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Human Deoxycytidylate Deaminase

Substrate and Regulator Specificities and Their Chemotherapeutic Implications

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SUMMARY

Deoxycytidylate deaminase isolated from blast cells of patients with acute myelocytic leukemia has been characterized as to the specificity of substrates and allosteric regulators. Neither CMP nor 1-β-D-arabinofuranosyl CMP was an effective substrate or inhibitor of the enzymatic reaction, while both CTP and 1-β-D-arabinofuranosyl CTP could activate this enzyme as well as dCTP. These discriminating properties for substrate and effector binding led us to postulate the therapeutic effectiveness of 1-β-D-arabinofuranosylcytosine through a "self-potentiation" mechanism. 5-Methyl dCMP, 5-bromo-dCMP, and 5-iodo dCMP were good substrates for dCMP deaminase in the absence of the allosteric regulators. The specificity of action of the allosteric regulators for these analogues was found to be different from that for dCMP. These observations suggest that the interaction between the substrate and regulatory sites of the enzyme can alter not only the ligand binding affinity but also the rate of catalysis for a particular substrate. Among all of the nucleoside monophosphates examined, GMP and dGMP were the most potent inhibitors of dCMP deamination. The inhibition by GMP or dGMP was attenuated by dCTP and potentiated by dTTP. This suggests that GMP and dGMP might also be involved in the regulation of dCMP deaminase activity under physiological conditions.

INTRODUCTION

The characterization of deoxycytidylate deaminase (EC 3.5.4.12), an important enzyme in *de novo* synthesis of thymidine nucleotides (1, 2), has revealed significant variations in physical and kinetic properties of the enzyme isolated from a number of sources (3–15). These observed differences may be related to either species specificity or artifacts due to the unstable nature of this enzyme. Since this enzyme plays a key role in nucleotide metabolism, it also may be involved in the action as well as the metabolism of the anticancer agent area-C[1] (16, 17). Therefore, it is important to examine the behavior of this enzyme toward the phosphorylated derivatives of ara-C.

Recent reports of deoxycytidylate deaminase isolated from cultured CCRF-CEM human lymphoblastic leukemic cells (13) and from human spleen (14) have shown that ara-CMP is an effective substrate for the enzyme. In a preliminary report (15), we observed that ara-CMP was neither an inhibitor nor a significant substrate for deoxycytidylate deaminase isolated from AML cells. This communication reports the detailed investigation of the substrate and regulator specificities of the AML enzyme and their chemotherapeutic implications.

MATERIALS AND METHODS

Nucleotide analogues ara-ATP, ara-CTP, 5-I-dUTP, and 5-I-dCTP were purchased from P-L Biochemicals, Inc. Milwaukee, Wisc.). 2′-Azido-ara-CMP and 2′-azido-ara-CTP were kindly provided by Dr. A. Bloch and Dr.

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[1] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CMP, 1-β-D-arabinofuranosylcytosine-5′-monophosphate; AML, acute myelocytic leukemia; ara-CTP, 1-β-D-arabinofuranosylcytosine-5′-triphosphate; 5-F-dUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; 5-Br-dUMP, 5-bromo, 2′-deoxyuridine-5′-monophoshate; 5-I-dUMP, 5-iodo-2′-deoxyuridine-5′-monophosphate; 5-Br-dCMP, 5-bromo-2′-deoxycytidine-5′-monophosphate; 5-I-dCMP, 5-iodo-2′-deoxycytidine-5′-monophosphate; 5-CH_3-dCMP, 5-methyl-2′-deoxycytidine-5′-monophosphate; 2′-azido-ara-CMP, 2′-azido-1-β-D-arabinofuranoside-5′-monophosphate; 2-F-ara-AMP, 2-fluoroadenine-9-β-D-arabinofuranoside-5′-monophosphate. For the triphosphates of the analogues, the M in the abbreviation is T. I_{50}, concentration of inhibitor needed to inhibit the enzymatic reaction by 50%.

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A. Schroeder, of Roswell Park Memorial Institute, and 2-F-ara-AMP by Dr. J. Montgomery and Dr. R. Brockman, of Southern Research Institute. All other nucleosides and nucleotides as well as cellulose phosphate were purchased from Sigma Chemical Company (St. Louis, Mo.). Blue Sepharose (CL-6B), phenyl Sepharose (CL-4B), and Sephadex G-15 were obtained from Pharmacia Fine Chemicals (Piscataway, N. J.). Dowex AG 50W-X4(hydrogen form) was purchased from Bio-Rad Laboratories (Richmond, Calif.). [U-^{14}C]dCMP (408 mCi/mmole), [U-^{14}C]CMP (517 mCi/mmole), [U-^{14}C]dAMP (574 mCi/mmole), and [U-^{14}C]dGMP (482 mCi/mmole) were obtained from Amersham/Searle (Arlington Heights, Ill.), and [5-^{3}H]ara-CMP (15 Ci/mmole) was purchased from Moravek Biochemicals (Brea, Calif.). All other chemicals were reagent-grade.

Enzyme preparation. A preliminary report of the purification scheme has been presented (18). Blast cells (obtained by leukophoresis) from untreated patients with AML were provided by Department of Medicine A of Roswell Park Memorial Institute. Contaminating erythrocytes were removed by hypotonic shock as described previously (19). Packed cells (3 g) were suspended in 15 ml of extraction buffer [10 mM Tris-HCl (pH 7.5)/3 mM dithiothreitol/1.5 mM $MgCl_2$] before being frozen and thawed three times. KCl was added to a final concentration of 0.15 M followed by sonication and centrifugation at 15,000 rpm for 30 min. The supernatant was treated by a 1% streptomycin sulfate precipitation followed by two ammonium sulfate fractionation steps similar to those described (20). The enzyme precipitated in the 35%–50% ammonium sulfate step was resuspended in Buffer A [50 mM Tris-HCl (pH 7.5)/2 mM dithiothreitol/10% Glycerol] before being applied to a phosphocellulose column (2 × 7 cm). The unadsorbed fraction was then applied to a Blue Sepharose column (1.5 × 9.0 cm) and treated as shown (Fig. 1A). Solid ammonium sulfate was slowly added to the pooled enzyme solution to a final concentration of 1 M before being applied to a phenyl Sepharose column (0.9 × 9 cm) equilibrated with Buffer B [1 M ammonium sulfate/2 mM dithiothreitol/10 mM Tris-HCl (pH 7.5)]. The enzyme was eluted by a linear gradient (80 ml) of Buffer B to Buffer C [10 mM Tris-HCl (pH 7.5)/2 mM dithiothreitol/50% ethylene glycol monoethyl ether] (Fig. 1B). The peak fractions were pooled and passed through a G-25 column equilibrated with Buffer A before being applied to a phosphocellulose column (2.5 × 8.0 cm). The enzyme was eluted by a linear gradient of Buffer A to Buffer D (50 mM Tris-HCl/2 mM dithiothreitol/10% glycerol/2 M KCl (Fig. 1C). Peak fractions were pooled and desalted before use in characterization studies. The total amount of enzyme recovered was consistently 35% (Table 1). The enzyme preparation was devoid of the potentially interfering enzymes Cyd-dCyd deaminase, dCyd kinase, dCMP kinase, and phosphatase activity. Under standard assay conditions the catalytic conversion of dCMP to dUMP using the purified preparation was found to be linear with respect to time (up to 60 min) and enzyme concentration.

Enzyme assays. The assay is a modification of a previously described method that uses radiolabeled substrates (21). The dCMP deaminase reaction mixture contained, in a volume of 0.1 ml: 50 mM morpholinopropanesulfonic acid (pH 7.5), 2 mM dithiothreitol, 2 mM $MgCl_2$, bovine serum albumin (250 μg/ml), 50 μM EDTA, the enzyme preparation, and the additives as indicated. The reaction was terminated by the addition of 50 μl of 1.3 N cold trichloroacetic acid. One unit of dCMP deaminase is defined as the amount of enzyme which catalyzes the formation of 1 nmole of dUMP from dCMP per minute at 37° under standard assay conditions.

When substrates were used for which radioactive material was not available, the enzymatic reaction was terminated by heating at 100° for 3 min. The substrate and product were separated by high-pressure liquid chromatography with a Partisil SAX anion exchange column(SAX 10/25) and an isocratic buffer, 50 mM NH_4HPO_4 (pH 3.35). UV absorption peaks at 254 and 280 nm were integrated and the ratios were determined. Retention times were accurate to ±0.05 min for all nucleotides. Substrate and product peak ratios were accurate to ±1.0%.

Enzyme stability. The enzyme preparation was extremely labile. However, in this study the particular ligand concentrations chosen for the enzyme assays yielded a linear rate of catalysis under the conditions described. All of the determinations were performed two or more times.

Protein determination and method for protein concentration. The Hartree (22) modification of the Lowry method or the Coomassie blue method (23) was used to determine protein, using bovine serum albumin as the standard. The purified enzyme was concentrated using a negative pressure p-Micro-Pro DiCon microprotein dialysis system (obtained from Bio-Molecular Dynamics, Beaverton, Ore.). The procedure was performed at 5° for 24 hr using a dialysate buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM mercaptoethanol, 0.2 mM $MnCl_2$, and 50 μM dCTP.

RESULTS

Behavior of dCMP analogues. The concentration of dCMP analogues in the reaction mixture was the same as that of dCMP. Their effects on deoxycytidylate deaminase are shown in Table 2. 5-CH_3-dCMP, 5-Br-dCMP, and 5-I-dCMP extensively inhibited dCMP deamination (competitively), whereas the other three analogues did not significantly inhibit the reaction. The degree of inhibition was not altered when 1 μM dCTP was added to the reaction mixture. Since the relationship of velocity to the concentration of dCMP of this enzyme was sigmoidal in the absence of dCTP, accurate K_I values could not be obtained in the absence of 2.5 μM dCTP. However, Michaelis-Menten kinetics was observed in the presence of 2.5 μM dCTP. Under this condition those inhibitors were competitive with dCMP, and the K_I values were estimated for 5-CH_3-dCMP 5-Br-dCMP, and 5-I-dCMP to be 12 μM, 5 μM, and 5 μM, respectively.

Since the reduction of dCMP deamination by these analogues could be achieved by either true inhibition or because of alternate substrate capability of the analogues for the enzyme, the behavior of the analogues (0.8 mM) in the presence of enzyme was examined. The results are shown in Table 3. All three analogues could serve as

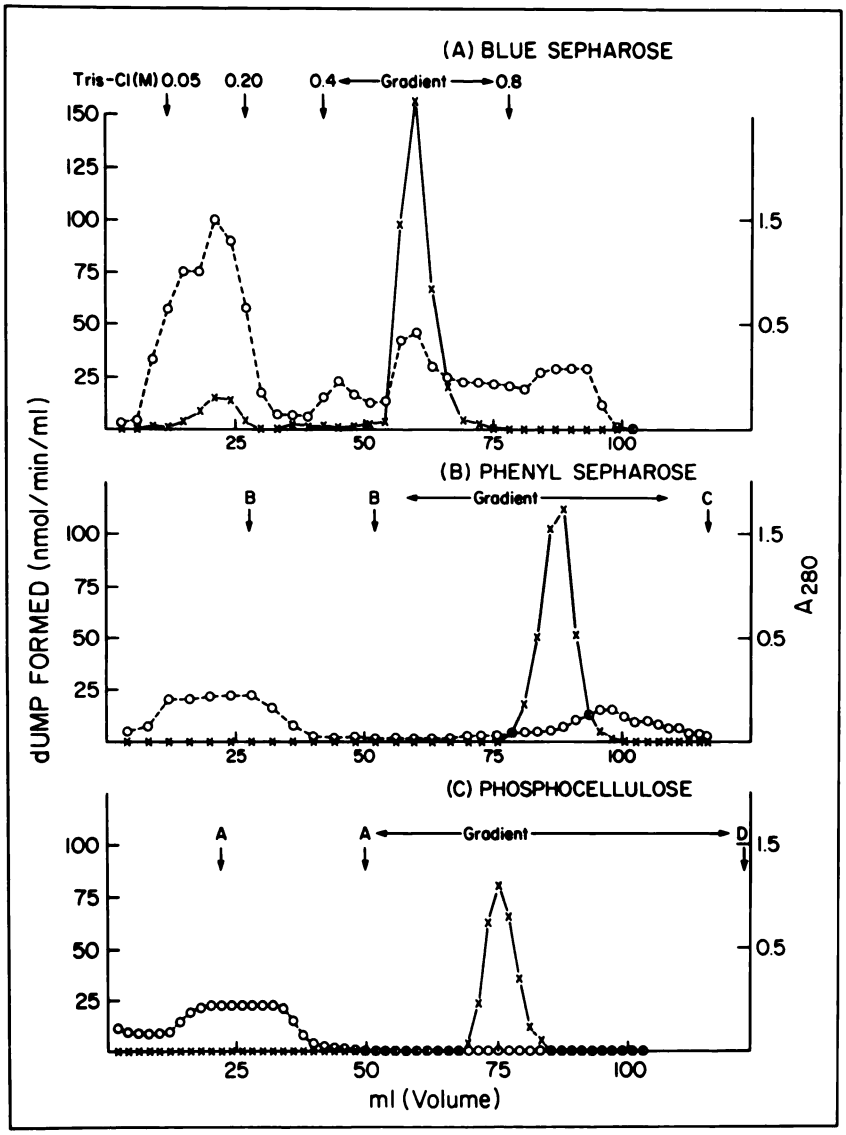

FIG. 1. *Column chromatography profile of dCMP deaminase activity*

A, Blue Sepharose column chromatography. The unadsorbed fraction from phosphocellulose column chromatography (Phosphocellulose 1) was applied to the column and developed as described. B, Phenyl Sepharose column chromatography. The enzyme preparation from Blue Sepharose was loaded onto the column after the addition of ammonium sulfate to a final concentration of 1 M and developed as described. C, Phosphocellulose column chromatography (Phosphocellulose 2). The enzyme solution from phenyl Sepharose was passed through a G-25 column equilibrated with Buffer A before being applied to the column. Fractions (2 ml) were collected and assayed as described. Enzyme activity, × —— ×; A_{280}, O- - -O.

substrate. The effect of dCTP and dTTP on the deamination of these analogues was different from that of dCMP. dTTP did not inhibit and dCTP did not activate the enzymatic reaction when these analogues were used as the substrates.

CMP and ara-CMP were examined for their ability to be deaminated by deoxycytidylate deaminase (Table 4). Ara-CMP and CMP were poor substrates in comparison with dCMP under all of the conditions examined.

Effects of the monophosphates of other nucleosides and their analogues. The monophosphate of several nucleosides and their analogues were examined for their effects on deoxycytidylate deaminase (Table 5). Among those occurring in nature, GMP and dGMP had potent inhibitory activity. The inhibitory action of dGMP (Fig. 2A) or GMP (data not shown) could be reversed by increasing the concentration of dCMP in the reaction mixture. When GMP and dGMP were examined as possible substrates for deoxycytidylate deaminase in the absence or presence of dCTP, dGTP, dTTP, or dATP, neither dGMP nor GMP was deaminated (data not shown). The inhibition of dCMP deamination by dGMP (Fig. 2B) or GMP (data not shown) could be potentiated by dTTP and attenuated by dCTP. At 0.8 mM dCMP, the I_{50} for dGMP was 105 μM. The I_{50} was decreased to 35 μM in the presence of 1 μM dTTP and increased to more than 200 μM with 1 μM dCTP in the reaction mixture.

The 5-substituted derivatives of dUMP also were found to inhibit dCMP deaminase. The potency of these

TABLE 1

Purification scheme

Step	Total activity	Specific activity	Purification yield	
			-fold	%
	units	*units/mg*		
Homogenate	1,160	17	—	100
Streptomycin sulfate (196)	532[a]	6.4	0.4	—
Ammonium sulfate (35–50%)	938	74	4.4	80
Phosphocellulose 1	1,123	142	8.4	97
Blue Sepharose	1,060	1,046	62	91
Phenyl Sepharose	1,026	3,931	232	88
Phosphocellulose 2	426[b]	142,400	8,376	36

[a] The assay was performed in the presence of streptomycin sulfate, which interfered with enzyme activity. It is believed that the removal of streptomycin sulfate in subsequent purification steps accounts for the increase in total units following this step.

[b] Peak tubes were pooled (6 ml) and concentrated more than 50-fold in order to determine the concentration of protein.

substitutions at position 5 is in the order of I > Br = CH_3 (thymidine) > H. The K_I of dTMP was estimated to be 12 μM.

Effects of the triphosphates of nucleosides and their analogues. Several triphosphate derivatives of the nucleosides and their analogues were examined for their effect on dCMP deamination by deoxycytidylate deaminase (Table 6). The choice of 0.5 mM dCMP in this study is due to the unstable nature of the enzyme at lower concentrations of dCMP at 37°. Thus the maximal activation of dCMP deamination by dCTP could only be 0.22-fold more than in its absence at 0.5 mM dCMP. All dCTP analogues could serve as the activator. Higher concentrations of ara-CTP or 2′-azido ara-CTP (i.e., 1 mM) also yield 0.22-fold activation. All of the dUTP analogues examined inhibited dCMP deamination. It should be noted that dTTP was not an inhibitor when 5-CH_3-dCMP, 5-Br-dCMP, or 5-I-dCMP was used as the substrate (Table 3).

DISCUSSION

We have analyzed the substrate specificity and allosteric regulators of purified deoxycytidylate deaminase from human blast cells of patients with AML. A number of interesting findings have been made with practical implications for cancer and antiviral chemotherapy. The dCMP deaminase could tolerate some modification of dCMP at position 5. 5-I-dCMP, 5-Br-dCMP, and 5-CH_3-dCMP could all serve as the substrate. It should be noted that 5-I-dCyd and 5-Br-dCyd are potent antiherpes simplex virus agents due to the ability of infected cells to phosphorylate these analogues (24, 25). Thus, this enzyme may play a role in the antiviral action of these analogues. The difference in the specificity of the regulators for the deamination of dCMP and these analogues is quite intriguing. The interaction of the regulator and substrate on the enzyme will alter not only the binding affinity but also the rate of catalysis of the substrate.

In constrast, the other dCMP analogues (ara-CMP, 2′-azido ara-CMP, and 2-F-ara-AMP) are not good inhibitors of the dCMP-catalyzed reaction (Table 2) and therefore are probably poor substrates of deoxycytidylate deaminase, as is clearly demonstrated for ara-CMP in Table 4. This is significant, since all three compounds at the nucleoside level are known cytotoxic agents which are

TABLE 2

Effect of the monophosphates of deoxycytidine analogues on deoxycytidylate deaminase

The assays were performed at 37° for 30 min, utilizing 0.8 mM [U-^{14}C]dCMP (specific activity 0.25 mCi/mmole) as the substrate and 0.33 unit of deoxycytidylate deaminase.

Additive (0.8 mM)	Activity[a]
	% control
	100
5-CH_3-dCMP	16
5-Br-dCMP	12
5-I-dCMP	11
ara-CMP	96
2′-azido-ara-CMP	97
2-F-ara-AMP	94

[a] The experimental error was within 5%.

TABLE 3

Deamination of the 5-substituted analogues of dCMP by deoxycytidylate deaminase

The assays were performed at 37° for 40 min, using 0.33 unit of dCMP deaminase. The reaction was terminated by heating at 100° for 3 min and was immediately analyzed by high-pressure liquid chromatography. The area of each peak was automatically determined by the data processor. Individual controls and standards were analyzed just prior to sample analysis.

Additive (2 μM)	Substrate (0.8 mM)			
	dCMP	5-CH_3-dCMP	5-Br-dCMP	5-I-dCMP
None	100[a] (13)[b]	100 (7)[b]	100 (6)[b]	100 (11)[b]
dCTP	103	66	64	47
5-I-dCTP	114	87	71	67
dTTP	55	101	104	114
5-Br-dUTP	65	89	121	112
5-I-dUTP	25	86	87	107

[a] Activity is expressed as percentage of control, which was the substrate in the absence of allosteric effectors. The experimental error was within 5%.

[b] Indicates nanomoles of product formed in 40 min.

TABLE 4

Comparative studies of dCMP, CMP, and ara-CMP deamination by deoxycytidylate deaminase

Assays were performed in duplicate at 37° for 40 min under standard assay conditions, with the radiolabeled analogue used in place of dCMP, using 0.34 unit of dCMP deaminase.

Additive (0.1 mM)	Substrate	Deamination[a]		
		dCMP[b]	CMP[c]	ara-CMP[d]
	mM	*nmoles/min*		
None	4	0.34	0	0
None	6	0.34	0.006	0.02
CTP	4		0.003	0
ara-CTP	4		0	0
dCTP	4	0.34	0.010	0.05

[a] The experimental error was ±0.01 unit.

[b] [U-^{14}C]dCMP (specific activity 0.25 mCi/mmole).

[c] [U-^{14}C]CMP (specific activity 0.22 mCi/mmole).

[d] [5-^{3}H]ara-CMP (specific activity 2 mCi/mmole).

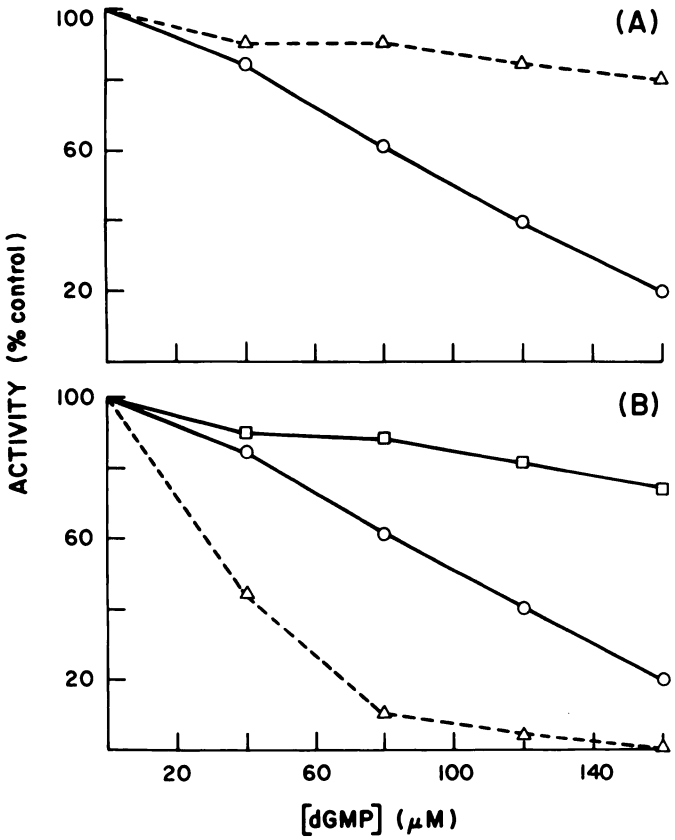

FIG. 2. *Inhibition by dGMP of dCMP deamination catalyzed by deoxycytidylate deaminase*

The assays were performed at 37° for 30 min. In A, 0.33 unit of enzyme was used and the concentrations of dCMP were 0.8 mM (○——○) and 1.5 mM (△- - -△). In B, 0.25 unit of enzyme was used and the concentration of dCMP in all assays was 0.8 mM. The assay was performed in the absence (0——0) or presence of 1 μM dCTP (□——□) or 1 μM dTTP (△- - -△). dTTP alone (1 μM) could inhibit the reaction by 10%, and 1 μM dCTP alone could increase the catalysis by 2% under these assay conditions.

converted to their respective monophosphates by deoxycytidine kinase (26–28). As the nucleoside monophosphate, these compounds will apparently not be rapidly deaminated by dCMP deaminase.

GMP and dGMP were found to be the most potent inhibitors among all of the naturally occurring nucleoside analogues examined. This purine-induced inhibition has also been observed for deoxycytidylate deaminase isolated from chick embryo (8), donkey spleen (10), monkey liver (11), and human spleen (14). However, the characteristics of this inhibition vary depending on the source the enzyme. In the studies using monkey liver, GMP was significantly more potent than dGMP (11). The dGMP inhibition could be reversed by dCTP for the enzyme isolated from chick embryo (8) but not for the enzyme from donkey spleen (10). Also, for the enzyme isolated from human spleen, the K_I for dGMP was the same in the absence or presence of dCTP (14). In our studies, it is clearly demonstrated that dGMP inhibition of dCMP deaminase is not only attenuated in the presence of dCTP but also is potentiated in the presence of dTTP. The physiological significance of this finding is unclear. However, it suggests that purine nucleoside monophosphates may play an important role in regulating deoxynucleotide metabolism through their action on dCMP deaminase. This requires further investigation.

TABLE 5

Effect of the monophosphates of other nucleosides and their analogues on deoxycytidylate deaminase

Assays were performed at 37° for 30 min with 0.8 mM [U-^{14}C]dCMP (specific activity 0.25 mCi/mmole) and 0.33 unit of dCMP deaminase.

Additive (0.8 mM)	Activity[a]
	% control
None	100
UMP	87
dUMP	62
5-F-dUMP	48
5-Br-dUMP	18
5-I-dUMP	12
TMP	20
AMP	91
dAMP	25
IMP	101
dIMP	39
GMP	3
dGMP	1

[a] The experimental error was within 5%.

Ara-C is clinically effective in the treatment of AML (16, 17). The active form of this agent is the triphosphate, which is believed to exert its cytotoxic effect by inhibition of DNA polymerase or its incorporation into DNA (29–33). Furthermore, patients with AML whose blast cells have a longer retention of the intracellular level of ara-CTP appear to have an increased duration of remission (34). Several enzymes are suggested to play key roles in

TABLE 6

Effect of the triphosphates of other nucleosides and their analogues on deoxycytidylate deaminase

Assays were performed at 37° for 15 min with 0.5 mM [U-^{14}C]dCMP (specific activity 0.4 mCi/mmole) and 0.28 unit of dCMP deaminase.

Additives (0.8 mM)	Activity[a]
	% control
None	100
dCTP	122
5-I-dCTP	123
ara-CTP	104
2′-azido ara-CTP	109
CTP	122
dUTP	7
5-Br-dUTP	0
5-I-dUTP	0
dTTP	0
ATP	97
dATP	88
GTP	98
dGTP	93

[a] The experimental error was within 5%.

the action of ara-C, whereas the role of dCMP deaminase in the metabolism of araC is still unclear.

In the blast cells of patients with AML we observed a high specific activity of dCMP deaminase.[2] If ara-CMP is an efficient substrate for dCMP deaminase from AML cells, as has been indicated for the enzyme isolated from cultured human lymphoblastic leukemic cells (13) and from human spleen (14), then one might expect this drug to be deaminated rapidly by this enzyme to the inactive ara-UMP. Therefore, cells which have higher activity of deoxycytidylate deaminase should be less sensitive to ara-C as compared with those with lower enzymatic activity. This apparently is not the case. In fact, cells deficient in dCMP deaminase are partially resistant to ara-C (35).

Our studies suggest that ara-CMP is apparently a very poor substrate in comparison with dCMP whether dCTP is present or absent (Table 4). Also, the binding affinity of ara-CMP is much less than that of dCMP (Table 2). Thus, given a mixture of dCMP, CMP, and ara-CMP, dCMP deaminase would preferentially catalyze the deamination of dCMP. In addition, ara-CTP could also activate dCMP deamination, although much less efficiently than dCTP. These observations suggest the possibility that deoxycytidylate deaminase may play an important role in the over-all action of ara-C through a "self-potentiation" mechanism of ara-C by which ara-CTP could augment its own action by depleting the competing deoxycytidine nucleotide pools. To reiterate, as long as dCMP is present, it will be preferentially deaminated, sparing ara-CMP which could be phosphorylated to the di- and triphosphates. Once the concentration of ara-CTP is increased, dCMP deaminase could remain in an activated form (deaminating dCMP preferentially), further depleting the deoxycytidine nucleotide pool and at the same time increasing the effective (therapeutic) concentration of the nucleotides of ara-C. Thus the presence of this enzyme may facilitate the action of ara-C.

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[2] W. R. Mancini and Y.-C. Cheng, unpublished results.

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