

Characteristics of a Pyrimidine-specific 5'-Nucleotidase in Human Erythrocytes*

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A 5'-nucleotidase with unique specificity has been identified in the soluble fraction of normal human erythrocytes. It mediates the hydrolytic dephosphorylation of pyrimidine 5'-ribosemonophosphates but is catalytically ineffective with purine nucleotides or with the 2', 3', or cyclic isomers of pyrimidine nucleotides. Activities at 37° in dialyzed hemolysates of normal human erythrocytes averaged 7.3 and 6.2 μmol of P_i liberated per hour per g of hemoglobin for the substrates UMP and CMP, respectively. Activity with TMP as substrate was approximately one-half as much as with UMP or CMP. Apparent Michaelis constants were 0.33 mM UMP, 0.15 mM CMP, and 1.0 mM TMP. Magnesium was required for optimal activity, and this cation could not be replaced by Mn²⁺. Maximum activity was obtained between pH 7.0 and 7.5 with rapid decreases in more alkaline media and moderate decreases with acidification.

The enzyme was quite sensitive to heat and was strongly inhibited by AMP, by some purine bases, and by both purine and pyrimidine nucleosides. Divalent cations of heavy metals were also strongly inhibitory, as were agents active against sulfhydryl groups. The presence of substrates and/or 2-mercaptoethanol provided considerable protection against some of these deleterious agents and conditions.

Pyrimidine 5'-nucleotidase activity in hemolysates was clearly distinguishable from erythrocyte acid phosphatase and from leukocyte and serum alkaline phosphatases and nucleotidases.

An unusual hereditary syndrome has been observed which includes, among other features, hemolytic anemia accompanied by pronounced basophilic stippling and markedly elevated erythrocyte nucleotides. The latter, initially presumed to represent ATP, ADP, and AMP (1, 2), were subsequently found to consist largely (>80%) of cytidine and uridine phosphates (3). Investigations undertaken to explain these remarkable accumulations of pyrimidine nucleotides revealed the normal existence of highly specific erythrocyte 5'-nucleotidase which was severely defective in patients with the syndrome (3). This enzyme catalyzes the hydrolytic dephosphorylation of pyrimidine 5'-nucleotides but not purine nucleotides or a variety of related analogs. While 5'-nucleotidases (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) are ubiquitous (4-6), they classically react with a broad spectrum of nucleotides as substrate (7-12), and a 5'-nucleotidase with such restrictive specificity appears unique. This paper presents some of the biochemical characteristics of the enzyme as it occurs in normal human erythrocytes.

METHODS AND MATERIALS

Preparation of Hemolysates—Venous blood obtained from normal healthy volunteers and from patients referred for investigation of

hemolytic anemias was anticoagulated with heparin. Leukocytes and platelets were removed by sedimentation with Plasmagel (Roger Bellon Laboratories, Neville, France) or by filtration through cotton (13). The harvested erythrocytes were alternately washed with 40 volumes of isotonic saline and packed lightly by centrifugation a total of three times, then resuspended in saline to approximately 3×10^8 per μl . Aliquots were removed to determine hemoglobin content and erythrocyte, reticulocyte, and contaminating leukocyte counts.

Cell suspensions were alternately frozen rapidly in a dry ice, Dowanol bath and thawed a total of three times. Resultant hemolysates were dialyzed 16 to 20 hours against 200 volumes or more of isotonic saline buffered to pH 8.0 by 0.01 M Tris-HCl and containing MgCl₂ and EDTA in final concentrations of 10 and 0.02 mM, respectively. Hemoglobin concentrations were then redetermined, allowing activities to be related either to amount of hemoglobin or number of cells.

Pyrimidine 5'-Nucleotidase Assay—The standard assay system for pyrimidine 5'-nucleotidase activity contained 0.5 ml of dialyzed hemolysate, 0.9 ml of 0.05 M Tris-HCl buffer, pH 8.0, 0.15 ml of 0.1 M MgCl₂, and 0.2 ml of 0.02 M UMP or CMP (all reagents obtained from Sigma Chemical Co., St. Louis, Mo.). Final pH of the reaction mixture was 7.7 to 7.8, remaining within ± 0.1 unit throughout the incubation period. In assay systems which included 2-mercaptoethanol (obtained from Calbiochem, Los Angeles, Calif.), 0.05 ml of this reagent, diluted 1:1000, replaced an equal volume of buffer to yield a final concentration of 0.4 mM.

Reaction mixtures were incubated at 37°, generally for 2 hours, then deproteinized by the addition of 1 ml of 20% trichloroacetic acid. Reagent blanks consisted of identical systems except for the absence of substrate, which was added after deproteinization. The acid extracts

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were filtered and assayed for P_i by the method of Fiske and SubbaRow (14). One unit of nucleotidase activity was defined as the amount of enzyme catalyzing the release of 1 μmol of P_i per hour per g of hemoglobin.

RESULTS

Preliminary experiments established that P_i release during incubation was dependent upon both an appropriate pyrimidine substrate and undegraded hemolysate. Denaturation by heat or acidification rendered hemolysates totally inactive. Observed nucleotidase activities were consistently a linear function of both hemolysate concentration and of incubation time up to 4 hours (Fig. 1). Destromatization of dialyzed hemolysates by high speed centrifugation did not diminish activity of the supernatants, nor was there perceptible activity in reconstituted stromata.

Reagent blanks uniformly exhibited less than 10% of total activities if hemolysates were dialyzed free of endogenous nucleotides and other phosphates prior to assay. Comparative studies had previously shown no diminution in nucleotidase activity secondary to the dialysis procedure *per se* (3).

Inclusion of the sulfhydryl reagent, 2-mercaptoethanol, in the incubation mixture was noted to increase nucleotidase activities by approximately 20% at concentrations between 0.2 and 1.5 mM. The standard assay system eventually incorporated 2-mercaptoethanol at a final concentration of 0.4 mM. Comparable levels of glutathione also resulted in activation, but cysteine was without perceptible effect until its concentration exceeded 1 mM. Ascorbic acid, an activator of serum 5'-nucleotidase, produced no observable effect even at a final concentration of 10 mM.

Normal Values for Pyrimidine 5'-Nucleotidase—Measurements of erythrocyte nucleotidase activities with either UMP or CMP as substrate were performed on hematologically normal individuals and on patients with various levels of reticulocytosis induced by causes other than demonstrable erythroenzymopathies. Table I records mean values for these assays. Enzymatic activities were approximately 20% less when CMP was the substrate rather than UMP, regardless of

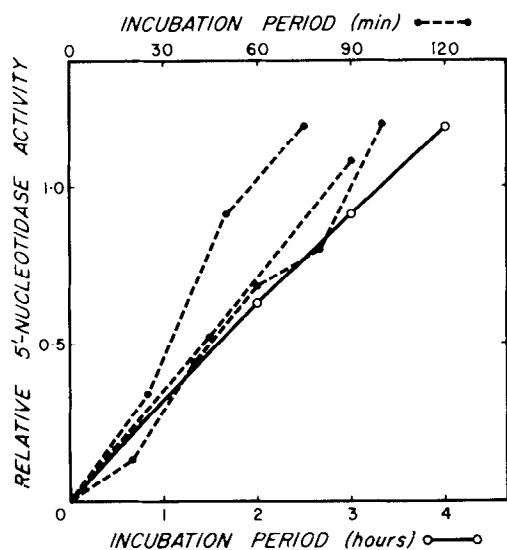


FIG. 1. Effect of incubation period on observed erythrocyte pyrimidine 5'-nucleotidase activity. The solid line represents the mean of 11 determinations on separate hemolysates at 2, 3, and 4 hours (lower abscissa) utilizing either UMP (seven experiments) or CMP (four experiments) as substrate. Broken lines were derived from individual hemolysates assayed at various time periods up to 100 min (upper abscissa) using only CMP as substrate.

the presence of reticulocytosis. Reticulocytosis was accompanied by a general increase in reaction rates, but there was no close correlation between activity and percentage of reticulocytes in this small group.

Substrate Specificity—Numerous preliminary experiments indicated that AMP was not a substrate for erythrocyte 5'-nucleotidase under the conditions of this assay. Values for P_i evolution from AMP were virtually identical with those from reagent blanks. In studies with 10 different hemolysates, the mean $A_{660\text{ nm}}$ of comparable aliquots of the P_i colorimetric solution was 0.026 (range = 0.014 to 0.032) in systems incubated with 0.23 to 2.3 mM AMP. Corresponding values from 20 reagent blanks assayed simultaneously ranged between 0.014 and 0.033, averaging 0.024. These means were less than 10% of the values obtained with 2.3 mM UMP or CMP.

The 5'-monophosphates of inosine, guanosine, and xanthosine and the 3'-monophosphate of adenosine were similarly ineffective substrates at 2.3 mM final concentrations, also yielding less than 10% of the P_i evolved from identical systems containing either UMP or CMP. In one experiment, adenosine 2'-monophosphate yielded 20% as much P_i as UMP or CMP.

Among pyrimidine analogs, cytidine 3'-monophosphate was totally ineffective as a substrate, but the cyclic 2':3'-monophosphate on one occasion yielded almost one-third as much P_i as did CMP. The 2'-deoxyribose analogs, deoxyuridine 5'-phosphate and deoxycytidine 5'-phosphate, were utilized almost as effectively as UMP and CMP at comparable concentrations.

Effects of Substrate Concentration—The substrate concentration (2.3 mM) selected for a standard assay system was found consistently to yield nucleotidase activities which were within ± 5 to 8% of those obtained at twice that concentration. The maximum reaction velocity attainable with CMP was consistently about 80 to 85% of that obtained with UMP, whether or not the system contained 2-mercaptoethanol (Table I). At comparable concentrations, TMP was approximately 50% as effective a substrate as UMP.

Activities were determined with UMP or CMP at various concentrations between 0.23 and 4.6 mM. Although reaction rate curves appeared hyperbolic, sigmoidal kinetics could not be ruled out, since substrate availability may vary considerably at the lower concentrations during such long incubations, making reaction velocity measurements unreliable in this region. Average values from such studies are presented as Wolff plots in Fig. 2, demonstrating an approximately 2-fold difference between apparent K_m (UMP) and K_m (CMP). Twelve separate determinations on hemolysates from 6 normal subjects yielded mean values for apparent K_m of 0.33 mM UMP (range = 0.25 to 0.39 mM) ($V_{\text{max}} = 9.3$ units) and 0.15 mM CMP

TABLE I
Erythrocyte pyrimidine 5'-nucleotidase activities in normal controls and in patients with reticulocytosis

Subjects	Pyrimidine 5'-nucleotidase activity			
	UMP		CMP	
	No additive	MCE ^a added	No additive	MCE ^a added
	$\mu\text{mol } P_i/\text{hr/g hemoglobin } (\pm 1 \text{ S.D.})$			
Normal controls	6.6 \pm 2.0 (n = 116)	7.3 \pm 1.9 (n = 50)	5.5 \pm 1.4 (n = 113)	6.2 \pm 1.4 (n = 50)
Reticulocytosis ^b	14.1 \pm 6.6 (n = 24)	15.1 \pm 7.5 (n = 9)	10.8 \pm 4.2 (n = 24)	12.2 \pm 5.1 (n = 9)

^a Mercaptoethanol.

^b Range of reticulocytosis = 2.4 to 36.0%.

(range = 0.13 to 0.18 mM) ($V_{max} = 6.9$ units). Two determinations with TMP as variable substrate yielded a K_m value of 1.0 mM ($V_{max} = 3.9$ units). Addition of mercaptoethanol to the assay system did not alter apparent K_m (UMP) or K_m (CMP), despite its effect on V_{max} .

Effect of pH—Under conditions of the standard assay system, nucleotidase activity peaked distinctly between pH 7.0 and 7.5 rather than exhibiting the broad flat curve noted in our preliminary studies (3). Fig. 3 shows curves obtained with UMP and CMP compared to those obtained with the acid and alkaline phosphatase substrates, α - and β -glycerophosphate.

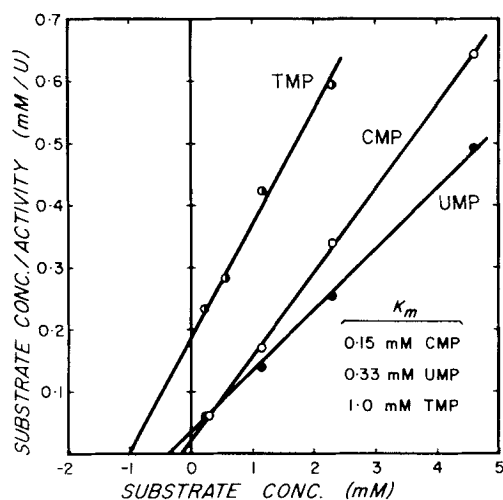


FIG. 2. Wolff plots of erythrocyte 5'-nucleotidase activities with various concentrations of pyrimidine nucleotides. Each point on the UMP and CMP curves is the mean of seven to eight determinations on eight specimens from five normal subjects. Points on the TMP curve are means of duplicate assays on two separate normal hemolysates.

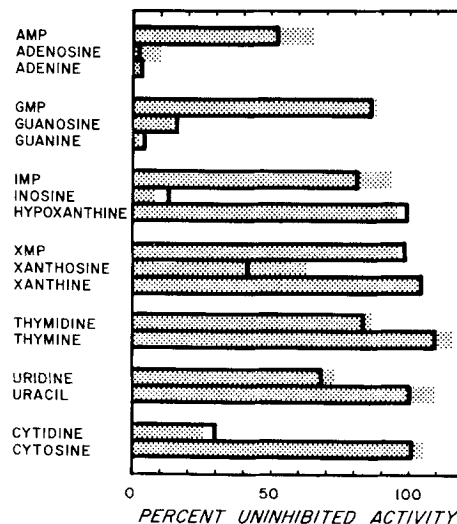
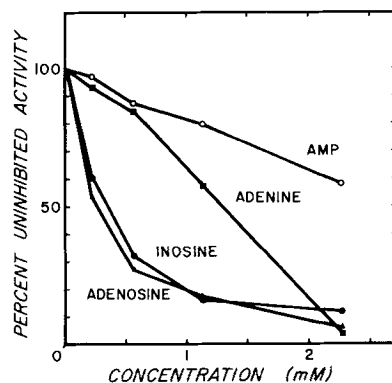
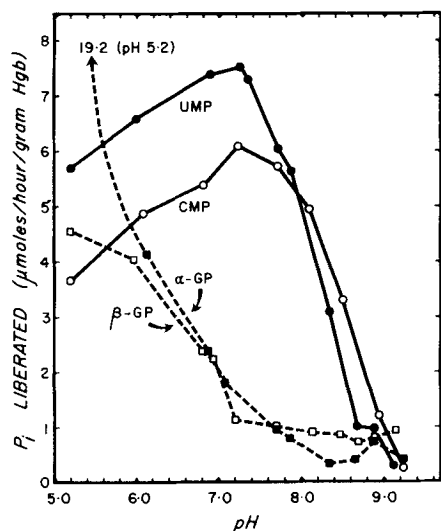


FIG. 3 (left). Effect of pH on the dephosphorylation of various substrates by dialyzed hemolysates. Acetate or acetate-barbital buffer was used below pH 6.0, Tris buffer for pH range 6.0 to 9.0, and glycylglycine buffer above pH 9.0. Final concentration of each buffer was 0.1 M, and each substrate, 2.3 mM. (α -GP = α -glycerophosphate; β -GP = β -glycerophosphate.) Each point is the mean of two to four assays with two separate blood samples. Reaction mixture pH was measured both before and after the 2-hour incubation period and averaged. These values never diverged by more than ± 0.1 pH unit.

FIG. 4 (center). Inhibition of erythrocyte 5'-nucleotidase by purine analogs. Each compound was present during the incubation phase of the assay at the final concentrations noted. Blanks contained both

The rapid decline of activity in more alkaline media is quite different from that observed with nucleotidases in serum (15, 16) and diverse other tissues (8–10, 17–19). Higher and more consistent values for erythrocyte nucleotidase activities might be expected if the pH of the incubation medium were lowered from pH 7.8 to approximately 7.4.

Inhibition by Purine and Pyrimidine Compounds—Although AMP was not utilizable as a substrate, it did interfere with the dephosphorylation of UMP or CMP. As shown in Fig. 4, this interference was concentration-dependent and was more pronounced if the nucleoside or purine base, rather than the nucleotide, was present during incubation. Adenosine produced an inhibition curve which was virtually identical with that resulting from its deaminated analog, inosine.

Relative inhibitory effects of these and other nucleosides, nucleotides, and free bases are presented in Fig. 5. Nucleosides in general, both purine and pyrimidine, were consistently inhibitory to varying degrees. Strong inhibition by guanosine was comparable to that by adenosine or inosine, yet the phosphorylated purine nucleosides, except AMP, had little or no effect on nucleotidase activity. Among the bases, guanine was as strongly inhibitory as adenine, but hypoxanthine, xanthine, and the pyrimidine bases were without perceptible effects. The presence of free ribose or ribose-5-phosphate did not significantly alter observed nucleotidase activities.

Inhibitory effects of adenine and AMP were completely abolished by redialysis. Dialyzed hemolysates were incubated for 20 min at 37° with 2.3 mM adenine, adenosine or AMP, then redialyzed for 45 min at 4° against 250 volumes of 0.01 M Tris, pH 8.0, containing 10 mM $MgCl_2$, 0.02 mM EDTA, and 0.5 mM 2-mercaptoethanol. These were redialyzed a second time against 250 volumes of the same medium for an additional 45 to 90 min prior to assaying for nucleotidase activity. Results of duplicate experiments with two different cell samples assayed

hemolysate and inhibitor, but substrate was not added until after acid precipitation. There were no apparent relative differences between values obtained with UMP or CMP. Each point is the mean of two to eight determinations on one to four separate hemolysates.

FIG. 5 (right). Inhibition of erythrocyte 5'-nucleotidase activity by various purine and pyrimidine compounds. Each inhibitor was present in the assay system at a final concentration of 2.3 mM. Values were derived from one to four determinations on one to four separate hemolysates. Solid lines are values obtained with UMP as substrate. Shaded areas indicate relative activities obtained with CMP as substrate.

in quadruplicate indicated that 102 to 105% of the original activity was present in systems incubated with adenine or AMP prior to redialysis. Those incubated with adenosine, however, retained only 52 to 82% of control activities, averaging 71%.

Effects of Sulfhydryl Inhibitors—Nucleotidase activity was 50% inhibited by *p*-chloromercuribenzoate or *N*-ethylmaleimide at approximately 0.1 mM concentrations, but inhibition was less pronounced with CMP as the substrate than it was with UMP. Mercaptoethanol largely abolished the inhibitory effects of both reagents at this concentration. The enzyme was relatively insensitive to iodoacetate, which reduced activity by only 10 to 15% at a final concentration of 1 mM.

Effects of Metal Cations—As shown in Fig. 6, nucleotidase activity was dependent upon Mg^{2+} concentration. Hemolysates dialyzed thoroughly against the Tris buffer devoid of $MgCl_2$ or EDTA exhibited optimum activity only when Mg^{2+} was added to final concentrations of 1 mM or more. Manganese could not replace Mg^{2+} as an activator and in fact was inhibitory. Some enzyme instability was apparent under these dialysis conditions as well, since the maximum activity obtained (approximately 5.0 units) was slightly less than that

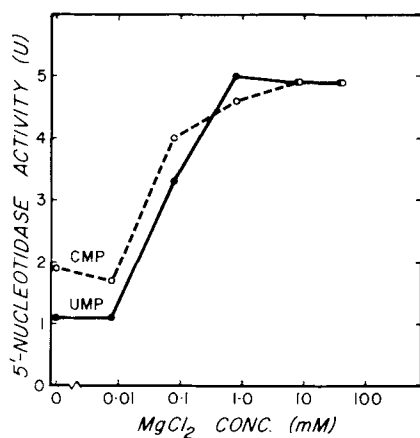


FIG. 6. Representative experiment demonstrating effect of Mg^{2+} concentration on erythrocyte nucleotidase activity. Hemolysate was prepared by the usual procedure, but the dialysis medium and buffer were devoid of Mg^{2+} or EDTA. Mercaptoethanol (0.4 mM) was present in the assay system. Nucleotidase activity was completely abolished by EDTA (2.3 mM final concentration) in systems devoid of added Mg^{2+} .

usually observed with hemolysates dialyzed in the presence of $MgCl_2$ and EDTA.

Alterations of nucleotidase activities induced by other metal cations at final concentrations between 10^{-6} and 10^{-3} M fell into four general categories (Table II). Trivalent chromium distinctly stimulated activity; some cations (Ni^{2+} and Ba^{2+}) were without significant effect; and others were moderately (Zn^{2+} and Mn^{2+}) or markedly (Cu^{2+} , Pb^{2+} , Hg^{2+} , and Cd^{2+}) inhibitory. When CMP rather than UMP was the substrate, both inhibitory and stimulatory effects of these cations were generally less pronounced. With either substrate, relative stimulation was increased and relative inhibition was decreased by the presence of mercaptoethanol. For example, with UMP as substrate, 0.4 mM mercaptoethanol increased nucleotidase activity in the presence of 1 mM Cr^{3+} from 123% to 139% of initial activity. The ability of mercaptoethanol to reduce enzymatic inhibition by Cd^{2+} and Cu^{2+} was less pronounced but readily apparent and consistent.

Heat Stability—Pyrimidine 5'-nucleotidase activity in dialyzed hemolysates was markedly sensitive to thermal inactivation. Preliminary screening studies indicated that activity was completely lost after 5 min at 100° . Residual activities following incubation at 60° for 10 min or 50° for 30 min were 0 to 8% of initial activities. Fig. 7A demonstrates the rapid deterioration of activity induced by hemolysate incubation at 45° . These experiments were performed before mercaptoethanol was a standard component of the assay system. In three separate experiments, however, addition of mercaptoethanol to the hemolysate to a final concentration of 0.4 mM either before, during, or after heat exposure did not appreciably alter the results.

Significant protection against thermal inactivation was afforded by the presence of substrate during the period of heat exposure. Observed nucleotidase activities in hemolysates protected by CMP were essentially identical, whether subsequently measured with UMP or CMP as substrate, perhaps reflecting the lower K_m for CMP carried over from the initial incubation. As shown in Fig. 7A, resistance to thermal inactivation was significantly enhanced by the presence of the pyrimidine substrate (CMP) and to some extent as well by the purine analog (AMP). The true effect of AMP on thermal sensitivity under these conditions is probably masked by the fact that AMP was also carried over into the assay mixture and

TABLE II

Effect of various cations on erythrocyte nucleotidase activity

Cations, as chloride salts (or acetate salt, in the case of Pb^{2+}), were present in the incubation mixtures at the final concentrations indicated. Each value is the mean of two to four assays on one to three separate hemolysates. All systems contained 10 mM $MgCl_2$ but were devoid of mercaptoethanol.

Effect	Cation	Pyrimidine 5'-nucleotidase activity							
		10^{-6} M		10^{-5} M		10^{-4} M		10^{-3} M	
		UMP	CMP	UMP	CMP	UMP	CMP	UMP	CMP
		% control activity							
Activation	Cr^{3+}	106	102	111	105	112	103	123	104
Minimal or no inhibition	Ni^{2+}							90	104
	Ba^{2+}							87	91
	Mn^{2+}	89	93	91	92	75	82	32	29
Moderate inhibition	Zn^{2+}	93	102	102	111	66	85	6	6
	Cu^{2+}	95	95	59	63	32	29	13	12
Marked inhibition	Hg^{2+}	87	91	26	23	21	17	1	1
	Pb^{2+}	93	94	45	64	7	12	8	8
	Cd^{2+}	84	93	20	35	7	7	6	6

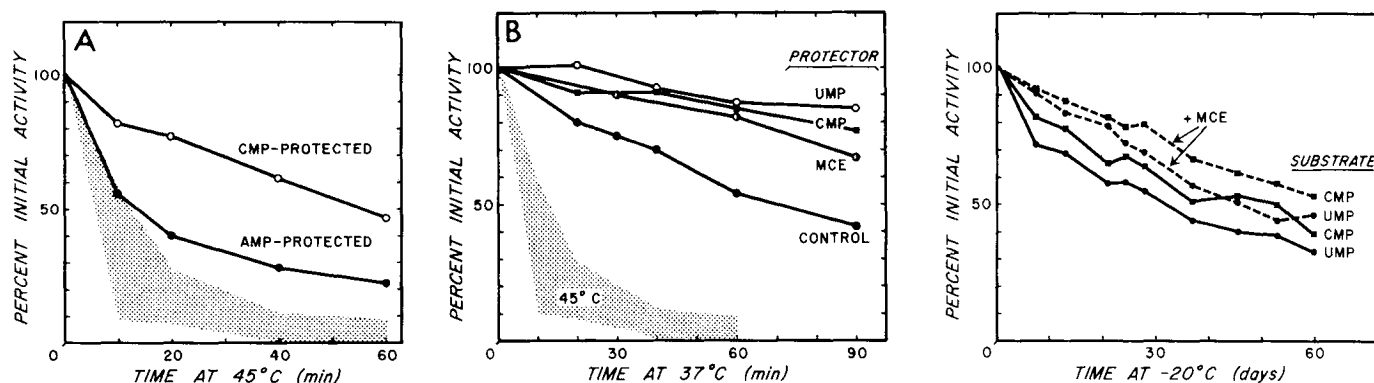


FIG. 7 (left and center). A, substrate protection of erythrocyte nucleotidase against thermal inactivation at 45°. Either CMP or AMP was added to hemolysate to a final concentration of 3.3 mM. Hemolysates were heated to 45° for the periods indicated, rapidly cooled, then assayed for nucleotidase activity using the standard assay system with UMP or CMP as substrate. Reagent blanks consisted of identical mixtures of hemolysate and stabilizer, which, after exposure to 45° for appropriate periods, were inactivated by heating quickly to 60° for 10 min. These were cooled rapidly to 37° for the incubation phase of the assay. Each point is the mean value of 6 to 10 determinations on four normal hemolysates. The shaded area encompasses two standard deviations on either side of mean nucleotidase activities in eight unprotected hemolysates assayed at the same time intervals. B, effects of mercaptoethanol and substrates in protection of erythrocyte nucleotidase against deterioration at 37°. The control curve depicts

in itself is an effective inhibitor of erythrocyte pyrimidine 5'-nucleotidase.

As shown in Fig. 7B, sensitivity to thermal inactivation remained apparent even at 37°. For comparative purposes, the decay limits at 45° are also included in this figure. Significant protection was afforded by the presence of either substrate, UMP or CMP. In contrast to the findings at 45°, addition of mercaptoethanol to the hemolysate exerted a distinct protective effect at 37°.

Storage Stability—Hemolysates dialyzed overnight to equilibrium against 0.05 M Tris buffer at pH 8.0 were stored under various conditions and assayed periodically to determine stability of the nucleotidase. As shown in Fig. 8, steadily progressive activity losses occurred when hemolysates were stored frozen at -20°. Enzyme alterations incurred under these conditions affected activities with UMP more severely than those with CMP. Decay to one-half initial values required at least 30 days. Addition of mercaptoethanol to the assay system produced comparable activation with either substrate. If dialyzed hemolysates were stored at -20° in the presence of 0.4 mM mercaptoethanol however, deterioration was twice as rapid (possibly due to the formation of mixed disulfides), even though the subsequent addition of mercaptoethanol to the assay mixture produced the expected relative activation. Dialyzed hemolysates stored in the refrigerator at 4° retained 90% or more of their initial activities for 6 days provided mercaptoethanol was added to the reaction mixture at the time of assay.

DISCUSSION

It has been known for over one-half a century that certain enzymes in mammalian tissues hydrolytically dephosphorylate nucleic acid derivatives (4, 20, 21). Some act selectively on nucleoside 5'-monophosphates (5, 22). Such 5'-nucleotidases are distinguishable from nonspecific acid or alkaline phosphatases by sharp differences in cation require-

ments, in activities as a function of pH, and in responses to certain activators and inhibitors (6, 9, 10, 12, 15-19, 23). Whether obtained from diverse mammalian tissue extracts or secretions, these enzymes are catalytically active with a broad range of substrates, including both purine and pyrimidine 5'-ribonucleotides, but not the 2', 3', or cyclic isomers (4, 5, 7-12).

Nucleosidases are known which cleave ribose from specific purine or pyrimidine bases (24), but there is apparently no such substrate selectivity among nucleotidases previously studied. In their studies with aqueous extracts of canine kidney and intestinal mucosa, Levene and Medigreceanu (4) noted that enzymatic hydrolysis of purine nucleotides proceeded more rapidly than hydrolysis of pyrimidine nucleotides. Conversely, it has also been observed that nucleotidase from some sources, such as bovine seminal plasma, may exhibit higher optimum activities with pyrimidine rather than with purine nucleotides (10). In all instances, however, the individual nucleotidase actively dephosphorylated either class of substrates.

It seems quite probable that the "UMPase" activity detected in soluble fractions of erythrocytes by Boninsegna *et al.* (25) was the same pyrimidine 5'-nucleotidase described herein. This remains uncertain, however, since their experiments were directed toward another purpose and did not include substrates other than uridine compounds. In any case, the present studies indicate that a pyrimidine-specific 5'-nucleotidase does exist within mature human erythrocytes. Indeed, their normal maturation and survival apparently is heavily dependent upon the proper function of this enzyme, since its hereditary deficiency is associated with premature hemolysis (3).

The erythrocyte enzyme was clearly distinguishable from human serum and other tissue nucleotidases or phosphatases by a number of criteria. In crude or dialyzed hemolysates, nucleotidase activity was exclusively limited to UMP, dUMP, CMP, dCMP, and TMP, in descending order of effectiveness.

the mean decay rate for nucleotidase activity in crude unprotected hemolysates exposed to 37° for the periods indicated prior to the addition of substrate for the incubation phase of the assay procedure. Points on the control curve are means of 4 to 10 determinations on three hemolysates. Points on the remaining curves are means of two to three assays on the same hemolysates, containing 1.1 mM UMP, 1.1 mM CMP, or 0.4 mM mercaptoethanol (MCE) during exposure to 37° prior to assaying nucleotidase activities.

FIG. 8 (right). Deterioration of erythrocyte nucleotidase activity as a consequence of hemolysate storage at -20°. Hemolysates were frozen after overnight dialysis in the usual manner except the dialysis medium, 0.05 M Tris, pH 8.0, was devoid of MgCl₂ or EDTA. Each point is the mean of two to five assays on three separate specimens from 2 normal subjects. Broken lines indicate values obtained with mercaptoethanol (MCE) in the assay system.

Purine nucleotides, 2', 3', and cyclic pyrimidine ribosemono-phosphates, and compounds commonly used as substrates for nonspecific phosphatase were not catalytically dephosphorylated under these conditions. The restriction to pyrimidine substrates demonstrated by these *in vitro* studies apparently extends to the physiologic role of erythrocyte nucleotidase. This may be inferred from one of the consequences of severe hereditary deficiency of this enzyme, specifically the 3- to 6-fold elevations in total intracellular nucleotide concentrations (3). These consisted largely (>80%) of cytidine and uridine phosphates. Adenine nucleotide concentrations were actually decreased below those expected for cells of comparable ages, and guanine nucleotides were not detectable. These compounds presumably represent degradation products of RNA derived during reticulocyte maturation, and relative accumulations of all four would be expected in hereditary deficiency states if the erythrocyte enzyme were catalytically effective with both purine and pyrimidine 5'-nucleotides *in vivo*.

Apparent Michaelis constants for UMP were identical with the K_m (AMP) reported for human serum (16), but were 10 times greater than those reported for partially purified nucleotidase from human liver (9). As with nucleotidases from most tissues, the erythrocyte enzyme required Mg^{2+} for optimum activity, but it was inhibited by Mn^{2+} , a cation which may activate the nucleotidases of human liver (9), aorta (23), and serum (26). Activity variations as a function of pH differed entirely from those characteristic of erythrocyte acid phosphatase or nonspecific alkaline phosphatase possibly introduced by contaminating leukocytes. Furthermore, there was no evidence of the double pH optimum of the hepatic enzyme induced by Mg^{2+} (9) nor of the relative insensitivity to alkaline pH usually exhibited by serum nucleotidases (15, 16, 18). Distinction from red cell acid phosphatase is emphasized by our previous observation that individuals hereditarily deficient in erythrocyte nucleotidase nonetheless have abundant phosphatase activities (3).

The enzyme in dialyzed hemolysates was highly susceptible to inactivation by a variety of agents and conditions, especially those affecting sulfhydryl groups. Nucleotidase activity was rapidly lost following exposure to certain heavy metals, organic sulfhydryl reagents, and elevated temperatures. Considerable protection against such inactivation was afforded by 2-mercaptoethanol, by pyrimidine substrates, and perhaps by some purine nucleotides as well.

It would be premature to attempt descriptions of the enzyme combining site based on studies with unpurified enzyme, yet several preliminary observations merit attention in this regard. Agents or conditions which either inhibited or stimulated nucleotidase activity (*e.g.* exposure to *N*-ethylmaleimide or to trivalent chromium ions, respectively) consistently produced greater relative effects when UMP was the substrate rather than CMP. This observation, coupled with the enzyme's lower V_{max} and apparent K_m for CMP and the inhibitory effects of certain nucleosides and free bases, suggests that the pyrimidine moiety itself (and probably the ribose) is intimately involved in the enzyme-substrate coupling mechanism. Such a conclusion is supported *a priori* by the clear exclusion of nonpyrimidine nucleotides as substrates. The existence of distinct combining sites for UMP as opposed to CMP seems improbable, since preliminary experiments to determine inhibition constants for AMP and other purine and pyrimidine

compounds indicate that competitive inhibition is involved.

Evolution of a nucleotidase with such unique specificity might be expected teleologically to occur in human erythrocytes. These cells lack the metabolic sophistication and redundancy of nucleated cells and must rely almost exclusively on anaerobic glycolysis to generate high energy phosphates in the form of ATP. Since they are not capable of *de novo* nucleotide synthesis, they must carefully preserve their adenine nucleotide pool against irrevocable loss. In this regard, AMP is in a specific position of jeopardy, for AMP deaminase is quite active in mammalian erythrocytes, and enzymatic systems to reverse or counter this reaction do not exist. Furthermore, adenosine may be irreversibly deaminated to inosine. In the absence of compensatory mechanisms, therefore, a nucleotidase which acted actively on AMP could be expected eventually to deplete the adenine nucleotide pool. Yet, maturing reticulocytes apparently require a nucleotidase to render diffusible uridine and cytidine phosphates resulting from degradation of RNA. These compounds were observed to accumulate in large amounts in those cells with hereditarily defective nucleotidase (3) and may seriously interfere with certain enzymes which are critical to erythrocyte glycolysis, such as pyruvate and phosphoglycerate kinases. Evolution of a pyrimidine-specific 5'-nucleotidase thus appears to represent another instance of erythrocyte adaptation to unique metabolic restrictions.

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REFERENCES

1. Valentine, W. N., Anderson, H. M., Paglia, D. E., Jaffé, E. R., Konrad, P. N., and Harris, S. R. (1972) *Blood J. Hematol.* **39**, 674-684
2. Valentine, W. N., Bennett, J. M., Krivit, W., Konrad, P. N., Lowman, J. T., Paglia, D. E., and Wakem, C. J. (1973) *Br. J. Haematol.* **24**, 157-167
3. Valentine, W. N., Fink, K., Paglia, D. E., Harris, S. R., and Adams, W. S. (1974) *J. Clin. Invest.* **54**, 866-879
4. Levene, P. A., and Medigreceanu, F. (1911) *J. Biol. Chem.* **9**, 389-402
5. Reis, J. (1940) *Bull. Soc. Chim. Biol.* **22**, 36-42
6. Reis, J. (1951) *Biochem. J.* **48**, 548-551
7. Levene, P. A., and Dillon, R. T. (1930) *J. Biol. Chem.* **88**, 753-769
8. Heppel, L. A., and Hilmoe, R. J. (1955) *Methods Enzymol.* **2**, 546-550
9. Song, C. S., and Bodansky, O. (1966) *Biochem. J.* **101**, 5c-6c
10. Levin, S. J., and Bodansky, O. (1966) *J. Biol. Chem.* **241**, 51-56
11. Widnell, C. C., and Unkeless, J. C. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **61**, 1050-1057
12. Drummond, G. L., and Yamamoto, M. (1971) in *The Enzymes* (Boyer, P. D., ed) Vol. IV, pp. 337-354, Academic Press, New York
13. Busch, D., and Pelz, K. (1966) *Klin. Wochenschr.* **44**, 983-984
14. Fiske, C. H., and SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375-400
15. Dixon, T. F., and Purdom, M. (1954) *J. Clin. Path.* **7**, 341-343
16. Belfield, A., and Goldberg, D. M. (1969) *J. Clin. Path.* **22**, 144-151
17. Bodansky, O., and Schwartz, M. K. (1963) *J. Biol. Chem.* **238**, 3420-3427
18. Schwartz, M. K., and Bodansky, O. (1964) *Am. J. Clin. Pathol.* **42**, 572-580

19. Song, C. S., and Bodansky, O. (1967) *J. Biol. Chem.* **242**, 694-699
20. Levene, P. A., and Medigreceanu, F. (1911) *J. Biol. Chem.* **9**, 65-83
21. Levene, P. A., and Medigreceanu, F. (1911) *J. Biol. Chem.* **9**, 375-387
22. Reis, J. (1934) *Bull. Soc. Chim. Biol.* **16**, 385-399
23. Ahmed, Z., and Reis, J. L. (1958) *Biochem. J.* **69**, 386-387
24. (1965) *Enzyme Nomenclature*, Elsevier, New York
25. Boninsegna, A., Deana, R., and Siliprandi, D. (1974) *J. Cell. Physiol.* **83**, 53-57
26. Campbell, D. M. (1962) *Biochem. J.* **82**, 34 P

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