ACID PHOSPHOMONOESTERASES OF HUMAN ERYTHROCYTES

A Thesis

Presented in Partial Fulfillment of the Requirements
for the Degree Master of Science

by

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1966

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Acknowledgements

I would like to express my appreciation to Dr. Keith E. Richardson for his support, encouragement, and guidance in this research project. I would also like to thank my Research Advisory Committee, Dr. Alben, Dr. McCluer, and Dr. Nuenke, for their interest in this project and their valuable suggestions in the writing of this thesis.

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Introduction

Orthophosphoric monoester phosphorylases (3.1.3.2) are enzymes which catalyze the hydrolysis of phosphate esters. These phosphomonoesterases, often called phosphatases, may be divided into two main groups according to their activity in an acid or an alkaline medium. Phosphomonoesterases active below pH 7.0 are classified as acid phosphatases; alkaline phosphatases exhibit maximum activity from pH 8.0 to 10.0. Human erythrocytes contain both acid and alkaline phosphatases.

Phosphomonoesterases are widely distributed in plant and animal tissues. The phosphatases from different species, as well as those from various tissues of the same species, vary in characteristic properties. Human prostatic acid phosphatase is inhibited by L(+)-tartrate, but is not inhibited by formaldehyde. The acid phosphatase of human erythrocytes is completely inhibited by formaldehyde; L(+)-tartrate has no inhibitory influence on this enzyme.[1]

Phosphatases have been implicated in various disease states. Increases in the level of the acid phosphatase have been observed in macrocytes of megaloblastic anemias, sickle cell disease, paroxysmal nocturnal hemoglobinurias, and other hemolytic anemias.[2] Human erythrocytic acid phosphatases have been linked to cases of chronic congenital non-spherocytic hemolytic anemia. A detailed study of the properties and characteristics of red cell acid phosphatases is necessary to elucidate the functions of acid phosphatases in normal

metabolism and in disease states. In this study three acid phosphatases have been isolated from the erythrocytes of human blood. A partial characterization of these isozymes, as well as a more detailed study of one of them, has been done.

Historical Review

Martland and associates in 1924 demonstrated the presence in human erythrocytes of a phosphatase with an optimum pH of 6.0.[3] This report was confirmed by Roche in 1931.[4] In later studies, Roche and co-workers identified two acid phosphatases with different pH optima in the red cells of rats and cattle.[5]

In 1934 Davies compared phosphatases from various sources including spleen, bone, kidney, plasma, and red cells. His results showed the presence of one pH optimum and activation of the enzyme by magnesium.[6] Behrendt also studied the acid phosphatase activity of human red cells and found the activity to lie between pH 4.8 and 6.1.[7] The optimum pH was near 5.3. The activity at this pH was inhibited only slightly by sodium fluoride. However, at pH 5.0, there was strong fluoride inhibition. There was no definite effect of magnesium chloride on this activity. King and co-workers[8] confirmed the work of Behrendt. The enzyme studied by this group was extremely active against the phosphoric esters of phenols, but exhibited little activity against phosphoric esters of alcohols, glycerol, and hexoses. Using phenyl phosphate as substrate, the enzyme was optimally active between pH 4.8 and 5.2. It was inhibited by magnesium, fluorine, and prolonged ethanol treatment, and was activated by manganese and cyanide.

The first indication of the presence of two acid phosphatases in human erythrocytes was reported by Abul-Fadl and King in 1949.[1] Their studies of the pH optima of the phosphatases found in human,

ox, rabbit, and sheep erythrocytes indicated the presence of two acid phosphatases, one with a pH optimum of 4.3 to 4.8, and a second with an optimum from 5.0 to 5.7. These optima varied slightly, not only from species to species, but even in different members of the same species. They reported that the enzyme with an optimum from pH 4.3 to 4.8 was very labile and could no longer be detected in crude hemolysates that had remained at room temperature for several hours. Freshly prepared crude hemolysates were found to hydrolyze alpha-glycerophosphate much faster than the beta isomer.

The enzyme with an optimum from pH 4.3 to 4.8 and the enzyme with an optimum from pH 5.0 to 5.7 were inhibited by magnesium. Inhibition also occurred in the presence of calcium, chromium, nickel, cobalt, or manganese at a concentration of 10^{-2} M. Zinc or iron, 10^{-3} M, as well as 2 x10^{-3} M copper, also caused inhibition. Copper inhibition could be partially overcome by the simultaneous addition of cysteine or reduced glutathione. Inhibition was also observed with arsenate, oxalate, tauroglycolate, iodoacetate, and formaldehyde.

The red cell acid phosphatase was shown to differ from that of the prostate gland. A 0.5 per cent formaldehyde solution had no effect on prostatic acid phosphatase, but completely inhibited acid phosphatase activity of red cells. Fluoride, 10^{-2} M, inhibited the prostatic enzyme almost 100 per cent, but had little or no effect on the red cell enzymes.

Tsuboi and Hudson did an extensive study of the acid phosphatase activity of human erythrocytes.[9-12] They detected only one pH optimum

in crude hemolysates. This optimum was found between pH 5.5 and 6.0. Hemolysates were assayed for activity immediately after preparation, after 24 hours standing at 0^{o} C and after 24 hours dialysis. The majority of hemolysates tested in this manner showed no change in enzyme activity. These authors concluded that human erythrocytes did not contain a second, labile enzyme.

Tsuboi and Hudson obtained an enzyme of approximately 1500 fold purity by repeated adsorption on calcium phosphate gel and repeated precipitation with ammonium sulfate.[10] The enzyme had a broad optimum with a maximum near 6.0, and was not affected by magnesium. It hydrolyzed the alpha isomer of glycerophosphate much more rapidly than the beta isomer. The Km for phenyl phosphate was found to be 9×10^{-4} M and that for alpha glycerophosphate 7×10^{-3} M.[12] The purified enzyme was found to be resistant to inactivation by fluoride and L-tartrate and was shown to be unstable to surface forces.[11] Small quantities of synthetic non-ionic detergents resulted in the stabilization of the enzyme against surface forces. The enzyme was also found to be rapidly inactivated by trace quantities of heavy metals. The marked susceptibility of the enzyme to trace quantities of metals suggested the presence of essential sulfhydryl groups.

In 1962 Angeletti and Gayle reported the fractionation of red cell hemolysates into three distinguishable peaks of acid phosphatase activity by DEAE-cellulose column chromatography.[13] Using p-nitrophenyl-phosphate as substrate, the first peak had an optimum pH near 4.5, and the second and third peaks both had an optimum near pH 5.5. The enzyme

of the first peak showed about 40 per cent inhibition with 20 mM sodium tartrate, while the enzyme of peaks two and three showed no tartrate inhibition, even up to a concentration of 40 mM.

The evidence for more than one acid phosphatase in human erythrocytes was strengthened by the electrophoretic studies of crude hemolysates as reported by Hopkinson and associates.[14] Hemolysates examined by starch gel electrophoresis showed more than one zone of acid phosphatase activity. Five distinct red cell acid phosphatase patterns were detected. These patterns were referred to as A, BA, B, CA, and CB. They were described in terms of the relative activity of three zones of acid phosphatase - 'slow', 'intermediate', and 'fast' (Table I). These patterns appeared to be characteristic for the individual and genetically determined. A 0.5 per cent formalin solution, added during the incubation of the starch gel with the substrate, prevented the appearance of any bands of enzymic activity, but 3 $x10^{-2}$ M D (+)-tartrate had no apparent effect on the activity. This reported effect of tartrate is meaningless due to the error in nomenclature. It is L(+)-tartrate that has been reported to inhibit certain acid phosphatases such as that of the prostate gland.

As a result of studies on the electrophoretic patterns of 42 families, Hopkinson postulated that the variations observed are determined by three allelic autosomal genes P^a, P^b, and P^c. This hypothesis predicts the occurrence of a sixth phenotype. The presence of this phenotype has been confirmed.[15] A seventh phenotype of low

Table I

Approximate Description of the Five Red Cell Acid Phosphatase Electrophoretic Patterns.[a]

Zone of Enzymic Activity	Red Cell Acid Phosphatase Type				
	A	BA	B	CA	CB
'Fast'	+	+	-	+	-
'Intermediate'	+	+ +	+ + +	+	+ +
'Slow'	-	trace	+ +	+ + + +	+ + + +

[a]Hopkinson, D. A., N. Spencer, and H. Harris, Nature, 199, 969 (1963).

frequency has also been reported when a formate buffer system was used in the electrophoretic procedure.[16]

Georgatsos has prepared an acetone powder from human erythrocytes which has yielded two acid phosphatase peaks when fractionated by column chromatography using Sephadex G-75.[17] It was established that the faster moving enzyme (E_1) was magnesium activated, while the slower enzyme (E_2) was inhibited by magnesium and activated by EDTA. Enzyme E_1 possessed two pH optima, one at pH 5.0, the other at pH 6.0. Enzyme E_2 exhibited maximum activity at pH 5.2. Enzyme E_2 is inhibited by 6.6×10^{-3} M formaldehyde, 6.6×10^{-3} M oxalate, and 1.0×10^{-2} M ethanol; while E_1 is inhibited by 6.6×10^{-3} M formaldehyde, but to a lesser extent than E_2. Enzyme E_1 is also inhibited by 1.0×10^{-2} M fluoride which did not affect E_2. A very slight activation is noted with E_1 in the presence of oxalate and ethanol. The two enzymes differed in molecular weight, pH optima, metal requirements, and also in the extent of inhibition by various inhibitory substances.

Erythrocytic acid phosphatases have been implicated in some cases of chronic non-spherocytic hemolytic anemia.[18] Caucasian patients with a deficiency of glucose-6-phosphate dehydrogenase also have a deficiency in erythrocytic acid phosphatase. This deficiency is not evident in Negro patients with a glucose-6-phosphate dehydrogenase deficiency. It has been suggested that the lower levels of red cell acid phosphatase reported in carriers of the glucose-6-phosphate dehydrogenase deficiency may be due to the excess of oxidized glutathione known to be present in the erythrocytes of these individuals.[19] A

complete separation and detailed study of the acid phosphatases of human red cells is necessary in order to determine the role of these enzymes in various pathological disease states.

Materials and Methods

Enzyme Source

Outdated blood containing acid-citrate-dextrose as an anticoagulant was obtained from the Ohio State University Hospital Blood Bank. The blood was centrifuged at 10,000 g for 10 minutes and the plasma removed by suction. The cells were washed with physiological saline, centrifuged at 10,000 g for 10 minutes, and the leucocytes removed by suction. This was repeated twice. The washed cells were lysed in four volumes of cold, distilled water in the presence of 0.025 per cent Triton X-100 to insure lysis and solubilization. After mixing for 30 minutes at 4°C, the preparation was centrifuged for 30 minutes at 30,000 g to remove the stroma. The stroma-free hemolysate was dialyzed for 18 to 20 hours at 4°C against 4 liters of 0.005 M Tris-phosphate buffer pH 6.0, containing 20 mg of reduced glutathione per liter. This preparation will be referred to as the crude hemolysate.

Enzyme Assay

The enzyme was assayed by measuring the release of p-nitrophenol from p-nitrophenylphosphate by a modification of the procedure for alkaline phosphatases[20], or by measuring the release of inorganic phosphate by the method of Fiske and SubbaRow.[21] The reaction mixture for the assay of crude hemolysates and column eluates contained 200 umoles of citrate buffer, pH 5.75, 30 umoles of p-nitrophenylphosphate, and sufficient enzyme to cause a change in the absorbance at 415 mu of 0.2 to 0.8 in a 30 minute incubation period in a total volume of 3.0 ml.

Incubations were performed at 37°C. The reaction rates were linear under the conditions employed for more than 60 minutes. For the determination of p-nitrophenol, the reaction was stopped by transferring a 1.0 ml aliquot to 5 ml of 0.2 M NaOH. The solution was mixed and the absorbance at 415 mu was determined. For phosphate determinations, the reaction was stopped by transferring a 1.0 ml aliquot to 1 ml of 10 per cent trichloroacetic acid. Any precipitated protein was removed by centrifugation and a 1.0 ml aliquot removed for assay. A zero time blank was prepared for each individual assay. All readings were done on a Beckman Model B Spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme needed to cause a change of 1.0 in absorbance per 30 minutes at 415 mu using p-nitrophenylphosphate as substrate.

Protein Determination

Protein was estimated by the method of Lowry, et al.[22]

Electrophoresis

Starch gel electrophoresis was done according to the method of Hopkinson, Spencer, and Harris[14], except that a pH of 6.2 instead of 6.0 was used. Hemolysates for electrophoresis were prepared by diluting the washed cells in 2 volumes of distilled water.

Preparation of DEAE-Sephadex

DEAE-Sephadex A-50 was allowed to swell in distilled water and the fines removed by decantation. It was equilibrated for 24 hours in 0.005 M Tris-phosphate buffer, pH 6.0.

Column Chromatography

The equilibrated DEAE-Sephadex was packed by gravity at 4°C to a height of 45 cm in a column 2 cm in diameter. Dialyzed crude hemolysate containing 300 units of phosphatase activity was applied to the column. The column was placed on an automatic fraction collector adjusted to collect 10 ml fractions. Hemoglobin was removed by washing with the equilibration buffer. Two linear gradients were used to elute the enzyme activity from the column. The column was first washed with 600 ml of NaCl between 0.00 M and 0.02 M and then washed with 900 ml of NaCl between 0.02 M and 0.25 M. Each of the NaCl gradients contained 0.005 M Tris-phosphate, pH 6.0, and approximately 2.0 mg reduced gluthathione per 100 ml.

Vacuum Ultrafiltration

Enzyme solutions from the columns were concentrated by vacuum ultrafiltration except when p-nitrophenylphosphate was used as substrate. Enzyme solutions were concentrated to insure that lack of activity with a particular substrate was not due merely to dilution of the enzyme. The solution in the vacuum flask contained 20 mg Cleland's reagent (dithiothreitol) per 100 ml and 0.025 per cent Triton X-100.

Preparation of p-Nitrophenylphosphate

The p-nitrophenylphosphate was prepared according to the method of Bessey and Love.[23] All other reagents were obtained commercially and were of reagent grade.

Results

Enzyme Isolation

Three separate and distinct peaks of acid phosphatase activity were obtained from column chromatography (figure 1). The combined activity of these peaks accounted for 100 per cent of the 300 acid phosphatase units applied to the column. The concentrations of the individual isozymes obtained on column chromatography were directly related to the isozyme content of the crude hemolysate as established by genetic type (Table II). The relative distribution of isozyme activity obtained by direct assay is in essential agreement with the approximations made by Hopkinson, et al., by starch gel electrophoresis.[14] The ratios of activity on direct assay for p-nitrophenylphosphate to phenolphthalein diphosphate were 58, 10.0 and 3.3 for slow, intermediate, and fast bands according to electrophoresis. All three isozymes were present in each sample of blood fractionated, but since the electrophoretic assay was much less sensitive, all three were not always detected by electrophoresis of the crude hemolysate.

Electrophoresis

The three acid phosphatase isozymes were subjected to starch gel electrophoresis and each peak gave only a single zone of phosphatase activity. The three peaks corresponded electrophoretically to the isozymes reported by Hopkinson, et al. The first peak from the column corresponded to the slow electrophoretic band, E_s, the second peak, to the intermediate electrophoretic band, E_i, and the

Figure 1

Chromatographic Separation of Three Acid Phosphatase Isozymes from Human Erythrocytes with DEAE-Sephadex.

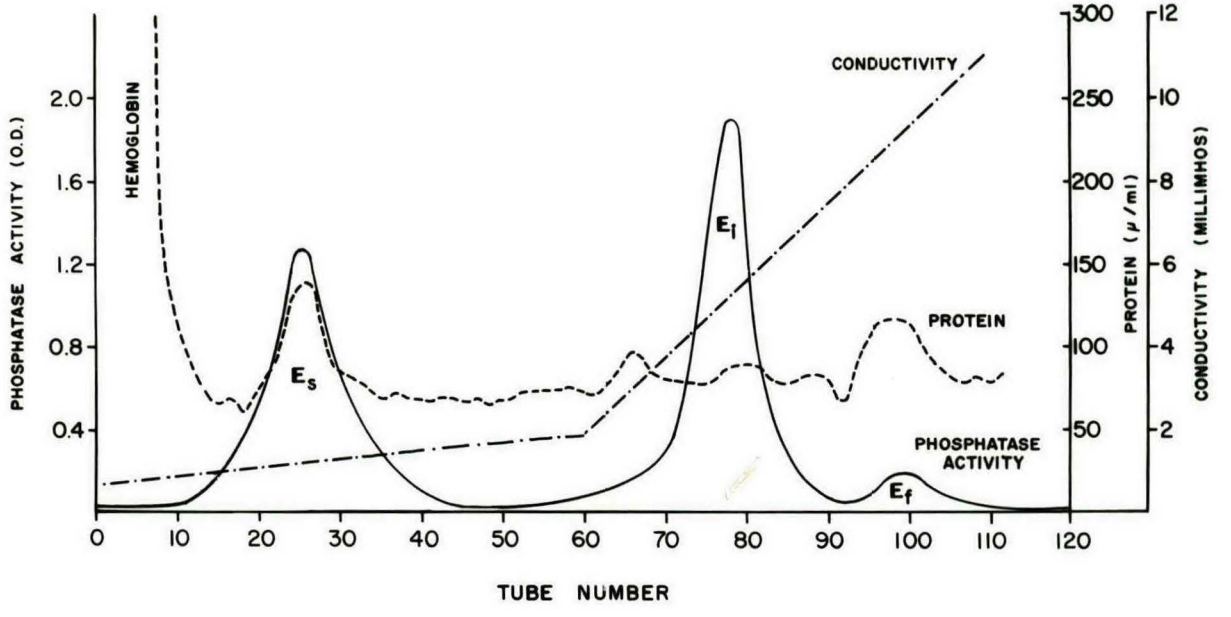


Table II

Distribution of Acid Phosphatase Isozymes from Human Erythrocytes of Different Phenotypes[a]

Phenotype	Acid Phosphatase Isozymes			
	E_s	E_i	E_f	Total Recovered
	(%)	(%)	(%)	(%)
BA	31.0	65.0	11.0	107
BA	31.0	65.0	10.6	107
BA	40.3	51.0	16.8	108
B	26.0	64.0	16.0	106
B	29.0	62.0	11.0	102
B	29.0	62.0	10.0	101
CB	52.0	42.6	7.3	102
CB	52.0	43.0	7.0	102
CB	57.0	35.0	4.0	96
CA	66.0	37.0	4.0	107
CA	66.6	33.5	2.0	102

[a]Crude hemolysates, classified according to phenotype by the electrophoretic procedure and nomenclature of Hopkinson, et al. (6), containing 300 units of acid phosphatase activity were resolved into individual isozymes by column chromatography on DEAE-Sephadex. Isozyme activity was assayed with p-nitrophenylphosphate as described under Methods. The per cent contribution of each isozyme to the total phosphatase activity recovered from the DEAE-Sephadex column was determined for each crude hemolysate.

third chromatographic peak, to the fast electrophoretic band, E_f. Hereafter, the isozymes will be referred to by the classification based on their electrophoretic mobility: E_s, E_f, E_i.

pH Optima

The three isozymes were characterized with respect to their optimum pH for the hydrolysis of p-nitrophenylphosphate (figure 2). At least 50 per cent of the optimum activity was observed between pH 4.0 and pH 6.5 for all three isozymes. The optimum pH for E_s was near 4.75, for E_i between 5.5 and 5.75, and for E_f near 5.75. Previously reported pH optima for erythrocytic acid phosphatases ranged between 4.5 and 6.0, but in some instances substrates different from the one employed here were used in determining the optimum pH.

Inhibition Studies

The effects of a number of inhibitors on the three phosphatase isozymes are summarized in Table III. Formaldehyde inhibited all three isozymes. $MgCl_2$, which was inhibitory to E_f at a concentration of 1.3×10^{-2} M, produced a 40 per cent activation of E_f at a concentration of 6.6×10^{-3} M and significantly inhibited E_s at pH 5.5 at a concentration of 6.6×10^{-3} M. Oxalate strongly inhibited E_s at all concentrations and pH's. E_i and E_f were inhibited about 20 per cent at an oxalate concentration of 1.3×10^{-2} M. NaF showed inhibitory effects against E_s and E_f only. Of the three isozymes, E_s is most readily inhibited, E_i was the most resistant to inhibition with only formaldehyde being highly effective, and E_f is intermediate with respect to susceptibility to inhibition and the only isozyme activated by compounds in this group.

Figure 2

pH Hydrolysis Curves for Human Erythrocyte Acid Phosphatase Isozymes

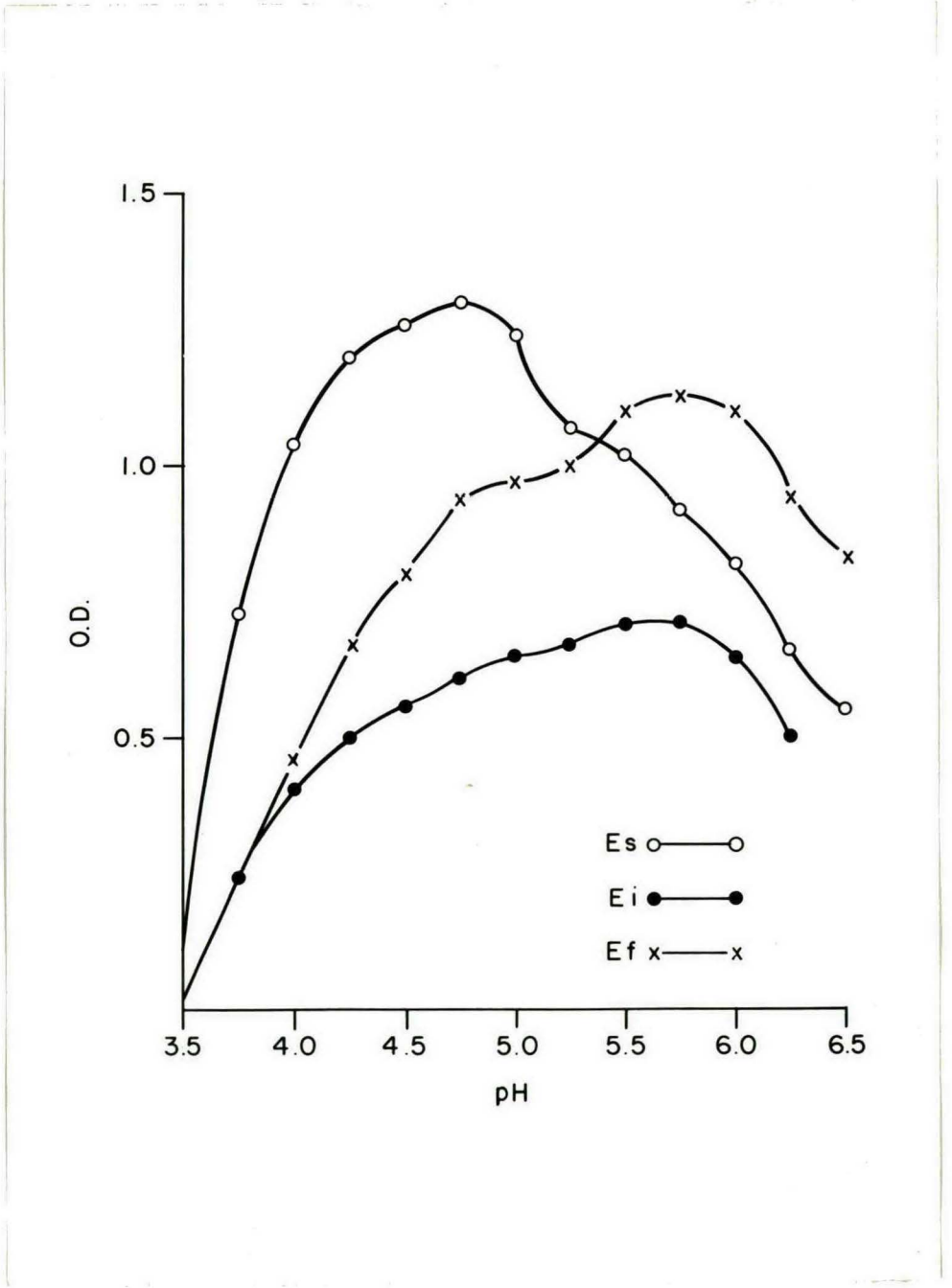


Table III

Effect of Various Compounds on the Activity of the Acid Phosphatase Isozymes

Addition	Concentration	% Activity[a]					
		E_s Phosphatase		E_i Phosphatase		E_f Phosphatase	
	M	pH 4.75	pH 5.5	pH 5.25	pH 5.5	pH 5.75	pH 5.5
None	--------	100	100	100	100	100	100
EDTA	3.3×10^{-3}	103	99	101	100	103	100
EDTA	6.6×10^{-3}	54	--	98	--	100	--
$MgCl_2$	6.6×10^{-3}	92	64	106	100	140	140
$MgCl_2$	1.3×10^{-2}	92	--	88	--	69	--
NaF	1.3×10^{-2}	97	73	100	100	98	93
NaF	2.0×10^{-2}	77	--	95	--	64	--
Formaldehyde	6.6×10^{-3}	84	72	86	59	60	53
Formaldehyde	1.3×10^{-2}	68	--	54	--	28	--
Oxalate	6.6×10^{-3}	58	54	95	83	102	84
Oxalate	1.3×10^{-2}	36	--	83	--	76	--
L-Tartrate	6.6×10^{-3}	106	100	104	100	113	96
L-Tartrate	1.3×10^{-2}	--	--	101	--	100	--

[a]Assays were performed at the pH indicated, as described under Methods with p-nitrophenylphosphate as substrate.

Inhibitors such as PCMB are thought to react with sulfhydryl groups on enzymes. This combination of inhibitors with sulfhydryl groups effectively blocks the free sulfhydryl groups which may be essential for enzymic activity. This inhibitory effect may be partially or totally blocked by sulfhydryl compounds such as reduced glutathione or cysteine. Therefore, to determine the actual effect of inhibitors, E_i was prepared in the absence of reduced glutathione. The absence of reduced glutathione had no effect on E_i activity.

Enzyme E_i was not inhibited by fluoride or L(+)-tartrate, but was almost completely inhibited by formaldehyde (Table IV). Inhibition by phosphate may be due to the fact that it is a product of the enzymic reaction, and the inhibition of molybdate, arsenate, and oxalate may be due to the similarity in structure of these compounds to phosphate.[24] Complete inhibition with PCMB indicates the possible necessity of a sulfhydryl group for enzymic activity.

Metal Ion Studies

Enzyme for metal ion studies was prepared in the absence of reduced glutathione since some metal ions can combine with free sulfhydryl groups of enzymes causing inhibition of enzyme activity. Reduced glutathione may block this inhibitory effect.

The substrate used for metal ion studies was p-nitrophenyl-phosphate. All solutions were prepared with glass distilled water. The per cent inhibition was determined by comparison to a standard incubated in the presence of glass distilled water in the absence of a metal ion (Table V). Enzyme E_i was inhibited completely by

Table IV

Inhibitor Study on E_i Prepared in the Absence of Reduced Glutathione

Inhibitor	Final Concentration in reaction mixture M	Per Cent Inhibition
Sodium Arsenate	0.01	82
KCN	0.01	20
NaF	0.01	0
Formaldehyde	0.01	91
Iodoacetic Acid	0.01	0
Sodium Molybdate	0.01	42
NaH_2PO_4	0.01	12
NaH_2PO_4	0.10	50
L(+)-Tartrate	0.01	0
Urea	0.01	0
Urea	1.0	30
Oxalate	0.01	40
PCMB	saturated	100

Table V

Effect of Metal Ions on E_i Prepared in the Absence of Reduced Glutathione

Metal Ion	Concentration M				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Co^{++}	100	0	0	0	0
Cd^{++}	100	100	70	10	0
Hg^{++}	100	100	100	60	0
Fe^{+++}	100	0	0	0	0
Zn^{++}	70	0	0	0	0
Ca^{++}	50	0	0	0	0
Mg^{++}	30	0	0	0	0

Results are expressed in per cent inhibition. Assays were performed as described under methods except for the addition of varying concentrations of metal ions.

cobalt, cadmium, iron, and mercury at a concentration of 10^{-1} M. At this same concentration it was only partially inhibited by calcium, magnesium, and zinc. Inhibition at such a high level of metal ion is not unusual in enzyme systems. At an inhibitor concentration of 10^{-2} M, E_i was inhibited completely by cadmium and mercury. At 10^{-3} M and 10^{-4} M, cadmium inhibited E_i 70 per cent and 10 per cent respectively. Mercury inhibited completely at 10^{-3} M and 60 per cent at 10^{-4} M. The effect of mercury was almost negligible at 10^{-5} M. Manganese, sodium, and potassium had no effect on E_i phosphatase activity.

Effect of Sulfhydryl Compounds

Reduced glutathione has been reported to be a possible cofactor for erythrocytic acid phosphatases.[18] The effect of sulfhydryl compounds on E_i activity with p-nitrophenylphosphate as substrate was therefore studied (Table VI). In A through D the enzyme used was prepared in the absence of reduced glutathione. The per cent inhibition in each case was determined by comparison to a standard which was enzyme without preincubation assayed in the normal manner without the addition of inhibitor or stabilizer. Cleland's reagent minimized the inhibition by cadmium and mercury. The stabilizing effect of this reagent in the presence of these heavy metals strengthens the evidence for a sulfhydryl group requirement for activity of E_i isozyme.

Determination of Michaelis Constant

The Michaelis constant which represents the substrate

Table VI

Effect of Sulfhydryl Compounds on Enzyme E_i Activity

Treatment	Per Cent Inhibition
A. 15 minute preincubation 37° C	45
B. 15 minute preincubation 37° C with 2 mg/ml Cleland's reagent added	0
C. Same as A, only 2 mg/ml reduced glutathione added	5
D. Same as A, only 2 mg/ml cysteine added	50
E. 10^{-3} M Cadmium	80
F. 10^{-4} M Mercury	60
G. 10^{-3} M Cadmium and 20 mg Cleland's reagent	20
H. 10^{-4} M Mercury and 20 mg Cleland's reagent	0

concentration at one-half maximum velocity is characteristic for each enzyme when determined under rigidly controlled conditions. Either FMN or p-nitrophenylphosphate was used as substrate. The determinations were done at two enzyme levels and on several different E_i preparations. The average K_m for p-nitrophenylphosphate was 1.4 $x10^{-3}$ M (figure 3). The K_m for FMN was 2.2 $x10^{-3}$ M (figure 4).

Effect of Temperature on Reaction Velocity

The activation energy of E_i was determined by use of the Arrhenius equation:

$$\frac{d \ln k}{dt} = \frac{E}{RT^2}$$

where k is the reaction velocity, T the absolute temperature, R the gas constant, and E is the activation energy. The reactions were carried out over a temperature range from 20°C to 50°C, using p-nitrophenylphosphate as substrate. A plot was made of ln k versus $^1/_T$. The slope of the straight line obtained is $-\ ^E/_R$. The energy of activation for E_i was 12,200 calories (figure 5). This value was the average of three separate determinations.

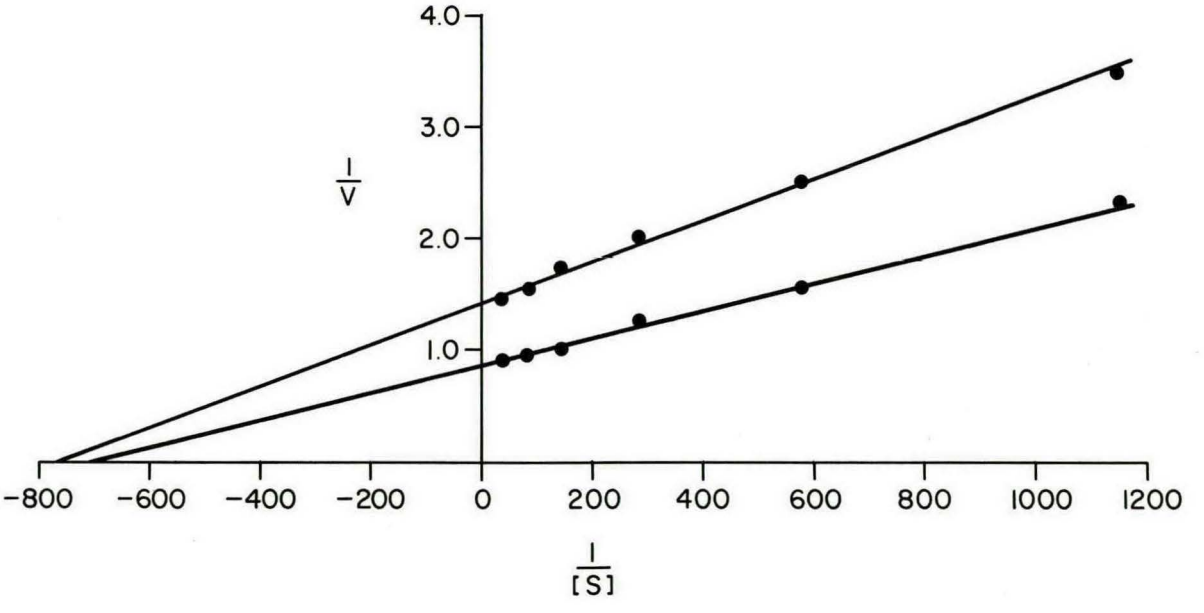
Determination of Substrate Specificity

To further differentiate the human red cell acid phosphatases, and to verify the isozymal nature of these enzymes, the substrate specificity of E_i was determined. All substrates used were at a final concentration of 10^{-2} M. When p-nitrophenylphosphate was used as substrate, enzyme directly from column chromatography was used. The release of p-nitrophenol was measured and related to

Figure 3

Michaelis Constant Determination for Two Levels of Isozyme E_i

With p-Nitrophenylphosphate as Substrate.

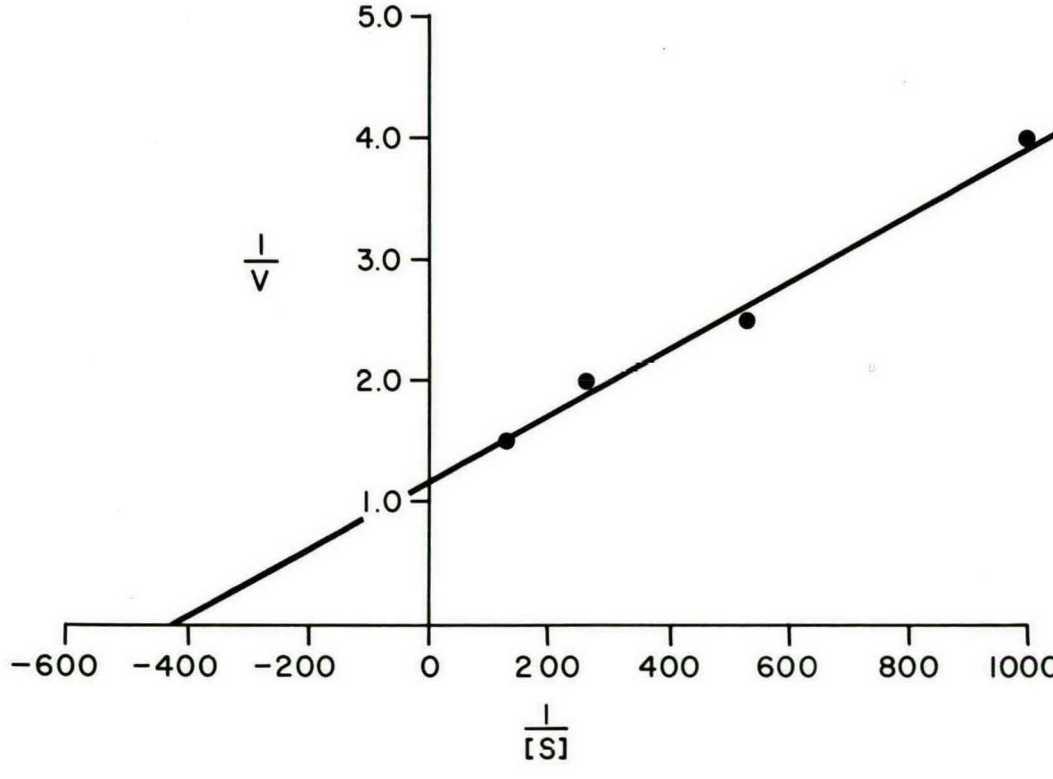


Assays were performed as described under Methods except for the change in substrate concentration.

Figure 4

Michaelis Constant Determination for Isozyme E_i

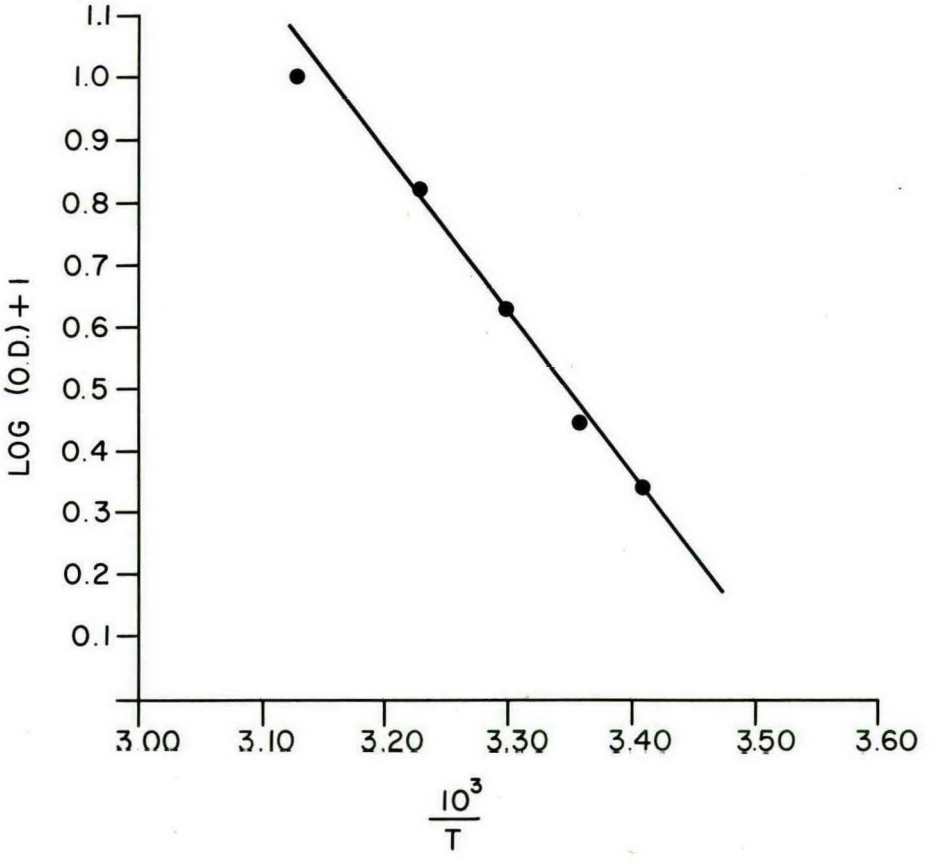
with FMN as Substrate



Assays were performed as described under Methods except for the change in substrate concentration. The release of inorganic phosphate was determined by the method of Fiske and SubbaRow.

Figure 5

Effect of Temperature on the Activity of Enzyme E_i.



the corresponding amount of inorganic phosphate. For all other substrates, the release of inorganic phosphate was measured by the method of Fiske and SubbaRow. Enzyme used with this method was prepared by column chromatography and then concentrated by vacuum ultrafiltration.

Enzyme E_i is rather non-specific in its action on the various substrates employed (Table VII). The enzyme hydrolyzed most rapidly the phosphoric esters of phenols. The enzyme exhibited extremely low activity toward organic and inorganic pyrophosphates. It appears that the enzyme is only slightly active in the hydrolysis of the phosphoric esters of hexoses. The substrate specificity results will be more valuable when a comparison can be made with E_s and E_f.

Table VII

Relative Substrate Specificity of Enzyme E_i

Substrate	Relative Rate of Hydrolysis
p-Nitrophenylphosphate	100
Phenyl phosphate	10
Phenolphthalein diphosphate	10
Flavin mononucleotide	7
alpha-Glycerophosphate	4
Inositol monophosphate	3
Glucosamine-6-phosphate	2
beta-Glycerophosphate	2
Propanediol phosphate	2
D-Galactose-6-phosphate	2
Guanine triphosphate	2
Glucose-6-phosphate	1
Uridine monophosphate	1
Inosine monophosphate	1
Guanine diphosphate	1
alpha-Naphthyl phosphate	1
Glucose-1-phosphate	1
Cytidine monophosphate	0.8
Adenosine diphosphate	0.4

Table VII (Continued)

Relative Substrate Specificity of Enzyme E_i

Substrate	Relative Rate of Hydrolysis
Pyridoxal phosphate	0.2
Pyrophosphate	0.2
Fructose-6-phosphate	0
Adenosine triphosphate	0
Creatine phosphate	0

Discussion

The acid phosphatase activity of human erythrocytes has been resolved into three isozymes. These isozymes differ in pH optimum, substrate specificity, and effects of inhibitors and metal ions. The mild isolation procedure minimizes the possibility of undesirable changes taking place in the structural configuration of the individual isozymes. While a number of other fractionations have been reported[13, 17], overlapping in the activity peaks occurred and the homogeneity of the phosphatase activity was not demonstrated by other methods. The direct relationship between the isozymes isolated by this procedure and the electrophoretic bands obtained from crude hemolysates, confirms the natural occurrence of the isozymes in the erythrocyte and the existence of the phenotype variations reported.[14] The quantity of individual isozymes recovered is in reasonable agreement with the concentration predicted for that phenotype by electrophoretic analysis.

There is considerable variation in the reported characterizations of erythrocyte acid phosphatase preparations.[9, 11, 13, 17] Differences in pH optima, activation, inhibition, substrate specificity, stability, and molecular weight are apparent. The limited studies of the isozymes indicates that conflicting results may be explained on the basis of three individual isozymes. The pH optima reported ranged from 4.4 to 6.0, a span readily covered by the isozyme pH optima of 4.75, 5.5-5.75, and 5.75. Previously reported activation or inhibition by $MgCl_2$, EDTA, formaldehyde,

NaF, oxalate, and L(+)-tartrate are characteristic of at least one of the individual isozymes, or as with formaldehyde, characteristic of all of the isozymes. The characteristics of acid phosphatase preparations could vary, depending on their individual isozyme components. The absence of a given characteristic would correlate with the absence or a decrease in the concentration of the isozyme possessing that characteristic response.

Attempts to correlate the characteristics of previously isolated acid phosphatases from erythrocytes with an individual isozyme were not successful. This supports the hypothesis that previous preparations have not been fully resolved into their isozyme components. The characteristics of the two acid phosphatase isozymes isolated by Georgatsos[17] could not be directly related to any one of the three individual isozymes. This indicates, as he suggests, that his preparations do not represent complete resolution. However, the possibility of the acetone treatment employed causing a structural change of the isozymes can not be disregarded.

The more detailed characterization of E_i is the beginning of a detailed study of the properties of each individual isozyme to correlate conflicting data reported in the literature with regard to these properties of erythrocytic acid phosphatases. Although complete evaluation is impossible until all isozymes are studied in more detail, it is seen that E_i differs distinctly from other reported isozyme preparations. The enzyme isolated by Tsuboi and Hudson[10] was unstable to the effects of metal ions. It was

inhibited by 10^{-5} M cobalt, 10^{-5} M cadmium, 10^{-5} M iron, 10^{-2} M magnesium, and 10^{-5} M manganese. Enzyme E_i is stable to these metal ions at these concentrations, being inhibited only by cadmium and mercury at concentrations of 10^{-2} to 10^{-4} M. It must be pointed out that E_i has been purified only 100 fold, whereas the enzyme of Tsuboi and Hudson was 1500-fold purified.

Georgatsos has isolated an enzyme (E_2) which has a pH optimum of 5.2. This enzyme is inhibited by formalin and oxalate, but is not affected by sodium fluoride. These properties are characteristic of E_i; however, Georgatsos reported his enzyme to be inhibited by magnesium, while E_i is not affected by magnesium at the concentration employed by Georgatsos. The other enzyme obtained by Georgatsos (E_1) has pH optima at pH 5.0 and pH 6.0. This enzyme differs from E_i. Enzyme E_1 is slightly activated by oxalate, and inhibited 50 per cent by fluoride. Enzyme E_i is inhibited by oxalate, but is not affected by fluoride.

The lack of activation of E_i by metal ions suggests that this isozyme does not have a metallo-protein structure as has been suggested for alkaline phosphatases. The inhibition by PCMB and the stabilizing effect of various sulfhydryl compounds as well as the inhibiting effect of heavy metals tend to support the hypothesis of a sulfhydryl group necessary for enzyme activity.

Summary

Three acid phosphatases have been isolated from crude hemolysates of human erythrocytes. Each isozyme yields a single electrophoretic band of phosphatase activity and is electrophoretically distinct from the other two isozymes. The isozymes have been designated E_s, E_i, and E_f according to their electrophoretic mobility. Differences in the isozymes have been observed with respect to pH optima and effects of inhibitors and metal ions. The pH optima are E_s, 4.75, E_i, 5.5-5.75, E_f, 5.75. Enzyme E_s is inhibited by fluoride, formaldehyde, and oxalate. Enzyme E_i is inhibited by formaldehyde. Enzyme E_f is activated by magnesium and inhibited by fluoride, formaldehyde, and oxalate. L(+)-tartrate has no effect on the isozymes.

Enzyme E_i has been studied in some detail. The K_m at 37°C with p-nitrophenylphosphate as substrate is 1.4×10^{-3} M. The K_m for FMN is 2.2×10^{-3} M. The substrate specificity of E_i has been considered.

The energy of activation for E_i has been determined by means of the Arrhenius equation. Eact was found to be 12,200 calories per mole.

Enzyme E_i is quite stable to the effects of metal ions, being inhibited only by the heavy metal ions such as cadmium and mercury. This inhibition can be partially overcome by Cleland's reagent. The inhibition by the heavy metal ions and stabilization by sulfhydryl compounds, as well as complete inhibition by PCMB, indicate the presence of a sulfhydryl group necessary for enzymic activity.

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