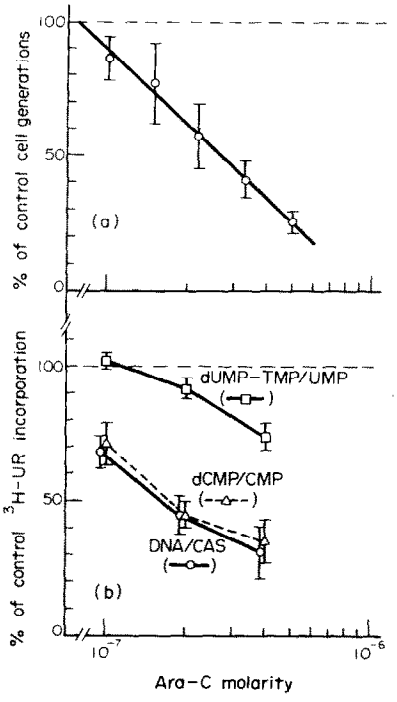

Fig. 2. Effect of arabinosylcytosine on intracellular pyrimidine nucleotide reduction, radioactive incorporation into DNA and L5178Y cell growth. (a) Effect of arabinosylcytosine on L5178Y cell growth in culture. Cells were incubated in increasing arabinosylcytosine concentrations for 96 hr, and the effect of arabinosylcytosine on growth was calculated as described in Methods. Each point represents the arithmetic mean of three experiments done in duplicate and the brackets indicate the standard error of the mean. (b) Effect of arabinosylcytosine on pyrimidine nucleotide reduction and radioactive incorporation into DNA. The data in Table 2 have been treated as described in the text and presented graphically. Each point represents the arithmetic mean of three experiments and the brackets indicate the standard error of the mean.

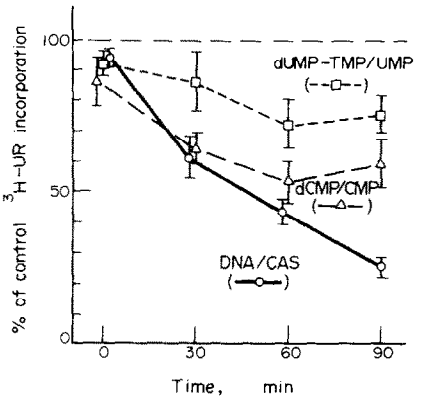

Fig. 3. Time course of arabinosylcytosine effect on intracellular pyrimidine nucleotide reduction and radioactive incorporation into DNA. The 5×10^6 to 9×10^6 cells were incubated at 37° in 40 ml FMS for 0, 30, 60 and 90 min. One set of tubes contained 2×10^{-7} M arabinosylcytosine and the control tubes contained no drug. This was followed by incubation of the cells in 2 ml FMS and 20 μCi [^{3}H]uridine for 15 min. The incubation was terminated by addition of 10 ml cold FM, and radioactive incorporation into pyrimidine nucleotide pools and into DNA was determined as described in Methods. These incorporation data have been treated as described in the text and have been presented graphically in this figure. Each point represents the arithmetic mean of three experiments and the brackets indicate the standard error of the mean.

DNA radioactivity to the radioactivity occurring in intracellular nucleotide pools has been employed as an indication of alteration of radioactive incorporation into DNA. By dividing the ratio occurring at each level of arabinosylcytosine by the control ratio and multiplying by 100, the per cent of control radioactive incorporation into DNA is obtained.

These data are illustrated graphically in Fig. 2b. As with deoxycytidine, arabinosylcytosine produces definite inhibition of cytidine phosphate reduction while having little effect on uridine phosphate reduction. In addition, this inhibition of deoxycytidine phosphate formation is closely correlated with inhibition of radioactive incorporation into DNA. A comparison of these inhibitions with arabinosylcytosine-induced L5178Y cell growth inhibition, depicted in Fig. 2a, reveals that arabinosylcytosine-induced inhibition of cytidine phosphate reduction, radioactive incorporation into DNA and L5178Y cell growth all occur over the same arabinosylcytosine concentration range.

The effect of 2×10^{-7} M arabinosylcytosine on intracellular pyrimidine nucleotide reduction and radioactive incorporation into DNA as a function of time is represented by Fig. 3. Inhibition of cytidine phosphate reduction and radioactive incorporation into DNA coincide at early time intervals, while at later time intervals inhibition of radioactive incorporation into DNA continues to increase while inhibition of cytidine phosphate reduction appears to level off.

Effect of hydroxyurea on intracellular pyrimidine nucleotide reduction, radioactive incorporation into DNA and L5178Y cell growth. The effect of hydroxyurea on the incorporation of [^{3}H]uridine into intracellular pyrimidine nucleotide pools and into DNA is depicted in Table 3. Hydroxyurea has little effect on [^{3}H]uridine incorporation into pyrimidine ribonucleotide pools and into the deoxycytidine phosphate pools while it decreases incorporation into dUMP-TMP and into DNA.

These data have been treated as described for deoxycytidine and arabinosylcytosine and are depicted graphically in Fig. 4b. Hydroxyurea-induced inhibition of uridine phosphate reduction and radioactive incorporation into DNA are closely correlated while this agent has little or no capacity to inhibit cytidine phosphate reduction. The effect of hydroxyurea on L5178Y cell growth (Fig. 4a) demonstrates that hydroxyurea-induced inhibition of uridine phosphate reduction, radioactive incorporation into DNA and inhibition of cell growth all occur over the same hydroxyurea concentration range.

A time course of hydroxyurea effect on pyrimidine nucleotide reduction and radioactive incorporation into DNA is illustrated in Fig. 5. Initially, inhibition of uridine nucleotide reduction and inhibition of

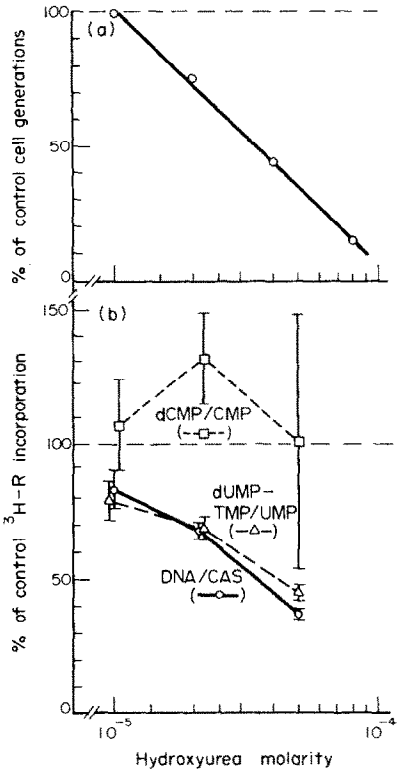

Fig. 4. Effect of hydroxyurea on intracellular pyrimidine nucleotide reduction, radioactive incorporation into DNA and L5178Y cell growth. (a) Effect of hydroxyurea on L5178Y cell growth in culture. Cells were incubated in increasing hydroxyurea concentrations for 96 hr, and the effect of hydroxyurea on cell growth was calculated as described in Methods. Each point represents the average of two experiments done in duplicate. (b) Effect of hydroxyurea on pyrimidine nucleotide reduction and radioactive incorporation into DNA. The data in Table 3 have been treated as described in the text and presented graphically. Each point represents the arithmetic mean of three experiments and the brackets indicate the stardard error of the mean.

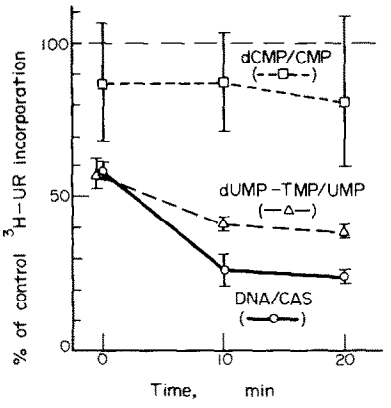

Fig. 5. Time course of hydroxyurea effect on intracellular pyrimidine nucleotide reduction and radioactive incorporation into DNA. The 4×10^6 to 1×10^7 cells were incubated at 37° in 2 ml FMS for 0, 10 and 20 min. One set of tubes contained 5×10^{-5} M hydroxyurea and one set contained no drug. The incubation was continued for 15 more min after the addition of 20 μCi [^{3}H]uridine. The incubation was terminated by addition of 10 ml cold FM, and radioactive incorporation into pyrimidine nucleotide pools and into DNA was determined as described in Methods. These incorporation data have been treated as described in the text and have been presented graphically in this figure. Each point represents the arithmetic mean of three experiments and the brackets indicate the standard error of the mean.

radioactive incorporation into DNA coincide, and inhibition of radioactive incorporation into DNA only exceeds inhibition of uridine nucleotide reduction at later time intervals.

DISCUSSION

Due to the complexity of cellular metabolism, it is difficult to draw definite conclusions from the intracellular effects of deoxycytidine, arabinosylcytosine and hydroxyurea described above. However, in addition to correlating some of these intracellular effects with the toxicity caused by these compounds, the data suggest certain interesting possibilities concerning intracellular nucleotide metabolism which are worthy of further investigation.

The inhibition of intracellular cytidine phosphate reduction by deoxycytidine suggests that the ribonucleoside diphosphate reductase of L5178Y cells, unlike that of Novikoff tumor cells [5, 8], may be sensitive to allosteric inhibition by deoxycytidine triphosphate, as was found to be the case in chick brain [13] and

Table 3. Effect of hydroxyurea on [^{3}H]uridine incorporation into intracellular pyrimidine nucleotide pools and into DNA*

Hydroxyurea concn	Activity (cpm/10^6 cells)					
	CMP	dCMP	UMP	dUMP-TMP	Total CAS	DNA
0	14,060	970	78,170	800	94,000	5,900
1×10^{-5} M	16,050	1,460	88,000	740	105,920	5,240
$2{\cdot}2 \times 10^{-5}$ M	13,730	1,300	87,910	610	103,550	4,340
5×10^{-5} M	11,180	860	78,050	360	90,450	2,070

* The 6×10^6 to 1×10^7 L5178Y cells were incubated for 10 min at 37° in 2 ml FMS containing increasing concentrations of hydroxyurea. Twenty μCi of [^{3}H]uridine was then added and the incubation was continued for 15 min. The incubation was terminated by addition of 10 ml cold FM, and radioactive incorporation into pyrimidine nucleotide pools and into DNA was determined as described in Methods. Each value represents the arithmetic mean of three experiments.

Yaba monkey tumor [14]. Another possible explanation is that deoxycytidine is metabolized to thymidine triphosphate within L5178Y cells and that intracellular inhibition of cytidine phosphate reduction is due to allosteric inhibition of the reductase enzyme caused by intracellular accumulation of thymidine triphosphate. If this were the case, however, intracellular uridine phosphate reduction would also be inhibited by deoxycytidine, since thymidine triphosphate has been shown to inhibit both cytidine and uridine diphosphate reduction by ribonucleoside diphosphate reductase [5, 8]. Thus, our inability to detect significant deoxycytidine-induced inhibition of uridine phosphate reduction within L5178Y cells, except at the lowest concentration employed, would tend to shed doubt on this explanation for deoxycytidine-induced inhibition of cytidine phosphate reduction. Kinetic studies of L5178Y cell ribonucleoside diphosphate reductase are necessary, however, to provide conclusive evidence that this enzyme is sensitive to allosteric inhibition by deoxycytidine triphosphate.

Arabinosylcytosine appears to have the capacity to inhibit the intracellular reduction of cytidine phosphate in L5178Y cells. Depletion of deoxycytidine phosphate pools is implicated in the mechanism of arabinosylcytosine toxicity by the close correlation of this depletion with inhibition of radioactive incorporation into DNA and by the fact that both inhibition of cytidine phosphate reduction and incorporation into DNA occur over the same arabinosylcytosine concentration range at which growth inhibition occurs.

It is possible that arabinosylcytosine inhibits deoxycytidine phosphate formation indirectly by feedback inhibition of ribonucleoside diphosphate reductase resulting from elevation of deoxyribonucleotide pools due to DNA synthesis inhibition. If this were the case, however, inhibition of radioactive incorporation into DNA would be initially greater than inhibition of radioactive incorporation into deoxyribonucleotide pools. Thus, the finding that arabinosylcytosine-induced inhibition of cytidine nucleotide reduction and radioactive incorporation into DNA coincide at early time intervals suggests that arabinosylcytosine may be acting directly to inhibit cytidine phosphate reduction. These time course data do not provide conclusive evidence, however, because of the necessity of employing a 15-min incubation period to detect significant accumulation of radioactivity in the intracellular deoxyribonucleotide pools; any complex effects of arabinosylcytosine during this period would be undetected. An investigation of the ability of phosphorylated derivatives of arabinosylcytosine to affect the ability of L5178Y cell homogenates to reduce cytidine diphosphate will hopefully provide such conclusive evidence.

The time course of arabinosylcytosine-induced inhibition of cytidine phosphate reduction and radioactive incorporation into DNA also reveals that at later time intervals the inhibition of radioactive incorporation into DNA continues to increase while the inhibition of cytidine phosphate reduction levels off. This suggests that, while at early time intervals inhibition of radioactive incorporation into DNA may be a function of inhibition of cytidine phosphate reduction, at later time intervals arabinosylcytosine-induced inhibition of radioactive incorporation into DNA may be independent of inhibition of cytidine phosphate reduction. A reasonable explanation for this finding is that depletion of deoxycytidine phosphate pools by arabinosylcytosine decreases the intracellular concentration of deoxycytidine triphosphate, an antagonist of arabinosylcytosine, thus allowing arabinosylcytosine to exert its effect at one or more of its various proposed sites of action to inhibit DNA synthesis. Thus, depletion of deoxycytidine phosphate pools may play a role in arabinosylcytosine toxicity by enhancing the action of arabinosylcytosine at other sites within the cell.

This hypothesis explains the finding that, while arabinosylcytosine initially decreased the intracellular deoxycytidine triphosphate pool of mouse embryo cells by 50 per cent, this pool gradually returned to normal [7]. The initial decrease of deoxycytidine triphosphate could have been due to a direct effect of arabinosylcytosine initially on cytidine phosphate reduction. However, when inhibition of DNA synthesis exceeded inhibition of reduction at later time intervals, the deoxycytidine triphosphate pools would have gradually increased due to prevention of deoxycytidine triphosphate incorporation into DNA.

Hydroxyurea has the capacity to inhibit intracellular uridine phosphate reduction and this inhibition is closely correlated with inhibition of radioactive incorporation into DNA and inhibition of cell growth. The time course data are consistent with the interpretation that inhibition of dUMP TMP formation is a primary effect of hydroxyurea and is not an indirect result of DNA synthesis inhibition. Thus, the inhibition of isolated Novikoff tumor ribonucleoside diphosphate reductase [8, 9], the depletion of purine deoxyribonucleoside triphosphate pools in mouse embryo cells [7], and the correlation we have found between intracellular inhibition of uridine phosphate reduction, radioactive incorporation into DNA and inhibition of L5178Y cell growth strongly suggest that the toxicity caused by hydroxyurea in mammalian cells is due at least in part to the intracellular inhibition of ribonucleoside diphosphate reductase.

Our inability to detect significant hydroxyurea-induced inhibition of intracellular cytidine phosphate reduction in L5178Y cells is in agreement with the finding that hydroxyurea is without effect on the deoxycytidine triphosphate pool of mouse embryo cells [7]. However, these results conflict with the finding that this drug inhibits cytidine diphosphate reduction by isolated Novikoff tumor ribonucleoside diphosphate reductase [8, 9] and with the strong evidence that hydroxyurea is capable of inhibiting ribonucleoside diphosphate reductase within mammalian cells, as described above.

Since hydroxyurea has been shown to inhibit isolated soluble mammalian ribonucleoside diphosphate reductase by preventing the actual reductive process [9], it is difficult to explain why intracellular uridine phosphate reduction but not cytidine phosphate reduction is inhibited by hydroxyurea unless the presence of a cytidine phosphate reductase is postulated which is either insensitive to hydroxyurea or is sequestered at an intracellular site which is relatively inaccessible to this drug. In this case, hydroxyurea-induced inhibition of soluble ribonucleoside diphos-

phate reductase within L5178Y cells would have little effect on deoxycytidine nucleotide levels, since the hypothetical cytidine phosphate reductase would continue to operate.

The possibility that more than one reductase is present in mammalian cells is supported by the finding that extracts of regenerating rat liver treated with actinomycin D lost the capacity to reduce cytidine diphosphate while retaining much of the capacity to reduce adenine diphosphate and that, in untreated cells, cytidine diphosphate- and adenine diphosphate-reducing ability could be separated somewhat during the purification of the enzyme [15]. Cytidine diphospate-reducing activity has also been found associated with smooth membrane in Novikoff tumor cells [16] and with nuclear and mitochondrial fractions of M1 sarcoma cells [17], and this enzymatic activity may have different allosteric and catalytic properties from that found in the cytosol.

On the other hand, the finding that hydroxyurea produced a distinct inhibition of intracellular deoxycytidine nucleotide formation in human leukemic cells [18] suggests that a separate, hydroxyurea-insensitive cytidine phosphate reductase does not exist, at least in these cells. The hydroxyurea concentration employed in this study, however, was 400-fold higher than the concentration employed in our investigation, and it is possible that this excessive amount may affect a cytidine phosphate reductase which is relatively insensitive to hydroxyurea or which is relatively inaccessible to this drug within the cell.

As a concluding remark, it should be mentioned that, due to the intricacy of intracellular nucleotide metabolism, our inability to demonstrate hydroxyurea-induced inhibition of intracellular cytidine phosphate reduction may be due to a complex effect of this drug rather than to the presence of a second reductase. Thus, while these intracellular studies suggest the possibility that such a reductase may be present in L5178Y cells, direct measurement of this enzyme activity is necessary to demonstrate conclusively the existence of such an enzyme.

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