Purification and Properties of Cytidine Deaminase from Normal and Leukemic Granulocytes

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A B S T R A C T Cytidine deaminase, an enzyme that catalyses the deamination of both cytidine and its nucleoside analogues including the antineoplastic agents cytosine arabinoside (ara-C) and 5-azacytidine (5-azaC), has been partially purified from normal and leukemic human granulocytes. The purification procedure included heat precipitation at 70°C, ammonium sulfate precipitation, calcium phosphate gel ion exchange, and Sephadex G-150 gel filtration. The enzyme has mol wt 51,000, isoelectric pH of 4.8, and maximum activity over a broad pH range of 5–9.5. The enzyme is stabilized by the presence of the sulfhydryl reagent, dithiothreitol.

Cytidine deaminase from normal human granulocytes has a greater affinity for its physiologic substrate cytidine ($K_m = 1.1 \times 10^{-5}$ M) than for ara-C (8.8×10^{-5} M) or 5-azaC (4.3×10^{-4} M). Halogenated analogues such as 5-fluorocytidine and 5-bromo-2'-deoxycytidine also exhibited substrate activity, with maximum velocities greater than that of the physiologic substrates cytidine and deoxycytidine. No activity was observed with nucleotides or deoxynucleotides. The relative maximum velocity of the enzyme for cytidine and its nucleoside analogues remained constant during purification, indicating that a single enzyme was responsible for deamination of these substrates.

Tetrahydrouridine (THU) was found to be a strong competitive inhibitor of partially purified deaminase with a K_i of 5.4×10^{-8} M.

The biochemical properties of partially purified preparations of cytidine deaminase from normal and leukemic cells were compared with respect to isoelectric pH, molecular weight, and substrate and inhibitor kinetic parameters, and no differences were observed. However, normal circulating granulocytes contained a significantly greater concentration of cytidine deaminase (3.52 ± 1.86

$\times 10^3$/mg protein) than chronic myelocytic leukemia (CML) cells ($1.40\pm0.70 \times 10^3$ U/mg protein) or acute myelocytic leukemia (AML) cells ($0.19\pm0.17 \times 10^3$ U/mg protein). To explain these differences in enzyme levels in leukemic versus normal cells, the changes in cytidine deaminase levels associated with maturation of normal granulocytes were studied in normal human bone marrow. Myeloid precursors obtained from bone marrow aspirates were separated into mature and immature fractions by Ficoll density centrifugation. Deaminase activity in lysates of mature granulocytes was 3.55–14.2 times greater than the activity found in the lysates of immature cells. Decreased enzyme activity was also found in immature myeloid cells from a patient with CML as compared to mature granulocytes from the same patient. These observations support the conclusion that the greater specific activity of cytidine deaminase in normal mature granulocytes as compared to leukemic cells is related to the process of granulocyte maturation rather than a specific enzymatic defect in leukemic cells.

INTRODUCTION

Cytosine arabinoside (ara-C)[1] and 5-azacytidine (5-azaC), both analogues of the naturally occurring nucleoside cytidine, have become important agents in the treatment of human acute leukemia (1, 2). These agents are converted to active triphosphate derivatives by kinases found in normal and tumor tissue (3, 4) and are subject to inactivation by the enzyme cytidine deaminase found in human liver, plasma, and red blood cells, as well as various tumors (5–8).

[1] Abbreviations used in this paper: AML, acute myelocytic leukemia; ara-C, cytosine arabinoside; 5-azaC, 5-azacytidine; CML, chronic myelocytic leukemia; dCMP, deoxycytidine monophosphate; DTT, dithiothreitol; PBS, sodium chloride solution, 0.15 M, buffered with potassium phosphate, pH 7.4; THU, tetrahydrouridine.

In addition, Silber, Gabrio, and Huennekens (9, 10) have described cytidine deaminase activity in homogenates of normal and leukemic granulocytes. Interest in the granulocyte enzyme has been heightened by a recent report by Steuart and Burke, which suggested that the pretreatment level of cytidine deaminase in leukemic cells may determine the response of patients to ara-C (11). These workers have also described a progressive rise in deaminase levels in leukemic cells during treatment with ara-C, a finding that correlated with the development of clinical resistance to this agent.

Because of the importance of ara-C and 5-azaC in the treatment of acute myelocytic leukemia (AML) and the possible role of deaminase in the development of resistance to these agents, we have undertaken the purification and biochemical characterization of this enzyme from normal and leukemic human granulocytes. In addition, the changes in deaminase levels associated with granulocyte maturation were investigated in an effort

to explain the higher levels of enzyme activity observed in normal granulocytes as opposed to leukemic cells.

METHODS

Enzyme isolation from granulocytes. Approximately 2 × 10¹⁰ granulocytes were obtained from normal human volunteers and from patients with chronic myelocytic leukemia (CML) and acute myelocytic leukemia (AML) by leukapheresis with a "Leukapak" unit containing a nylon fiber filter (Fenwal, Morton Grove, Ill.) as previously described (12). Granulocytes were released from the filter at the conclusion of the run by washing with a solution of plasma anticoagulated with acid citrate dextrose. The recovered cells included greater than 99% granulocytes as determined from Wright stain smears.

Leukemic granulocytes were also obtained from the peripheral blood of patients with AML or CML either before treatment or during periods of marked leukocytosis while receiving hydroxyurea or busulfan therapy. No patient had received ara-C or 5-azaC at any time before this study. All leukemic patients had more than 20,000 leukocytes/mm³ of peripheral blood, and 67–98% of these

TABLE I

Cytidine Deaminase Activity in Circulating Granulocytes and Leukemic Cells

Group	Age	Peripheral WBC	Differential* (Mature/Int/Imm)	Cytidine deaminase activity
	yr	*cells/mm³*		*U/mg protein × 10⁻³*
AML				
1. E. M.	48	78,000	6/0/91	0.17
2. M. W.	55	33,200	5/0/70	0.00
3. R. H.	28	104,000	7/3/88	0.30
4. K. T.	14	130,000	0/0/96	0.00
5. L. D.	23	23,000	13/0/67	0.44
6. W. C.	36	53,000	5/0/69	0.36
7. G. P.	42	186,000	8/0/92	0.21
8. R. B.	18	650,000	0/0/98	0.02
Mean	33			0.19±0.17‡
CML				
1. D. M.	29	181,000	49/12/27	0.74
2. W. F.	18	221,000	61/20/5	1.70
3. E. G.	61	102,000	76/18/4	1.09
4. M. M.	53	105,000	66/8/9	0.87
5. R. J.	40	370,000	60/17/12	0.75
6. F. A.	61	32,000	62/0/0	1.08
7. C. M.	53	760,000	46/7/42	2.53
8. M. G.	61	23,500	32/1/2	1.0
9. E. R.	41	54,000	68/14/21	2.62
10. M. S.	34	49,000	31/15/44	1.64
Mean	35			1.40±0.70‡
Normal volunteers (10 subjects)	29	6,400	—	3.52±1.86

* Polys, bands, metas/myelocytes/promyelocytes, blasts.
‡ Mean ±1 SE.

cells were of the myeloid series (Table I). Samples of peripheral blood were anticoagulated with heparin, 20 U/ml.

Cells from the granulocytic series were separated from red blood cells and lymphocytes by gravity sedimentation in dextran, 1.5 g/100 ml. The white cell-rich supernate was concentrated by centrifugation at 600g and cells were resuspended in 0.15 M sodium chloride buffered with sodium phosphate, pH 7.4 (PBS). The residual red cells were lysed by addition of 3 vol of sodium chloride solution, 0.05 M, and normotonicity restored after 30 s by addition of one volume of 0.6 M sodium chloride solution. The cells were collected by centrifugation, resuspended in PBS, lysed by freeze-thawing three times, and further disrupted with a Dounce homogenizer. Cell debris was removed by centrifugation at 40,000g for 30 min, and the supernate was saved for further studies.

Enzyme assay. Two methods for assay of cytidine deaminase activity were used in this study. The first, which was used in all assays for which radiolabeled nucleoside substrates were available, was described by Maley and Maley (13) and modified by Steuart and Burke (14). In this procedure enzyme was incubated with 0.2 μmol [^{14}C]-cytidine (0.05 μCi/μmol) (New England Nuclear, Boston, Mass.) and 0.015 mmol Tris-Cl, pH 7.5, at 37°C in a total volume of 0.3 ml. The reaction was linear for 1 h, but was usually terminated at 15 min by addition of 0.2 ml of 0.1 N HCl; the solution was then applied to 0.75 × 5-cm column of Dowex 50 H$^+$ resin previously equilibrated with water. The column was eluted with 2 ml of distilled water and the eluant, containing [^{14}C]uridine, was counted in 18 ml of Aquasol (New England Nuclear). Recovery of [^{14}C]uridine averaged 83% in the first 2.5 ml of column eluate. This assay procedure was also used for determination of deaminase activity with [^{3}H]ara-C and [^{14}C]deoxycytidine.

In the second assay procedure, ammonia production was measured by a coupled enzyme reaction with glutamic acid dehydrogenase as previously described (8, 14). This assay was used for determining reaction velocities of other substrates as compared to cytidine, and in the determination of Michaelis constants for 5-azaC and the halogenated substrates, 5-fluorocytidine (Hoffman-La Roche, Inc., Nutley, N. J.), 5-bromo-2'-deoxycytidine and 5-iodo-2'-deoxycytidine (both from Schwarz-Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.).

Enzyme units. 1 U of enzyme activity was defined as the hydrolysis of 1 nmole of substrate per hour at 37°C.

Enzyme purification. The crude leukocyte extract was immersed in a 70°C water bath for 6 min with constant agitation, and denatured protein was removed by centrifugation at 5,000g for 15 min. The supernate of approximately 10 ml was diluted to 20 ml with 0.1 M Tris-Cl, pH 7.5, containing 5 × 10^{-3} M dithiothreitol (DTT). Ammonium sulfate, 36.1 g/100 ml, was added with stirring to bring the solution to 60% saturation, and the precipitate, containing the cytidine deaminase activity, was collected by centrifugation at 27,000g for 20 min. The precipitate was resuspended in 2 ml of 0.01 M Tris-Cl buffer, pH 7.0, with 2.5 × 10^{-3} M DTT, and dialyzed against 500 vol of this buffer for 18 h. 250 mg of aged calcium phosphate gel (Sigma Chemical Co., St. Louis, Mo.) was added to the enzyme solution and the supernate discarded after centrifugation at 5,000g for 10 min. The enzyme was then eluted by resuspending the gel in 2 ml of 0.05 M phosphate buffer, pH 7.0, with 2.5 × 10^{-3} M DTT; the calcium phosphate gel was removed by centrifugation and discarded. The supernate was subjected to a second ammonium sul-

fate precipitation at 60% saturation and the precipitate dissolved in 0.5 ml of 0.05 M Tris-Cl buffer, pH 7.5, with 2.5 × 10^{-3} M DTT (Buffer A). The enzyme was then applied to a downward flow column (1 × 40 cm) of Sephadex G-150 equilibrated in Buffer A. 3-ml fractions were collected at a rate of six fractions per hour. The enzyme eluted after 25 ml and was stored at 4°C.

Protein determination. Protein concentrations of the crude leukocyte extract, the supernate obtained by heating, and ammonium sulfate precipitate were determined according to the method of Lowry, Rosebrough, Farr, and Randall (15). Because of the small quantities of enzyme and protein remaining during the final stages of purification, the protein content of the second ammonium sulfate precipitate and Sephadex G-150 fractions was determined by measuring absorbance at 280 nm, assuming an absorbance of 1 OD unit/mg protein.

Isoelectric focusing. The isoelectric pH of enzyme from normal and CML cells was determined by isoelectric focusing. A 110-ml LKB column (LKB Instruments, Inc., Rockville, Md.) was loaded with the first 50 ml of a 110-ml linear sucrose gradient (2–50% sucrose) containing 1% ampholytes, pH 4–6. The next 5 ml of gradient solution was collected in a separate tube and mixed with 0.3 ml of enzyme solution containing 6 × 10^4 units of enzyme activity, sp act 8 × 10^4 U/mg protein. This 5.3 ml sample was then carefully layered on the sucrose gradient in the electrofocusing column, and the remaining 55 ml of gradient solution was then loaded. A starting current of 500 v and 3.5 mA was applied for 18 h and at the completion of the run, 3-ml fractions were collected for enzyme assay and determination of pH and protein content.

Molecular weight determinations. The molecular weight of cytidine deaminase was estimated by gel filtration on a 28 × 0.6-cm column of Sephadex G-200 as previously described (16). Carboxypeptidase G$_1$ (17), dihydrofolate reductase from L1210 murine leukemia cells (18), and bovine serum albumin (19) were used as standard proteins of known molecular weight.

Analysis of reaction products. The identity of reaction products for the substrates cytidine, deoxycytidine, and ara-C, which were assayed by the Dowex 50 H$^+$ method, was confirmed by thin-layer chromatography on silica gel plates. Chromatographic systems used were isopropanol: HCl:water, 65:16.7:18.3 and methanol:chloroform:1 M PO$_4$≡, pH 7.5, 30:70:4. In addition, the UV spectral shifts accompanying the deamination reaction with these substrates corresponded to those described by Camiener and Smith (5).

Other substrates. All nucleotides were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Nonradiolabeled nucleosides were supplied by Sigma Chemicals, St. Louis, Mo., except where indicated. 2,2'-anhydro-1-(β-D-arabinosyl) cytidine (cyclocytidine), 5-azaC, and THU were kindly supplied by Dr. Harry Wood of the Drug Research and Development Branch, National Cancer Institute, Bethesda, Md. All other chemicals were reagent grade and were supplied by Sigma Chemicals. [^{14}C]deoxyuridine and [^{14}C]-cytidine were obtained from Schwartz-Mann, Orangeburg, N. Y. All other radiolabeled nucleosides were obtained from New England Nuclear, Boston, Mass.

Cytidine deaminase levels in human bone marrow. Changes in enzyme level during myeloid maturation were studied in cells from normal human bone marrow. 15 ml of bone marrow were aspirated from two sites on the posterior iliac crest and immediately dispersed, without anticoagulant, into 135 ml of 0.15 M sodium chloride

TABLE II
Purification of Granulocyte Cytidine Deaminase

	Normal			CML			AML		
	Units × 10⁻⁶	Sp act*	% recovery	Units × 10⁻⁵	Sp act	% recovery	Units × 10⁻³	Sp act	% recovery
Cell extract	14.9	3.54	—	8.36	2.01	—	1.66	0.010	—
Heat −70°C for 6 min	11.2	9.19	75	6.40	4.71	77	1.63	0.035	98
Ammonium sulfate I	8.9	67.9	59	4.07	14.9	49	0.79	0.062	48
Calcium Phosphate	5.59	126.5	37	3.76	79	45	0.59	0.25	35
Ammonium sulfate II	3.41	450	23	2.78	141	34	—	—	—
Sephadex G-150 peak	2.67	2530	18	0.63	253	8	—	—	—

* Units per milligram protein × 10⁻³.

buffered with sodium phosphate, pH 7.4 (PBS). Cells were washed free of plasma with PBS and red cells were subjected to hypotonic lysis. Residual leukocytes were then separated into predominantly mature and immature cell fractions by Ficoll (Pharmacia, Piscataway, N. J.) density centrifugation, by a modification of previously described techniques (20) that will be the subject of a separate communication.[3] The cells in each fraction were collected by centrifugation at 1,000g, resuspended in 2-3 ml of PBS, and lysed by freeze-thawing and Dounce homogenization as described above.

Subcellular localization of cytidine deaminase. Peripheral blood granulocytes from a patient with CML were isolated by dextran sedimentation. Red cells were lysed by hypotonic exposure and the remaining 2.8×10^8 granulocytes with a differential count of 52% granulocytes and bands, 20 metamyelocytes, 17 myelocytes, 9 promyelocytes, 1 basophil, and 1 monocyte were divided into two equal portions. The first was lysed by the usual procedure of freeze-thawing, followed by Dounce homogenization, while the second was resuspended in 0.34 M sucrose and gently disrupted by Dounce homogenization, breaking cell membranes but leaving cytoplasmic granules intact. Cell debris, including nuclei, cell membrane fragments, and undisrupted cells, was collected by centrifugation at 200g for 10 min and the supernate, containing soluble protein and granules, was removed and subjected to a second centrifugation at 100,000g for 60 min. The supernate, representing cytosol, was saved for enzyme assay, while the granules, which formed the precipitate, were resuspended in PBS and disrupted by three cycles of freeze-thawing. This latter fraction was then centrifuged at 40,000g for 30 min and the supernate, containing soluble granular enzymes, was saved for enzyme assay. In order to judge the completeness of recovery of granular enzymes, all fractions were assayed for myeloperoxidase activity (21) as well as cytidine deaminase.

RESULTS

Enzyme isolation. Enzyme activity was readily detectable in homogenates of normal human granulocytes. With the method described above for preparing homogenates of granulocytes, approximately 2×10^{10} mature granulocytes obtained by leukapheresis yielded 1.0–1.5 ×

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[3] Evans, W. H., M. M. Wolf, and B. A. Chabner. 1973. Concentration of immature and mature granulocyte precursors obtained from human bone marrow. Submitted for publication.

10^6 U of enzyme activity in the crude extract. The mean specific activity of deaminase in granulocytes from 10 normal volunteers was $3.52 \pm 1.86 \times 10^3$ U/mg protein (Table I) with a somewhat higher mean value for women than for men (4.54 vs. 2.84×10^3 U/mg protein). In contrast, the mean enzyme level in granulocytes from 10 patients with CML was $1.40 \pm 0.70 \times 10^3$ U/mg protein, and the mean for eight patients with AML was $0.19 \pm 0.17 \times 10^3$ U/mg protein. No significant difference between enzyme levels in men and women was seen in the leukemic patients. The differences in deaminase activity between normal granulocytes and CML cells, between normal granulocytes and AML cells, and between CML and AML cells were significant at the $P < 0.005$ level.

Enzyme purification. A summary of typical purification of the enzyme from normal granulocytes is presented in Table II. The peak fractions from Sephadex G-150 gel filtration had a specific activity of greater than 2.5×10^6 U/mg protein, representing 700-fold purification, with a recovery of 18% of original activity in these fractions. The limited amount of protein present in peak fractions ($A_{280} = 0.02$ or less) prevented further definition of enzyme purity by disk electrophoresis. The purification of the deaminase from CML and AML cells is also depicted in Table II. The behavior of these enzymes during purification closely resembles that of the deaminase from normal granulocytes. The small quantity of enzyme found in AML cells prevented purification beyond the calcium phosphate step.

Substrate specificity. As shown in Table III, cytidine deaminase from normal granulocytes showed a broad specificity for cytidine derivatives, including cytidine, deoxycytidine, the pharmacologic agents ara-C and 5-azaC, and the 5-halogenated analogs. Among the naturally occurring nucleosides, the partially purified deaminase had greatest affinity for cytidine (Michaelis constant 1.1×10^{-5} M); the Michaelis constants for ara-C and 5azaC were 8.8×10^{-5} M and 4.3×10^{-4} M, respectively. The enzyme's greatest maxium velocity was demonstrated for 5-bromo-2'-deoxycytidine and 5-fluoro-

TABLE III
Michaelis Constants for Substrates for Human Granulocyte Cytidine Deaminase

Substrate	K_m
	μM
Cytidine	11
Deoxycytidine	26
Ara-C	88
5-AzaC	430
5-Fluorocytidine	30
CMP, dCMP	In.*
CTP, dCTP	In.
Cytosine	In.

* Inactive as substrate.

cytidine, while no activity was detected for the free base, cytosine, or the nucleotides CMP, dCTP, and CTP. The relative rate of deamination of deoxycytidine, ara-C, 5-azaC, and the halogenated derivatives as compared to cytidine did not change during purification (Table IV) suggesting that a single enzyme was responsible for the deamination of these compounds. Deamination of dCMP was detected when this substrate was incubated with the crude extract; however, this activity was eliminated by heating to 70°C during purification of cytidine deaminase; in addition dCMP did not competitively inhibit the deamination of cytidine, a further indication that these two substances were not substrates for the same enzyme.

Other enzyme properties. The enzyme from normal and leukemic cells was stable as a crude extract for several months at 4°C without loss of activity. However, more highly purified fractions rapidly lost activity unless protected by a sulfhydryl reagent such as dithiothreitol. Peak fractions obtained from the final purification steps were stable for only 2–3 days at 4°C or −90°C despite the inclusion of DTT. No dialyzable cofactors appear to be present, as judged by the enzymes stability during an 18-h dialysis before the calcium phosphate step.

FIGURE 1 Effect of pH on cytidine deaminase activity. 320 U of enzyme were incubated with 0.20 μmoles [^{14}C]-cytidine and 15 μmoles buffer at the indicated pH at 37°C, and the product formed was determined by Dowex 50 H$^+$ ion exchange. Buffers were: ▲——▲, sodium acetate; ●——●, sodium phosphate; △——△, Tris-Cl; and ○——○, glycine-sodium hydroxide.

A molecular weight of 51,000 was indicated for the enzyme from normal granulocytes and CML cells by gel filtration on Sephadex G-200. The enzyme displayed a broad range of maximum activity between pH 5 and pH 9.5 (Fig. 1). The isoelectric point of both normal and CML enzyme was 4.8 as determined by isoelectric focusing in a pH gradient of 4–6 (Fig. 2). In the peak fractions obtained by isoelectric focusing, enzyme specific activity was increased only four-fold over starting activity. This considerable loss of enzyme activity with little change in purity prevented the use of this procedure in the enzyme purification scheme.

Inhibition of cytidine deaminase by THU. THU, a known inhibitor of human liver cytidine deaminase (22), strongly inhibited the partially purified deaminase from normal granulocytes; the K_i's for THU determined with cytidine (Fig. 3) and deoxycytidine as substrates were 5.4×10^{-8} M and 5.5×10^{-8} M, respectively. The inhibition appeared to be competitive in that the V_{max} with

TABLE IV
Relative Substrate V_{max} During Purification

Fraction	Relative V_{max}*						
	5-BrCdR‡	5-FlCR‡	5-I-CdR‡	CR‡	CdR‡	Ara-C	5-azaC
Crude extract	2.29	2.22	1.29	1.0	1.09	0.83	0.44
G-150 Peak	2.28	1.85	1.12	1.0	1.04	0.73	0.50

* V_{max} for substrate indicated/V_{max} for cytidine. All substrates were used at 3.3×10^{-3} M in the deaminase assay. NH$_3$ production was determined by the coupled reaction with glutamic dehydrogenase.

‡ Abbreviations: 5-BrCdR, 5-bromo-2'-deoxycytidine; 5-FlCR, 5-fluorocytidine; CR, cytidine; CdR, deoxycytidine.
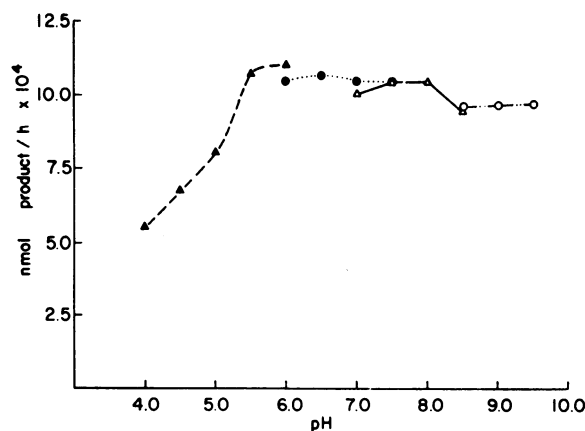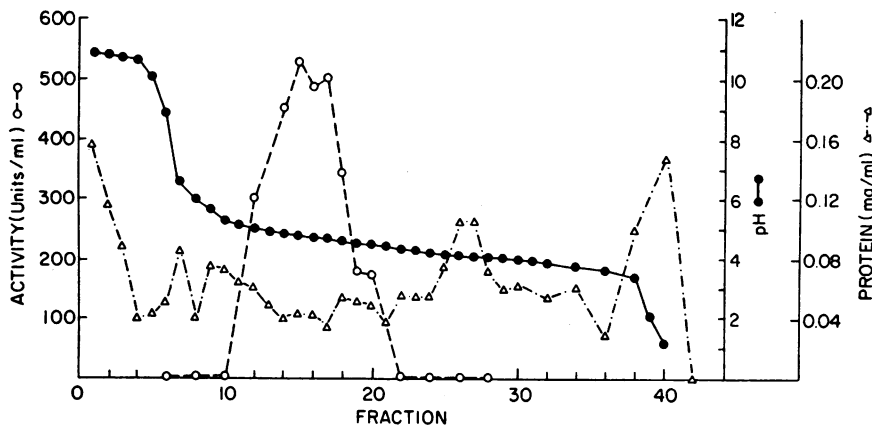
FIGURE 2 Isoelectric focussing of cytidine deaminase in a 1 g/100 ml ampholyte gradient, pH 4–6. The current was 3.5 mA at the beginning of the run and 1.5 mA at the conclusion. Fractions of 2.5 ml were collected after 18 h of focusing at 500 v. O——O = enzyme activity, ●——● = pH, △——△ = absorbance at 280 nm.

cytidine as substrate was not changed in the presence of a range of THU concentrations from 10^{-5} to 10^{-7} M. Inhibition by THU was slightly enhanced by preincubation of enzyme and inhibitor, as shown in Table V. This enhancement of inhibition was most noticeable at high concentrations of substrate relative to inhibitor.

A number of other compounds, including uridine, deoxyuridine, UMP, CTP, and UTP, weakly inhibited the deamination of cytidine, but only when the inhibitor concentration was severalfold greater than substrate. The apparent K_i for UTP inhibition was 1.2×10^{-4} M.

Further comparison of normal and leukemic enzymes. The enzymes from normal and leukemic cells were further compared with respect to substrate specificity and kinetic properties. The enzyme from CML cells was active against the same spectrum of compounds, including the halogenated derivatives and deoxynucleosides that served as substrates for the enzyme from normal granulocytes. A comparison of the kinetic properties of the most highly purified fractions (see Table II) of normal and CML and AML deaminase revealed no apparent differences in the deaminase enzymes from these three cell types. The apparent K_m's for the substrate cytidine were 1.1, 1.1, and 1.8×10^{-5} M respectively for the normal, CML, and AML enzymes, while the K_i's for
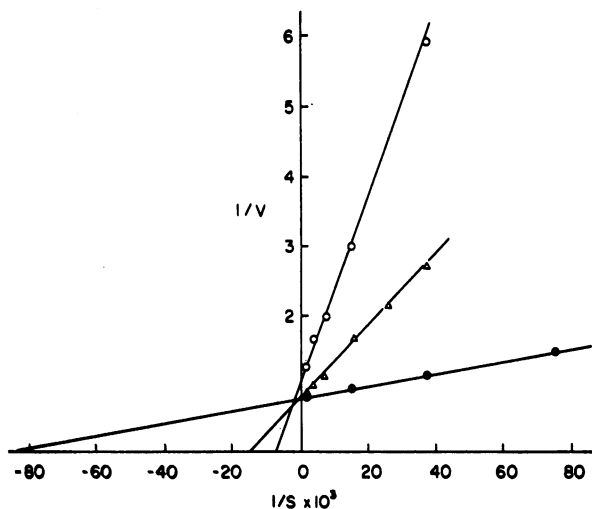

FIGURE 3 Lineweaver-Burk plots of the reciprocal of substrate concentration versus reaction velocity for the substrate cytidine without inhibitor (●——●) and in the presence of 6×10^{-7} M THU (O——O) and 1×10^{-7} M THU (△——△). 188 U of enzyme, sp act 188 U/mg protein, were used in each assay.

TABLE V
Effect of Preincubation of THU with Cytidine Deaminase on Inhibition *

	Inhibition (percent of control)	
[S]	Pre-incubation	No pre-incubation
mm		
1.34	48.6	29.3
0.67	59.6	48.6
0.268	74.0	62.6
0.134	81.6	74.0
0.067	99.9	96.7

* 160 u of cytidine deaminase were preincubated with THU, 5×10^{-6} M, at 37°C for 5 min before addition of substrate. In a duplicate series of tubes, THU and substrate were added to the enzyme assay solution simultaneously. A control rate was also determined with substrate and enzyme in the absence of Thu.

TABLE VI

Cytidine Deaminase: Changes in Activity During Granulocyte Maturation

Experiment source	Fraction	Differential				Cytidine deaminase	Relative activity (mature/immature)
		Mature	Myelo-cytes	Promyelo-cytes, myeloblasts	Other*		
						u/mg protein × 10⁻³	
1. Normal marrow	Mature	78	17	0	5	3.19	>4.15
	Immature	28	48	11	13	0.77	
2. Normal marrow	Mature	56	22	7	15	3.71	>3.88
	Immature	3	42	25	30	0.96	
3. Normal marrow	Mature	86	10	0	4	3.85	>3.55
	Immature	7	50	30	12	1.08	
4. Normal marrow	Mature	88	9	0	3	3.19	>14.2
	Immature	2	48	32	18	0.22	
5. CML peripheral blood	Mature	50	36	4	10	4.56	>13.8
	Immature	3	28	53	16	0.33	

* Lymphocytes, basophils, eosinophils.

THU were 5.4, 6.1, and 3.8×10^{-8} M for these same enzymes.

Relationship of cell maturity to enzyme levels. The decreased deaminase levels in leukemic cells could be due to a disease-related deficiency or structural alteration of the deaminase of leukemic cells, or alternatively might simply be due to the immaturity of leukemic cells. To determine whether normal granulocyte maturation is associated with an increase in intracellular deaminase levels, normal human bone marrow aspirates were separated into mature and immature myeloid fractions by centrifugation in a Ficoll density gradient. These studies revealed a 3.55- to 14.2-fold greater specific activity of cytidine deaminase in the mature cell fraction (predominantly polymorphonuclear leukocytes, bands, and metamyelocytes) as compared to the immature fraction (predominantly myelocytes, promyelocytes, and myeloblasts) (Table VI). A similar separation of granulocytes into mature and immature fractions was performed with circulating granulocytes from a patient with CML (Table VI, experiment 5); the results disclosed a similar pattern of greater activity in mature cells than in the less mature fraction. The absolute levels of enzyme activity in the CML mature and immature fractions corresponded closely to those of similar fractions from normal human marrow in experiments 1 through 4. The specific activity of deaminase in the mature cell fractions of experiments 1 through 4 ($3.19–3.84 \times 10^3$ U/mg protein) also corresponded closely to the activity found in normal circulating granulocytes (Table I), while that of the immature fractions varied from 0.22 to 1.08×10^3 U/mg protein, a range which lies between the average values for AML and CML cells (Table I).

These data suggest that deaminase activity appears during or before the myelocyte stage of maturity, but achieves maximal levels only in the later stages of maturation, both in normal and leukemic granulocytes.

Subcellular localization of cytidine deaminase. Because the appearance of deaminase activity parallels the development of cytoplasmic granules, the subcellular localization of this enzyme in mature granulocytes was examined. 85% of the activity present in the crude cell homogenate was recovered in the soluble portion of the cell, and only 0.8% in the granule fraction that contained the lysozomal enzyme, myeloperoxidase.

DISCUSSION

The present report has described the purification and properties of cytidine deaminase from normal and leukemic granulocytes. This granulocyte enzyme was first noted by Silber, Gabrio, and Huennekens in 1963 (9) and was differentiated from deoxycytidylate deaminase in a subsequent report (10). The separate identities of cytidine deaminase and deoxycytidylate deaminase were confirmed in the present study by selective heat inactivation of the nucleotide deaminase and the failure of dCMP to competitively inhibit deamination of cytidine.

Comparison of the deaminase enzymes from normal, CML, and AML granulocytes failed to disclose biochemical or kinetic differences, but showed significantly lower levels of enzyme in leukemic cells as opposed to normal granulocytes, as was suggested by earlier studies (10). This difference in enzyme activity appears related to the immaturity of the leukemic cells, in that the present experiments have shown an increase in the intracel-

lular deaminase levels associated with maturation of both normal and CML granulocytes.

Previous studies have shown differences in specific enzyme levels in leukemic as opposed to normal granulocytes. Increased levels of DNA polymerase (23), dihydrofolate reductase (24), aspartate transcarbamylase (25), dihydro-orotase (25), and dihydro-orotic dehydrogenase (25) have been observed in AML cells as compared to normal granulocytes. Rabinowitz (23) related the increased activity of DNA polymerase to immaturity of the leukemic cells by showing a decrease in polymerase activity during maturation of normal granulocytes; a similar correlation was not attempted in the other enzyme studies. Gallo (26) has partially purified and characterized thymidine phosphorylase from human granulocytes and has found a lower level of this enzyme in CML cells as compared to normal granulocytes. The possibility was raised that this finding represented a specific enzyme deficiency related to the leukemic state. It is apparent from the present work, however, that marked increases may occur in enzyme levels during maturation of the normal or CML granulocyte, and that these changes must be taken into consideration in investigations of the biochemistry of malignant cells.

The granulocyte deaminase characteristized in this study shares several characteristics with the deaminase previously described in human liver homogenates by Camiener, including its heat stability, substrate specificity, and a Michaelis constant for ara-C of ca. 10^{-4} M (5, 27–29). However, certain differences in substrate affinity and THU inhibition are apparent. The liver homogenate activity described by Camiener had approximately equal affinity for ara-C and 5-azaC, as defined by the competitive inhibition of ara-C deamination by 5-azaC. This contrasts with the fivefold difference in Michaelis constants for ara-C and 5-azaC observed with the granulocyte enzyme in the present study.

In addition, the inhibition of human liver deaminase by THU was described as partially noncompetitive (22); a threefold decrease in maximum velocity for ara-C was observed in the presence of 3.3×10^{-6} M THU. In the present study, THU in the same concentration did not alter the maximum velocity of the normal human granulocyte enzyme with either cytidine or deoxycytidine as substrates and appeared to inhibit the granulocyte deaminase competitively. Preincubation of the liver deaminase with THU in the absence of substrate markedly enhanced inhibition, while in the present experiments, preincubation of granulocyte deaminase and THU for 5 min only slightly enhanced inhibition. The affinity constant, or K_i, of THU for the partially purified granulocyte deaminase in the present study was 5.4×10^{-8} M, while that reported for the crude liver deaminase was between 10^{-4} and 10^{-5} M. It is possible, although unlikely, that differences in the enzyme purity, pH (7.5 vs. 8.0) and buffer (Tris-Cl vs. glycylglycine in Krebs-Ringer) used in these two studies could account for the marked differences in THU inhibition of the granulocyte and liver cytidine deaminase. It is also possible that these differences are due to the presence of multiple enzymes in the liver homogenate or to a basic difference in deaminases in these two tissues. Further studies of purified liver deaminase would be required to resolve this problem.

It is unusual that a reduced uridine derivative such as THU would function as a powerful competitive inhibitor of cytidine deaminase. However, this inhibition may result from the structural similarity that THU bears to cytidine-3,4-monohydrate, a possible transition state compound in the deaminase reaction (30). The reaction mechanism of cytidine deaminase has not been defined in detail, but analogous hydrated intermediates and inhibitors have been identified in the adenosine deaminase reaction (31, 32).

Other cytidine deaminases from leukemic mouse spleen (33), sheep liver (34), and mouse kidney (35, 36) have been partially purified and characterized. The enzyme from human granulocytes has a distinctly lower Michaelis constant for cytidine and a broader pH maximum than the other enzymes. Sufficient data from earlier studies were not available to allow comparison of other properties such as molecular weight, isoelectric point, and inhibition by THU.

Cytidine deaminase is responsible for the inactivation of two important pharmacologic agents, ara-C (5) and 5-azaC (8), both of which are widely used in the treatment of AML. The mechanism of clinical resistance to these agents has not been defined; most resistant animal tumors appear to lack the kinase necessary for ara-C activation (37, 38), but a recent study suggested a decreased response rate to ara-C in cases of AML having elevated deaminase activity before therapy, or showing a rise in deaminase levels during therapy with ara-C (11). In addition, Meyers et al have demonstrated the induction of cytidine deaminase in vitro in HeLa cells exposed to low levels of ara-C (39). High deaminase levels, which would be a disadvantage in treatment with ara-C or 5-azaC, might provide a selective advantage for agents such as the 5-halogenated derivatives of cytidine. Thus, 5-fluorocytidine, which has a greater maximum velocity than the physiologic substrates and ara-C, would be readily converted to the active antineoplastic agent, 5-fluorouridine (40), by cells with increased deaminase activity, and might be a therapeutic agent in patients resistant to ara-C or 5-azaC on the basis of increased deaminase.

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