

Identification of thymidine nucleotidase and deoxyribonucleotidase activities among normal isozymes of 5'-nucleotidase in human erythrocytes

(pyrimidine nucleotidase/erythrocyte enzymes)

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ABSTRACT The persistence of normal thymidine nucleotidase (ThyNase) activity in subjects with pyrimidine nucleotidase (PyrNase) deficiency suggested the possible existence of separate isozymes in normal human erythrocytes. This hypothesis was confirmed by studies of PyrNase-deficient individuals from five unrelated families. Erythrocytes deficient in PyrNase retained normal activity of an enzyme system preferentially active at pH 6.2 with a variety of 2'-deoxyribonucleoside 5'-monophosphate substrates, including those of uridine, thymidine, and cytidine. Lesser activities were observed with the corresponding ribonucleotides. Normal control hemolysates were also found capable of effectively dephosphorylating purine nucleotides (dAMP > AMP) when pH was lowered sufficiently from the pH 7.4-8.0 region commonly used in conventional assays. Variations in substrate specificity, pH optima, kinetics, and sensitivity to inactivation by Pb²⁺ indicated the existence of multiple 5'-nucleotidase isozymes in normal erythrocytes: PyrNase and deoxyribonucleotidase(s) that might function physiologically in the conversion of DNA-derived nucleotides to diffusible nucleosides. Evolution of such a unique 5'-nucleotidase suggests that normal erythroblast maturation and nuclear extrusion is accompanied by a degree of karyolysis sufficient to require dephosphorylation and clearance of DNA degradation products.

Nucleotidases (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) constitute a large class of heterogeneous isozymes widely distributed among animals, plants, and microorganisms. They mediate hydrolysis of monophosphate ester linkages with the fifth carbon of ribose or deoxyribose in various 5'-nucleotides, producing inorganic phosphate (P_i) and the corresponding nucleoside. Nucleotidase isozymes from different sources vary in substrate preferences, kinetics, cation requirements, pH optima, and other characteristics, but virtually all are active with 5'-nucleotides of both purine and pyrimidine bases.

A decade ago, we observed that lysates of normal human erythrocytes contained nucleotidase activity that was apparently limited to pyrimidine substrates (uridine, cytidine, and thymidine 5'-monophosphates; UMP, CMP, dTMP) (1, 2). Unlike nucleotidases from other sources, the human erythrocyte isozyme was inactive with purine analogues when assayed in the region of pH 7.5. This unique substrate restriction seemed a logical evolutionary development in anucleate red cells, whose viability critically depends upon a limited reservoir of high-energy ATP, the purine base of which cannot be synthesized *de novo*. An erythrocyte nucleotidase that was also capable of dephosphorylating AMP to diffusible adenosine theoretically should impose an inexorable

drain on the adenine nucleotide pool, the components of which are in rapid equilibrium through the mediation of adenylate kinase.

Recognition of the existence of pyrimidine-specific nucleotidase (PyrNase) evolved from studies of patients who shared an unusual hemolytic syndrome in which affected erythrocytes (i) retained 10% or less of normal PyrNase activity, (ii) accumulated enormous quantities of cytidine and uridine nucleotides, and (iii) exhibited prominent basophilic stippling with Wright's stain (1). None of these alterations occurs in heterozygous relatives, who have 40-60% of control PyrNase activity and are clinically and hematologically normal.

Marked hereditary deficiency of PyrNase is now recognized as one of the most common erythrocyte enzyme defects associated with chronic hemolytic anemia (3, 4). The hematologic alterations of the syndrome may be precisely duplicated by severe lead intoxication (5, 6), since PyrNase is exquisitely sensitive to inactivation by lead (2, 7).

In recent attempts to detect molecular heterogeneity among PyrNase deficiencies, we added dTMP to other substrates (UMP, CMP, AMP) used in our standard PyrNase assay. We had previously found dTMP to be an effective substrate for PyrNase activity in normal hemolysates (2), but it has not been used routinely in studies of patients with PyrNase deficiency. In the first case so studied, we found that dTMP was actively dephosphorylated by hemolysates that had less than 10% of normal mean PyrNase activity with UMP or CMP (8). Similar results were observed independently by Swallow *et al.* (9) in a Japanese subject with PyrNase deficiency.

Although such findings in an individual case could be explained by variations in substrate specificity of a mutant enzyme protein, it seemed more likely that PyrNase in human erythrocytes might consist of multiple isozymes, one of which could be a highly specific thymidine nucleotidase (ThyNase), a deoxypyrimidine nucleotidase, or a less specific deoxyribonucleotidase (dNase). This hypothesis is confirmed by the present study, in which five unrelated subjects with severe hereditary PyrNase deficiency and several of their heterozygous relatives and normal controls were evaluated for characteristics of erythrocyte ThyNase and dNase.

METHODS

Venous blood was obtained from subjects for whom we had previously established a diagnosis of severe PyrNase deficiency. Blood samples were prevented from coagulating by adding heparin and were sent by express under refrigeration to Los Angeles along with specimens from available relatives and volunteer normal controls. Saline suspensions of

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Abbreviations: PyrNase, pyrimidine nucleotidase; ThyNase, thymidine nucleotidase; dNase, deoxyribonucleotidase; U, enzyme unit.

washed erythrocytes were prepared free of leukocytes and platelets (10) and lysed by sonication for nucleotidase assays. Endogenous phosphates were removed by overnight dialysis at 4°C against 200 vol of isotonic saline buffered to pH 8.0 with 10 mM Tris-HCl containing 10 mM MgCl₂, 0.02 mM EDTA, and 1 mM 2-mercaptoethanol. Hemolysates were redialyzed against fresh solutions for 2 hr prior to assay.

Nucleotidase activities were measured by slight modifications (8) of procedures previously described (1, 2). Aliquots of dialyzed hemolysate were incubated at 37°C in 25 mM Tris-HCl with 8.5 mM MgCl₂, 0.5 mM dithiothreitol, and 2.3 mM AMP, UMP, CMP, dUMP, dCMP, or dTMP as substrate at a final pH of 7.4. Reactions were terminated by deproteinization with Cl₃CCOOH, and P_i was assayed by the Fiske and SubbaRow technique (11). Enzyme units were defined as μmol of P_i liberated per hr per g of hemoglobin. Assays were routinely performed in duplicate with reagent blanks that were incubated with hemolysate alone, the appropriate substrate being added after deproteinization.

Studies to determine pH optima substituted 0.3 M Tris/maleate for Tris-HCl buffer between pH 5.0 and 9.0. Actual pH of the incubation mixture was determined both before and after the 2-hr incubation period by using a Beckman model pH 41 digital pH meter calibrated against Beckman standard phosphate buffers.

Kinetic constants were determined from Lineweaver-Burk plots of nucleotidase activities measured in duplicate with substrates between 0.35 and 4.54 mM final concentrations.

Effects of lead on PyrNase activity were studied by using hemolysates dialyzed overnight against 1.0 M acetate buffer, pH 8.0 (in place of Tris-HCl), containing 10 mM MgCl₂ or Mg(C₂H₃O₂)₂ but devoid of EDTA. The overnight dialysis was performed in the presence of 1 mM mercaptoethanol, but that compound was deleted from a second 2-hr dialysis prior to lead exposure and assay. Pb(C₂H₃O₂)₂·3H₂O was added directly to aliquots of hemolysate to achieve final concentrations of Pb²⁺ between 10⁻¹ and 10⁻⁷ M. After 20 min at 37°C, each was assayed in 0.3 M acetate buffer with 8.5 mM MgCl₂ or Mg(C₂H₃O₂)₂, but devoid of dithiothreitol, using pH 6.0 or 7.0 buffers with dTMP or UMP, respectively, as substrates.

RESULTS

Substrate Specificity. Data presented in Table 1 demonstrate distinct and consistent segregation of nucleotidase activities dependent upon substrate. Hemolysates from subjects with marked PyrNase deficiency (homozygous or compound heterozygous) exhibited minimal activity with UMP, CMP, or dCMP but were briskly active with both dTMP and dUMP. All heterozygous relatives studied had intermediate activities with UMP, CMP, and dCMP and entirely normal activities with dTMP and dUMP.

Individual values shown in Table 1 are means of two to four separate determinations utilizing the standard pH 7.4 assay system with substrates at 2.3 mM final concentration. Neither pH nor substrate concentration was necessarily optimal for deoxyribonucleotides, but conditions were adequate for comparative purposes. Young cells and reticulocytes have significantly increased PyrNase activities with UMP and CMP (2) but not with dTMP under these suboptimal conditions.

The observation of normal nucleotidase activity for dTMP and dUMP confirms similar findings in single cases of PyrNase deficiency reported by Swallow *et al.* (9) and ourselves (8).

pH Optima. Similar segregation of activities occurred when PyrNase was measured as a function of pH. As shown

Table 1. Erythrocyte nucleotidase activity with various substrates assayed at pH 7.4 in subjects with PyrNase deficiency

Subject	Nucleotidase activity, U*				
	UMP	CMP	dCMP	dUMP	dTMP
Homozygous PyrNase deficiency					
M.J.	1.4	1.3	1.9	14.0	8.7
C.B.	1.2	0.6	1.2	11.6	7.5
V.T.	1.0	0.8	1.2	11.6	9.4
M.R.	0.9	1.4	2.9	13.9	8.1
C.T.	1.5	1.2			10.4
Mean	1.2	1.1	1.8	12.8	8.8
Heterozygous PyrNase deficiency					
C.B. mother	6.1	4.5	7.2	11.3	7.6
C.B. father	8.0	5.7	8.4	11.5	8.0
C.T. mother	7.8	5.5			10.1
Mean	7.3	5.2	7.8	11.4	8.6
Normal controls					
Mean	12.9	9.1	10.7	12.3	10.0
SD	±2.9	±1.7	±1.9	±3.6	±1.5

*Expressed as μmol of P_i released per hr per g of hemoglobin. Individual values are means of two to four separate determinations.

in Fig. 1, dephosphorylation of dTMP and dUMP in normal control hemolysates was optimal around pH 6.2, whereas UMP and CMP produced broadly plateaued curves with maxima in the region of pH 7.2. The activity curve with dCMP was identical to that of UMP in the lower pH range, but peaked at pH 6.8 and decayed more rapidly under alkaline conditions. Activities with UMP, dUMP, dCMP, and dTMP were quantitatively comparable at pH 7.2–7.4 used in the standard assay system.

In sharp contrast to previous studies with PyrNase, the purine substrates, AMP and dAMP, were both effectively dephosphorylated by normal control hemolysates if assayed in the region of pH 5.5–6.7. The contour of the dAMP curve in Fig. 1 differed distinctly from the contours of dTMP/dUMP on one hand and UMP/CMP/dCMP on the other. Activities with AMP as substrate were approximately half

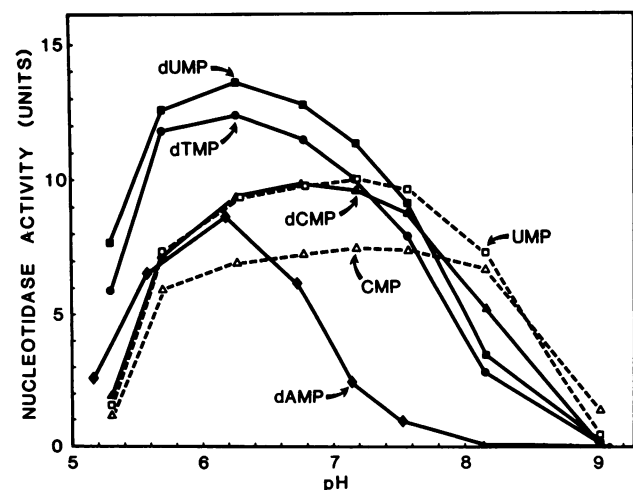


FIG. 1. Normal control erythrocyte nucleotidase activity as a function of pH with various pyrimidine substrates. Values are means of single or duplicate assays from each of four to six normal control subjects.

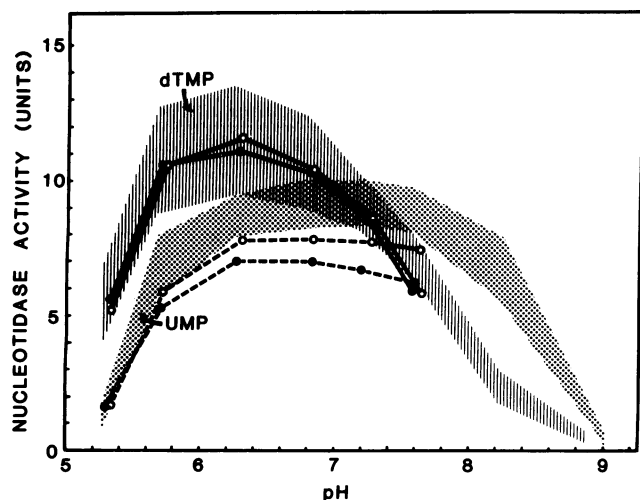


FIG. 2. Nucleotidase activity with UMP and dTMP as a function of pH in hemolysates from subjects with heterozygous PyrNase deficiency: mother (●) and father (○) of subject C.B. Solid lines are curves obtained with dTMP as substrate; broken lines are those with UMP. Shaded areas encompass means \pm 1 SD for six normal controls.

those obtained with dAMP and were identical in pH dependence.

As shown in Fig. 2, heterozygous relatives with partial PyrNase deficiency had entirely normal ThyNase activity. Their PyrNase activity curves with UMP were normal in configuration but reduced in magnitude.

In subjects with severe PyrNase deficiency, however, there was a distinct shift in optimal pH for UMP dephosphorylation. As shown in Fig. 3, optimal pH was approximately 6.2 for both dTMP and UMP, and activity with the latter substrate was markedly reduced. One deficient subject (M.R.) tested additionally with dUMP, dCMP, and CMP (Fig. 4). In

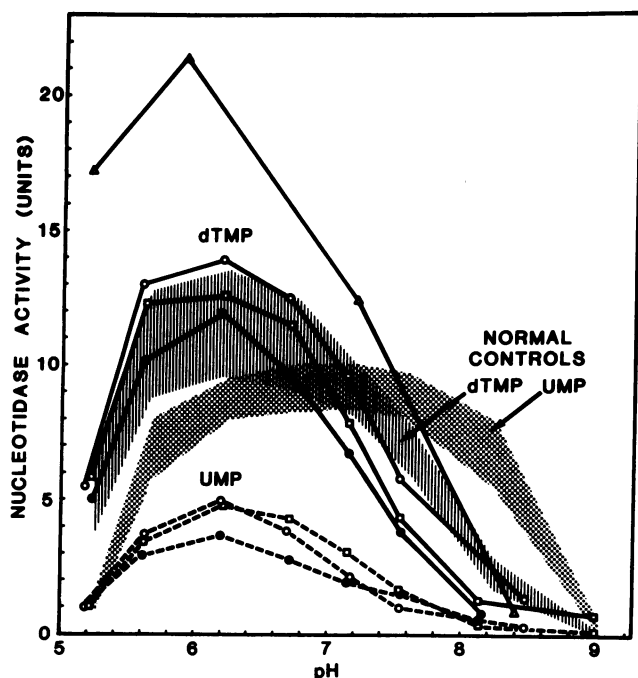


FIG. 3. Nucleotidase activity with UMP and dTMP in hemolysates from subjects with severe PyrNase deficiency: ●, C.B.; □, M.R.; △, M.J.; ○, V.T. Solid lines are curves obtained with dTMP as substrate; broken lines are those with UMP.

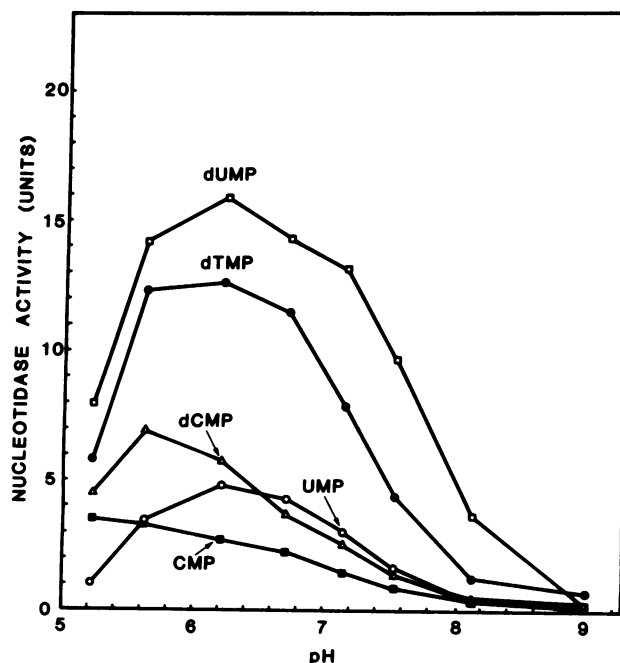


FIG. 4. Nucleotidase activity as a function of pH in a subject (M.R.) with severe PyrNase deficiency with various pyrimidine substrates.

this case, CMP was poorly but progressively dephosphorylated as pH was decreased, possibly as a result of acid phosphatase activity.

It is clear from these curves that ThyNase activity measured in the standard system at pH 7.4 may be only half that obtainable at its more optimal acidic pH. The data in Table 1, therefore, while valid for comparative purposes, do not necessarily reflect maximal activities obtainable with each substrate under ideal *in vitro* conditions. In addition, quantitative assays performed at neutral or alkaline pH will measure suboptimal activities with UMP or CMP as substrates in PyrNase-deficient subjects.

Substrate Kinetics. Determinations of kinetic constants in incubation systems are inherently imprecise, since substrate concentrations are variable during the prolonged incubation periods. Nonetheless, relative differences in kinetic characteristics can be reliably detected. We have previously observed that the apparent Michaelis-Menten constant (K_m) for dTMP in normal hemolysates assayed by this technique was 3–6 times greater than the K_m for UMP or CMP (2). Similar determinations in two subjects with severe PyrNase deficiency (C.B., M.J.) are presented in Fig. 5. Unlike normal controls, hemolysates from these subjects showed no significant differences between K_m (UMP) and K_m (dTMP), which were between 3.3 mM and 4.0 mM.

Inhibition by Pb^{2+} . The marked sensitivity of PyrNase to heavy-metal inactivation (2, 7) was verified by studies shown in Fig. 6. Normal hemolysates incubated with Pb^{2+} for 20 min prior to assay exhibited diminished activity with UMP as substrate when Pb^{2+} exceeded 10^{-5} M. By contrast, activities with dTMP were only mildly affected. The Pb^{2+} concentration required for 50% inhibition of ThyNase was about two orders of magnitude greater than that for PyrNase with UMP as substrate.

DISCUSSION

These studies establish a clear distinction between two (or more) major isozymes of erythrocyte 5'-nucleotidase. One isozyme, PyrNase, is principally active with UMP and CMP at neutral pH. This is the isozyme presumably responsible

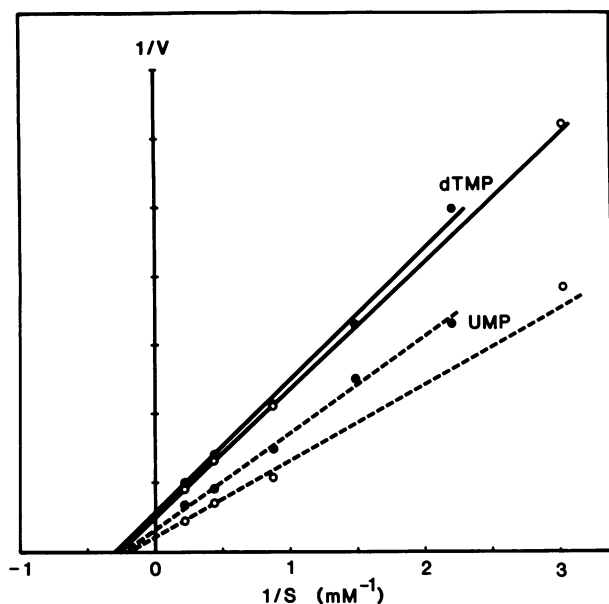


FIG. 5. Lineweaver-Burk plots of nucleotidase activities with dTMP (solid lines) and UMP (broken lines) in PyrNase-deficient subjects C.B. (●) and M.J. (○).

for clearance of pyrimidine degradation products of RNA during normal reticulocyte maturation (1), and it is the one that is defective in cases of hereditary or Pb^{2+} -induced PyrNase deficiency (5–7). Another isozyme (or isozymes), which we had tentatively designated ThyNase (8), operates optimally at acidic pH and is apparently active not only with dTMP but also with dUMP, dAMP, and dCMP. The relative resistance to *in vitro* inactivation by Pb^{2+} suggests that this isozyme will likely retain significant activity in patients with sufficient lead intoxication to induce deficiency of PyrNase.

The distinction between these isozymes was confirmed by studies of heterozygous and compound heterozygous or homozygous subjects with PyrNase deficiency. Diminished activity of the PyrNase isozyme unmasked the existence of the second isozyme and permitted partial characterization on the basis of substrate specificity and kinetics, pH optima, and heavy-metal inhibition.

The persistence of ThyNase activity that we observed previously in a single case of hereditary PyrNase deficiency (8), and which was observed independently by Swallow *et al.* (9) in another case, remained consistent in seven additional subjects with severe or intermediate deficiencies of PyrNase. Such findings in isolated instances conceivably could result from genetically induced alterations in molecular structure that affected substrate specificity or avidity, but the persistent presence of highly active ThyNase in all of these unrelated PyrNase-deficient subjects effectively eliminates any reservations about the existence of separate isozymes. This conclusion was additionally supported by the distinct difference between PyrNase and ThyNase in their sensitivities to Pb^{2+} inactivation (Fig. 6) and by the loss of normal distinctions between $K_m(\text{UMP})$ and $K_m(\text{dTMP})$ in PyrNase-deficient hemolysates (Fig. 5).

When other nucleoside monophosphates were tested as potential substrates, it became clear that residual nucleotidase activity in PyrNase-deficient hemolysates was not limited to dTMP alone. In our standard assay system at a final pH of 7.4, dUMP was also actively dephosphorylated, but dCMP was not (Table 1). Assays at lower pH, however, showed dCMP to be moderately effective as substrate (Fig. 4).

The marked difference between dCMP curves in Figs. 1 and 4 indicates that dCMP can serve as substrate for both Pyr-

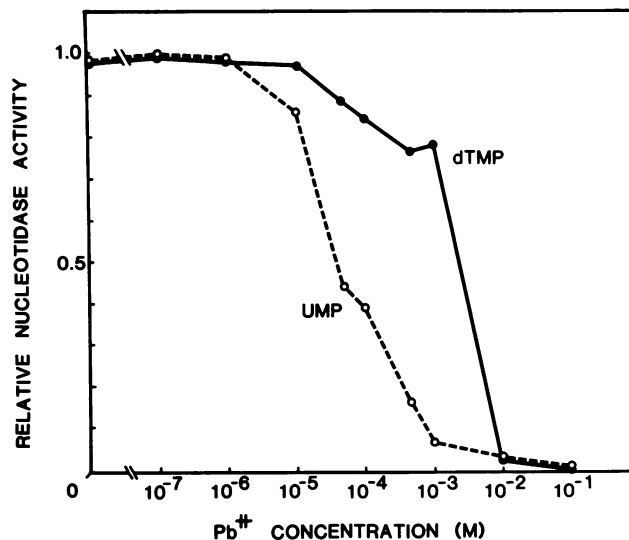


FIG. 6. Relative nucleotidase activities in normal hemolysates incubated with lead at various concentrations. Each point is the mean from four to eight determinations performed on four control subjects at pH 6.8–7.2 (UMP) or pH 6.2–6.4 (dTMP).

Nase and the residual isozyme in PyrNase-deficient cells. Data in Fig. 3 additionally suggested that the PyrNase isozyme had some degree of crossreactivity for dTMP, since most of the deficient subjects exhibited slightly more rapid decrease of ThyNase activity at alkaline pH than did normal controls.

An unexpected, but highly significant, finding was the effectiveness with which normal control hemolysates dephosphorylated dAMP (and, to a lesser extent, AMP) in the region of pH 6 (Fig. 1). To our knowledge, such vigorous activity with a purine substrate has not been demonstrated previously in normal erythrocytes, and it clearly removes the responsible isozyme from the class of pyrimidine-specific nucleotidases.

All of these data are consistent with the hypothesis that normal human erythrocytes contain two distinct 5'-nucleotidases, PyrNase and a second isozyme with the following characteristics: (i) variably active with both purine and pyrimidine substrates (dUMP > dTMP > dAMP > dCMP > AMP > UMP); (ii) principally dependent upon (but not restricted to) the 2'-deoxyribose moiety for substrate recognition; (iii) optimally active in the region of pH 6.2; and (iv), relative to PyrNase, significantly (10–100 times) more resistant to sulfhydryl inhibition by Pb^{2+} . Such an isozyme might best be classified as a deoxyribonucleotidase (2'-deoxy-5'-ribonucleotide phosphohydrolase, dNase). Compared to PyrNase, dNase is less limited in substrate specificity and so more closely resembles the 5'-nucleotidases found ubiquitously throughout nature in terms of being variably active with a variety of both purine and pyrimidine substrates. Although it was catalytically more effective with deoxyribonucleotide substrates, dNase appeared to crossreact significantly with each corresponding ribonucleotide, possibly excepting CMP. The present data do not exclude the possibility that multiple isozymes exist that are more or less specific for each substrate or class of substrates—e.g., ThyNase, deoxy-pyrimidine nucleotidase, etc.

Our findings further suggest that the slight residual activities observed in cases of severe hereditary deficiency of PyrNase are most likely due to dNase acting on UMP or CMP, with little or no contribution by the defective PyrNase isozyme. The variations in pH optima, kinetic constants, thermostability, and electrophoretic migration reported by Rosa *et al.* (12), Fujii *et al.* (13), Shinohara and Tanaka (14), and

Ishida *et al.* (15, 16) in other deficient cases may not be due to molecular alteration of residual PyrNase as presumed, or to a minor PyrNase isozyme as postulated by Rosa *et al.* (12), but more likely represent characteristics of the normal dNase isozyme no longer obscured by significant PyrNase activity. Their studies employed partially purified enzyme preparations and UMP or CMP as substrate, and they found pH optima variously shifted from the 7.0–8.0 region to approximately pH 5.7–6.1 and K_m (UMP) or K_m (CMP) increased severalfold, consistent with our findings for the dNase isozyme.

Similarly, the biphasic pH optima noted by Torrance and Whittaker (17) with PyrNase purified from normal erythrocytes may also have been due to the dNase isozyme. They found UMP and CMP to be optimally dephosphorylated at pH 7.2 with a minor peak at pH 6.3. They also detected slight activity with purine substrates (AMP and GMP) that was optimal at pH 6.1 with a minor peak at pH 7.2. Shinohara and Tanaka (14) additionally found purified preparations from PyrNase-deficient erythrocytes to have comparable activities with UMP and AMP at pH 8.0.

The extreme susceptibility of erythrocyte PyrNase to inactivation by Pb^{2+} is well documented (4) and is presumed to be due to its dependence on sensitive sulfhydryl groups for proper molecular conformation. PyrNase is inactivated *in vitro* and *in vivo* by Pb^{2+} concentrations too low to have significant effects on other erythrocyte enzymes of glycolysis, glutathione, and nucleotide metabolism (7). It therefore seems possible that PyrNase might not tolerate many genetically induced alterations in primary structure without virtually complete loss of catalytic effectiveness. If this were the case, it would leave only the dNase isozyme, which inefficiently hydrolyzes UMP or CMP at alkaline pH, the conditions traditionally used for quantitative assays of PyrNase. This might account for the apparent absence of disparate mutant PyrNase isozymes that reflect the phenotypic heterogeneity so characteristic of other erythroenzymopathies, such as those of pyruvate kinase and glucosephosphate isomerase.

The evolution of an enzyme system as specific as erythrocyte dNase suggests specific physiologic functions. Since dNase is active principally with deoxyribonucleotides, including dAMP, it might logically be expected to dephosphorylate degradation products of DNA in a manner analogous to that presumed for PyrNase in RNA degradation. This would imply that nuclear pyknosis and pitting, phenomena that are well documented morphologically in maturing erythroblasts (18–20), might be accompanied by sufficient karyolytic degradation of DNA to require a mechanism for dephosphorylation of resultant deoxyribonucleotides to diffusible nucleosides. Awai *et al.* (21) cited the absence of detectable [3H]thymidine label in reticulocytes as evidence against even

partial karyolysis, but dNase activity might account for their observations and negate their conclusion.

A deficiency state for dNase remains undetected. Should one be identified, the study of its effects should provide valuable insights into the physiologic function of this unique enzyme system.

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