HUMAN ERYTHROCYTE

ACID PHOSPHOMONOESTERASES

A Thesis

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

by

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Introduction

Phosphomonoesterases are enzymes which catalyze the liberation of inorganic phosphate from organic phosphate esters. Although these enzymes occur in many plant and animal sources, their comparative biochemistry and specific physiological roles have not been completely investigated.

Phosphatases have been divided into two main classes depending on their optimum pH--alkaline phosphatases which have an optimum pH of 8.0 to 10.0 and acid phosphatases which have an optimum pH of 4.0 to 7.0.

The presence in mammalian erythrocytes of one or more acid phosphatase has been known for several years. It was postulated in 1931 on the basis of pH optima that the enzyme within the erythrocyte was different than the phosphatase of plasma.

In recent years the erythrocyte acid phosphatases have been implicated in some cases of chronic congenital non-spherocytic hemolytic anemia, a general term for congenital and sometimes familial hemolytic

disorders which are not associated with any characteristic alteration of the red cell morphology. Very little is known concerning the role of erythrocyte acid phosphatases in relation to the biochemical defects of this disease.

In this investigation the number of human erythrocyte acid phosphatases and their individual properties have been studied. A partial purification of one of these enzymes has also been accomplished.

Historical Review

In 1911 Hans Euler and Yngve Funke attempted, without success, to prove the presence of phosphatase enzymes in animal organ extracts.[1] They did show, however, in feeding experiments that three-fourths of administered ethyl phosphate was hydrolyzed.

The presence of phosphatases was shown in maple leaves, barley[2], and malt.[3] In the case of malt, at least two kinds of phosphatases were postulated. One of the enzymes solubilized insoluble organic phosphates but lost its activity in five hours; the other catalyzed the formation of organic phosphates and was active for fourteen hours. These phosphatases were more sensitive to base than acid and were classified as secretion enzymes, i.e. they were soluble and worked outside of the cell.

In 1922 phosphatases were demonstrated in animal tissues.[4] Extracts of muscle, liver, kidney, spleen, pancreas, and brain were shown to be active against sodium sucrosephosphate. The enzymes were called saccharophosphatases although they acted on barium

hexosemonophosphate more readily than sucrose phosphate.[5] A series of organ powders were shown to have activity towards fructose-1,6-diphosphate.[6]

Elemer Forrai in 1924 proposed that all phosphatases were not alike since organ powders differed in their extent of hydrolysis and their relative activities were dependent on the substrate involved.[7]

Bamann and Riedel[8] were among the first to note that certain tissues which hydrolyzed glycerophosphate at an alkaline pH also exhibited a second pH optimum in the acid range. The alkaline phosphatase was activated by magnesium while the enzyme at the acid pH was unaffected. The alkaline phosphatase activity of dried liver was inactivated by treatment of the tissue with dilute acetic acid, but acid phosphatase activity remained.[9]

Histochemical studies have indicated that acid phosphatases are attached to a special type of cytoplasmic granule, which differs from either the mitochondria or the microsomes.[10,11] C. deDuve has termed these cellular particulates lysosomes.[12] There have also been reports that some of the enzymes exist in the cell wall.[13]

Erythrocyte Phosphatases

In 1931 Roche compared The phosphatases of the plasma and the erythrocyte. She observed that the optimum pH for the plasma enzyme was 8.8 to 9.1 and that of the erythrocyte was 6.0 to 6.8. On this basis she postulated that these two enzymes were separate entities.[14]

A phosphatase was isolated in 1934 from horse erythrocytes which was most stable at 37C at pH 6.0.[15] This enzyme was shown to be active on both alpha and beta glycerophosphates at pH 6.5. After adsorption on kaolin at pH 6.5 followed by elution with 0.05 N NH_4OH, the optimum pH for the alpha glycerophosphate was 8.0 and that for the beta isomer was 9.0.

Freudenberg studied the concentration of inorganic phosphate and phosphate esters in erythrocytes.[16] He found that erythrocytes contained significantly more inorganic phosphate than serum or plasma. In addition, they contained phosphate in the form of esters. In his investigation of erythrocyte phosphatases, he found the enzyme to be fully active at pH 7.1 but inactive at pH 7.3.

The first report which indicated the presence of two phosphatases in erythrocytes was from Roche and Bullinger.[17] In horse erythrocytes they found one phosphatase which acted faster on alpha than on beta glycerophosphate and was most active at pH 6.2. The

enzyme was not affected by magnesium. A second enzyme, which acted faster on beta than on alpha glycerophosphate, had an optimum pH of 8.5 and was activated by magnesium. It was located in the stroma.

The erythrocytes of dogs were also reported to contain two phosphatases.[18] Roche et. al. reported the presence of two phosphatases in the red cells of the ox and rat[19], one with a pH optimum of 4.6 to 4.8 and another with a pH optimum of 5.0 to 5.5. The first enzyme was unstable and was inhibited by magnesium and manganese while the second was relatively stable and was activated by these metals.

In 1949 Abul-Fadl and King studied the erythrocyte and prostatic acid phosphatases.[20] Their studies of the pH optima using the enzymes found in the erythrocytes of humans, oxen, rabbits, and sheep indicated the presence of two acid phosphatases, one with a pH optimum of 4.3 to 4.8 and the other with a pH optimum of 5.0 to 5.7. The pH optimum for each enzyme was found to vary slightly not only from one species to another but even in different members of the same species. They reported that the enzyme with the pH optimum of 4.3 to 4.8, was very labile and could no longer be detected in enzyme solutions which had been left to stand at room temperature for a few hours. Magnesium inhibited both enzymes, especially in the more acid range. Alpha

glycerophosphate was hydrolyzed much faster than the beta isomer. Phenyl phosphate was acted on even more rapidly. Inhibition occurred in the presence of 0.01 M calcium, 0.01 M chromium, 0.01 M cobalt, 0.01 M manganese, 0.01 M nickel, 0.001 M zinc, 0.002 M copper, and 0.001 M iron. Copper inhibition could be partially overcome by the simultaneous addition of cysteine or glutathione with the copper. However if the copper were allowed to react with the enzyme prior to the addition of cysteine or glutathione, the inhibition could not be reversed. Inhibition was also observed with arsenate, oxalate, tauroglycolate, iodoacetate, and formaldehyde.

Tsuboi and Hudson studied human erythrocyte acid phosphatases.[21-25] In erythrocyte hemolysates they detected only one pH optimum, 5.5 to 6.0, in contrast to the two reported by Abul-Fadl and King. After twenty-four hours of standing at 0°C followed by twenty-four hours of dialysis, no appreciable change in acid phosphatase activity was detected. They concluded that the presence of a labile second enzyme within the red cells was not justified. Alpha glycerophosphate was hydrolyzed more rapidly than the beta isomer. Although 0.01 M magnesium activated the enzyme, 0.1 M magnesium was inhibitory. Studies on the phosphotransferase activity of the crude hemolysate showed considerable transfer of phosphate groups. They concluded that the

hydrolysis of the ester and transfer of the phosphate group were mediated by the same enzyme.

An erythrocyte acid phosphatase has been partially purified by repeated adsorption on calcium phosphate gel and by ammonium sulfate precipitation.[22] Approximately a fifteen hundred fold purification was achieved, and kinetic studies were carried out on the enzyme. An optimum pH of 6.0 was found. No appreciable activation by magnesium was indicated. The enzyme was found to hydrolyze phenyl phosphate, alpha glycerophosphate, propanediol phosphate, and riboflavin-5-phosphate. A K_m of 9×10^{-4} M was obtained for phenyl phosphate and a K_m of 7×10^{-3} M was obtained with alpha glycerophosphate. No inhibition occurred with either fluoride or tartrate. The activation energy from 26°C to 40°C was 8.8 kcal. per mole and from 10°C to 26°C it was 16 kcal. per mole. The enzyme was very sensitive to metals, and dialysis tubing had to be treated with ethylenediaminetetraacetic acid prior to use to prevent inactivation. Silver, copper, mercury, palladium, cadmium, and iron showed inhibition. Complete inactivation with cysteine indicated the presence of a sulfhydryl group at the active site of the enzyme. Mild oxidation with iodoacetate also inactivated the enzyme.

Angeletti and Gayle reported the chromatographic separation of three peaks with acid phosphatase activity

using diethylaminoethyl cellulose column.[26] The first peak of activity had an optimum pH of 4.4, while the second and third peaks had an optimum pH of around 5.5. Peak one showed approximately a forty per cent inhibition with tartrate but peaks two and three showed no inhibition.

A recent report[27] has indicated the presence of three electrophoretically distinct species of acid phosphatases in erythrocytes. Five phosphatase patterns consisting of these phosphatases have been found in human erythrocytes, with a sixth pattern being predicted. It appears that the phosphatase patterns in erythrocytes are transferred genetically in the expected Mendelian ratios.

Phosphatases and Metabolism

Specific phosphomonoesterases are believed to play a significant role in intermediary metabolism.[28,29] In the liver glucose-6-phosphatase liberates glucose which is then passed into the blood. Phosphatidic acid phosphatase is involved in the pathway in which diglycerides are formed from glycerophosphates. The hydrolysis of adenosine triphosphate is known to play an important role in many biological processes involved in energy transfer, including muscle contraction and oxidative phosphorylation.

The role of the non-specific acid phosphatases is

not so apparent. Macrophages and giant cells associated with lytic phenomena have long been known to exhibit relatively high acid phosphatase activity which could suggest that there is some relationship between the enzyme and protein synthesis.[30] Barka has concluded that the acid phosphatase of various cells is related to the development and actual activity of the pinocytotic and reverse pinocytotic apparatus, including secretion vacuoles. Variations of acid phosphatase in intestinal epithelium in relation to fat absorption were also noted.[31]

Phosphatases and Disease

The first suggestion that phosphatases were involved in pathological conditions was made in 1930, when Roberts suggested a definite correlation between bone disease and abnormal phosphatase distribution in the body.[32] Later it was definitely shown that the phosphatase activity of the blood is of special clinical significance in diseases of the bone.[33] It is now known that the level of alkaline phosphatase of serum is related to bone disease. An increase in serum acid phosphatase is often the first indication of prostatic cancer. Erythrocyte acid phosphatases have been implicated in some cases of chromic non-spherocytic hemolytic anemia.[34] Caucasian patients with glucose-6-phosphate-dehydrogenase deficiency also have a deficiency in the

acid phosphatase. This phosphatase deficiency does not appear to be present in the glucose-6-phosphate-dehydrogenase deficient negroes.

Experimental

Methods and Materials:

Enzyme Source

Outdated human blood was obtained from The Ohio State University Hospital Blood Bank. The blood was centrifuged at 10,000 G for 10 minutes, and the plasma and leucocytes were removed by decantation. The remaining erythrocytes were washed three times with one per cent sodium chloride solutions. The washed erythrocytes were lysed in four volumes of distilled water. To insure complete lysis, the freeze-thaw technique was applied to the hemolysate. The stroma was removed by centrifugation at 12,500 G for 20 minutes, and the hemolysate was dialyzed overnight against three changes of 0.005 M Tris-phosphate buffer, pH 8.0. This solution shall be referred to as the crude hemolysate.

Enzyme Assays

All assays were performed using a Beckman Model B spectrophotometer. When para-nitrophenylphosphate was used as substrate, a modification of the method of

Bessey, Lowry, and Brock was employed.[35] One milliliter of 0.015 M substrate, one milliliter of 0.1 M buffer (acetate and citrate buffers, pH 5.75 were used), and one milliliter of enzyme solution were incubated for 10 to 30 minutes, depending on the concentration of the enzyme. The readings were made at 415 mu. One unit of enzyme was defined as the amount of enzyme which liberated one micromole of para-nitrophenol in one minute at 37°C. Figure 1 represents a standard curve for para-nitrophenol. With other substrates the standard Fiske-Subbarow phosphate analysis[36] was used after one milliliter of 0.02 M substrate,one milliliter of the enzyme, and one milliliter of 0.1 M acetate or citrate buffer, pH 5.75, were incubated for one hour. A one milliliter aliquot was removed and immediately added to one milliliter of ten per cent trichloroacetic acid. Figure 2 represents a standard curve for inorganic phosphate with the Fiske-Subbarow analysis.

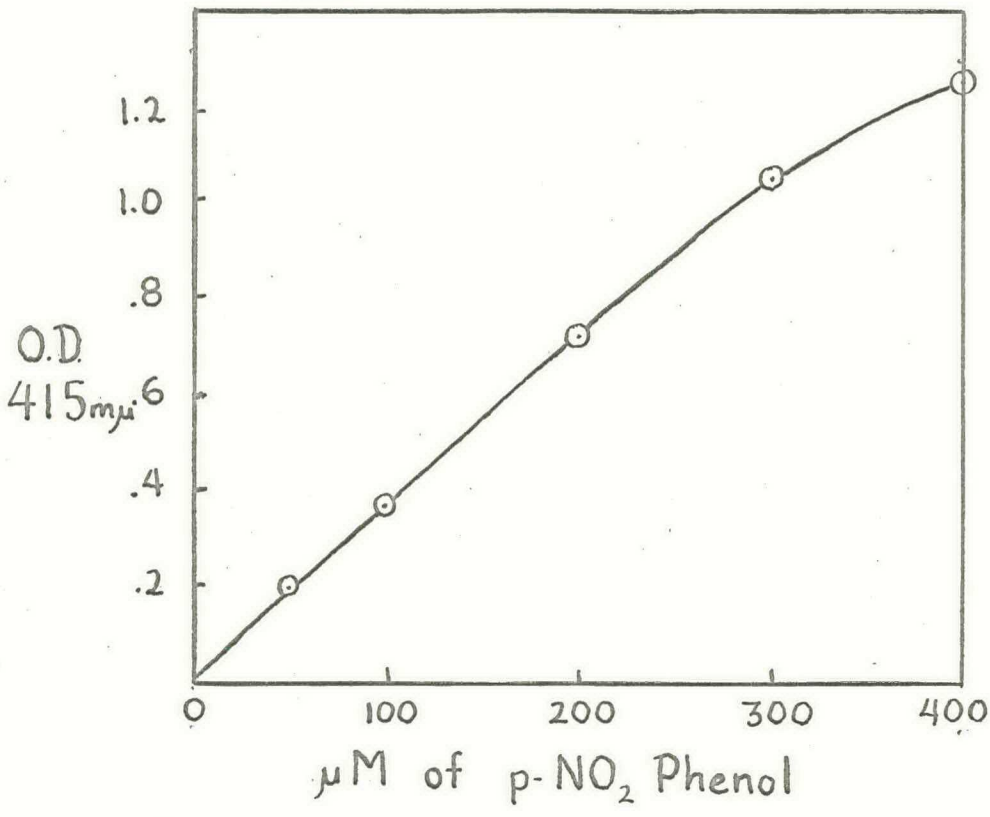
Protein Determinations

Protein was estimated by Kjeldahl nitrogen determinations or by measuring the absorption at 260 and 280 mu.[37]

Preparation of the calcium phosphate gel

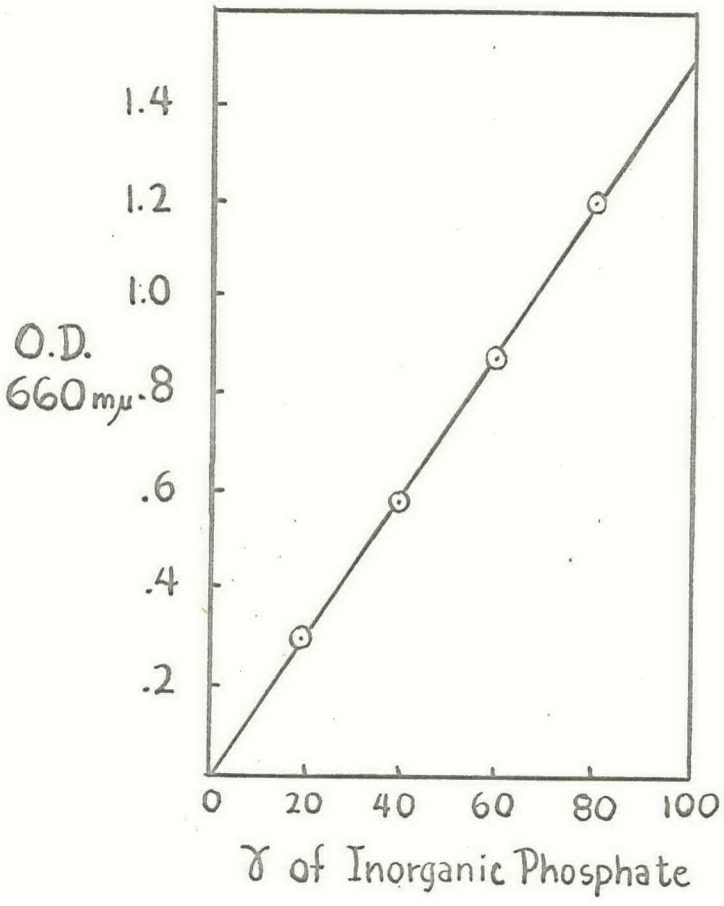
The calcium phosphate gel used in the purification procedure was prepared according to the procedure of Tsuboi and Hudson.[22]

FIGURE 1



Standard Curve for p-NO_2 Phenol

FIGURE 2



Standard Curve for Fiske-Subbarow

Diethylaminoethyl cellulose

Diethylaminoethyl cellulose (DEAE) was obtained from Eastman Chemical Co. It was prepared for use by consequetive washing with 0.5 N NaOH, water, ethyl alcohol, 0.5 N NaOH and water. After protein fractionation the DEAE could be regenerated by treating in the same manner. The DEAE was resuspended in distilled water for storage.

Column Chromatography

The columns were packed by gravity using the DEAE prepared as above. The size of the column depended on the amount of blood being used. A column 5 cm in diameter by 45 cm long was used for the lysed erythrocytes from three pints of blood. The columns were equilibrated overnight with 0.005 M sodium phosphate buffer, pH 8.0, and the dialyzed hemolysate was applied slowly, being sure not to disturb the top layer of DEAE. The hemoglobin was then washed through the column with 0.005 M phosphate buffer, and a linear gradient elution from 0 to 1 M NaCl was carried out as follows:
Two 1000 ml flasks were placed at equal heights above the column in order to have equal hydrostatic pressures. The one connected directly to the column was the mixing flask and contained 1000 ml of 0.005 M phosphate buffer at the beginning of the gradient. The second flask, which siphoned into the mixing flask, contained 1000 ml

1 M NaCl in 0.005 M buffer and was open to the air. The level of liquid in the two flasks dropped at equal rates. Fifteen milliliter fractions were collected using a Gilson Medical Electronics V15[2] fraction collector.

Preparation of Para-nitrophenylphosphate (Na salt)

Para-nitrophenylphosphate, which was used as a substrate, was prepared according to the method of Bessey and Love.[38]

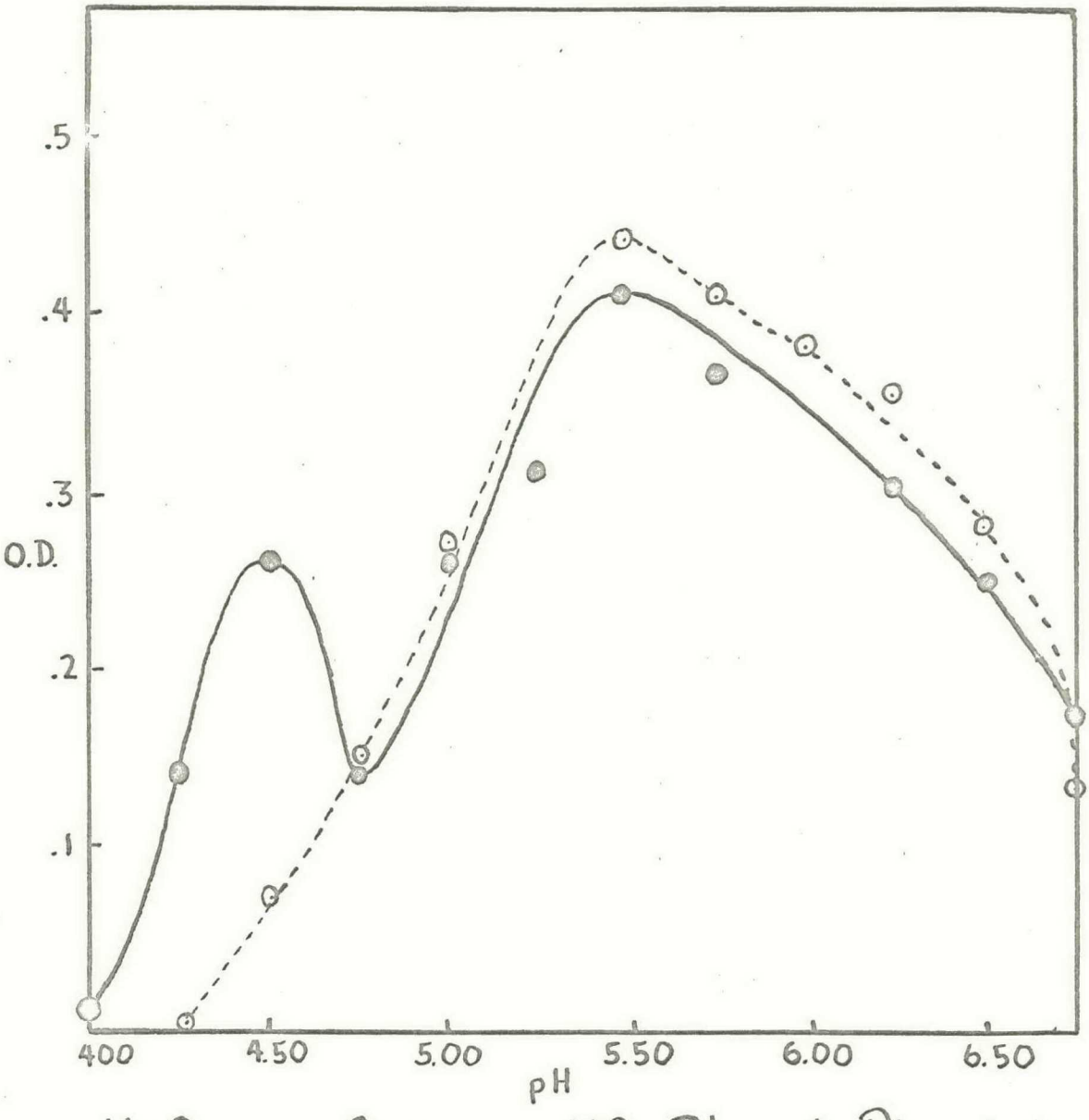
Results:

pH and Acid Phosphatase Activity

Contradictory results in the literature indicated the need for an investigation of the pH optima of erythrocyte acid phosphatases. Observations of the effect of pH on phosphatase activity were made in 0.1 M citrate buffer over a pH range of 4.0 to 6.75. Determinations were made employing freshly lysed blood and lysed blood which had been allowed to stand at room temperature for three hours (incubated blood) , Two pH optima, pH 4.5 to 4.74 and 5.5 to 5.75, were observed with freshly lysed erythrocyte hemolysates with either para-nitrophenyl-phosphate (Figure 3) or flavin mononucleotide (FMN) (Figure 4) as substrate, but only one pH optimum, pH 4.5 to 4.75, was observed with the alpha-beta glycerophosphate mixture (Figure 5). With incubated blood only one pH optimum, pH 5.5 to 5.75, was observed with either para-nitrophenylphosphate or FMN. No activity was detected with the glycerophosphate mixture.

The presence of two pH optima does not necessarily

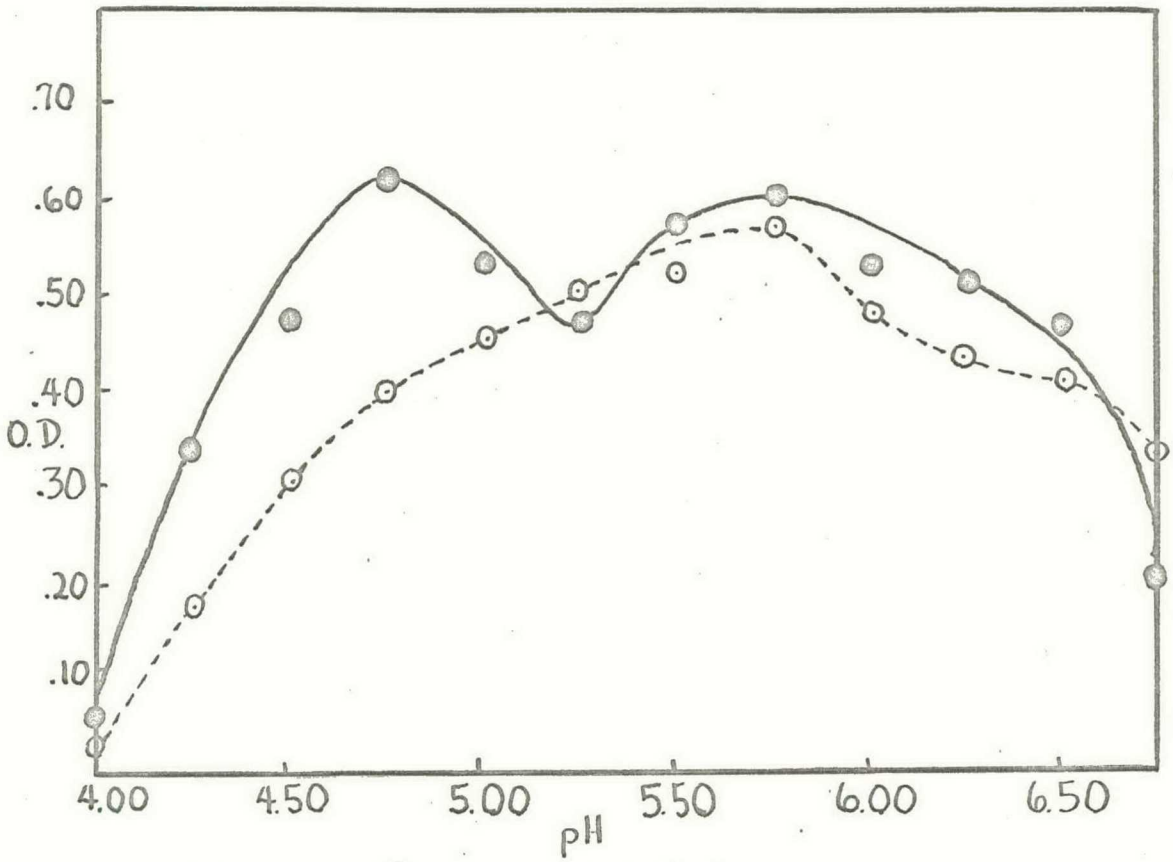
FIGURE 3



pH Optima Curve : p-NO_2 Phenyl Phosphate

Phosphatase activity against para-nitrophenylphosphate at 37° C in 0.1 M citrate buffer was measured spectrophotometrically at 415 mu. Determinations were made using freshly prepared hemolysate and hemolysate which had been allowed to stand three hours at room temperature (incubated crude hemolysate). ●——● Fresh crude hemolysate; ⊙------⊙ Incubated crude hemolysate

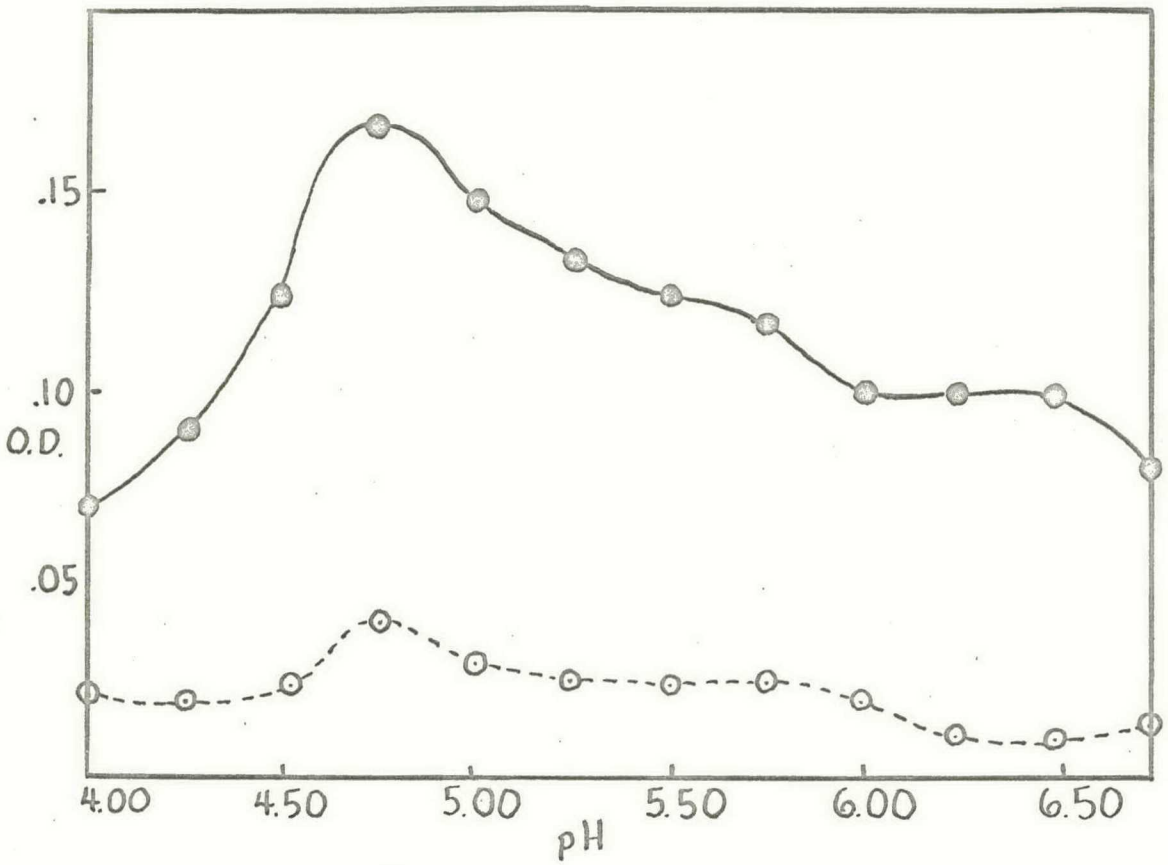
FIGURE 4



pH Optima Curves: FMN

Phosphatase activity against flavin mononucleotide at 37°C in 0.1 M citrate buffer was measured spectrophotometrically at 660 mu. Determinations were made using freshly prepared hemolysate and hemolysate which had been allowed to stand three hours at room temperature (incubated crude hemolysate). ⊙——⊙ Fresh crude hemolysate; ⊙- - - - -⊙ Incubated crude hemolysate

FIGURE 5



pH Optima Curves: α and β Glycerophosphate

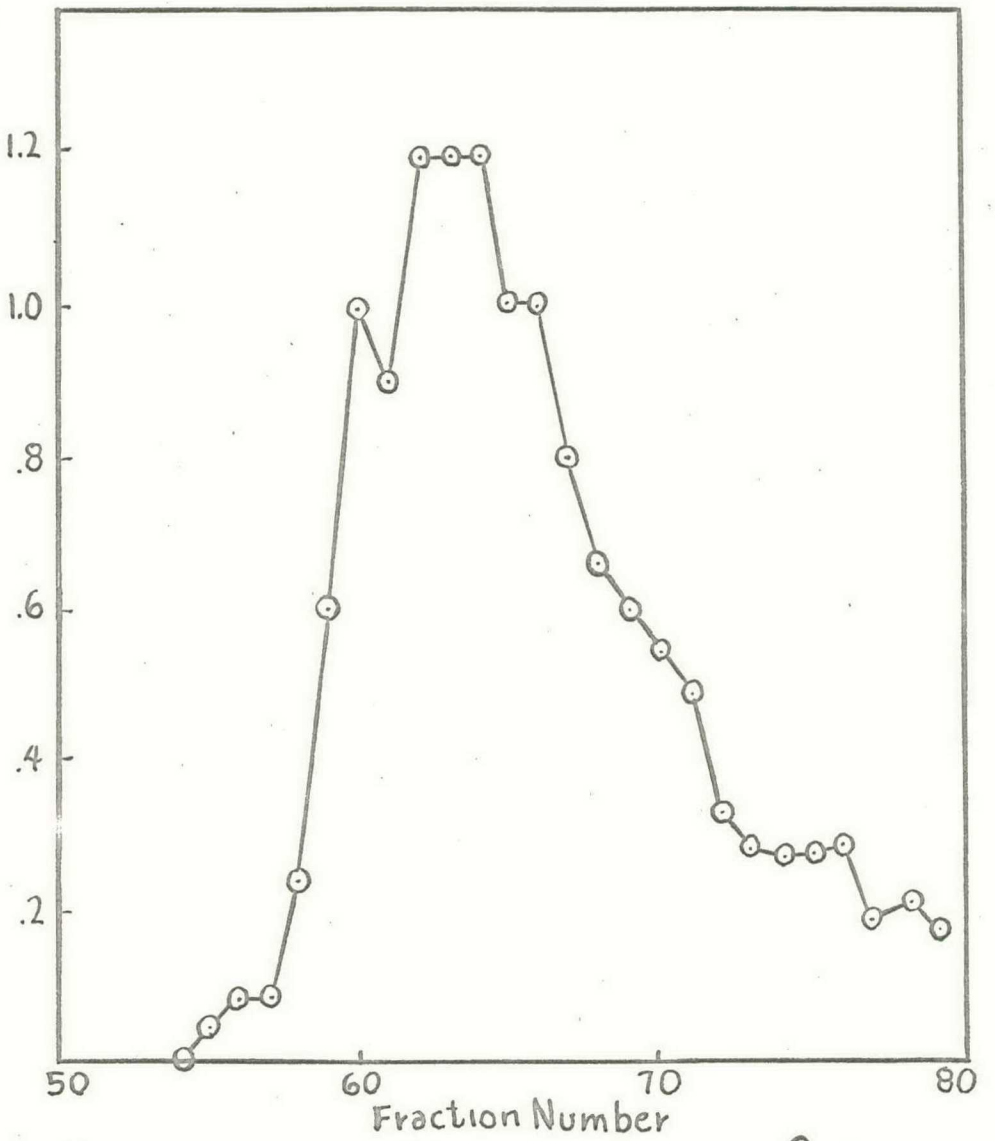
Phosphatase activity against α and β glycerophosphate at 37°C in 0.1 M citrate buffer was measured spectrophotometrically at 660 mu. Determinations were made using freshly prepared hemolysate and hemolysate which had been allowed to stand three hours at room temperature (incubated crude hemolysate). ●——● Fresh crude hemolysate; ⊙------⊙ Incubated crude hemolysate

indicate the presence of two distinct enzymes. Two forms of the same enzyme could also give this effect. Additional evidence for the presence of two enzyme species is derived from the observation that as the activity at pH 4.5 to 4.75 was lost there was no corresponding increase in the activity at pH 5.5 to 5.75 as would be expected if this were two forms of the same enzyme in equilibrium. In addition, the activity toward the glycerophosphate was completely lost in the incubated blood although the activity toward para-nitrophenylphosphate and FMN remained. The presence of at least two acid phosphatases in human erythrocytes appears certain.

Partial Purification of an Erythrocyte Acid Phosphatase

Dialyzed hemolysate (prepared as given in methods) was applied to a DEAE column. The hemoglobin, which had no affinity for the DEAE at the pH employed, was washed through the column with 0.005 M Tris-phosphate buffer and was discarded. The column was placed on the fraction collector, and a linear salt gradient from 0 to 1 M NaCl was used to fractionate the proteins. Phosphatase activity was measured in each 15 ml fraction (Figure 6), with para-nitrophenylphosphate as substrate. Those fractions which contained activity were pooled. Enough $(NH_4)_2SO_4$ was added to the pooled fractions so that the

FIGURE 6



Phosphatase Elution from Column

Phosphatase activity in each 15 ml. fraction was measured spectrophotometrically with para-nitrophenylphosphate as the substrate. Determinations were made in 0.1 M citrate buffer, pH 5.75, at 37° C. Activity was found in fractions 54-80.

solution was 70% saturated. The precipitate was collected by centrifuging at 10,000 G for 10 minutes and was redissolved in 100 ml of 5% $(NH_4)_2SO_4$. Overnight dialysis against 0.001 M EDTA yielded a precipitate which was discarded. The enzyme solution was retreated with $(NH_4)_2SO_4$, and the fraction between 25% and 55% saturation was collected, resuspended in 25 ml of 5% $(NH_4)_2SO_4$, and dialyzed as before. Calcium phosphate gel was added in two ml aliquots. After each addition the suspension was centrifuged and the phosphatase activity of the supernate was checked. The fractions of the gel which contained the bulk of the phosphatase activity were combined and washed with distilled water. The enzyme was eluted with a mixture of 0.15 M acetate and 0.015 M citrate buffers, pH 4.5. The eluate was treated with $(NH_4)_2SO_4$, and the fraction between 40% and 55% saturation was collected by centrifugation at 10,000 G for 10 minutes, resuspended in 25 ml of 5% $(NH_4)_2SO_4$ and dialyzed as before.

The enzyme was purified 2700 fold (Table 1) and represented a specific activity of 2400. This preparation shall be referred to as the 2700 fold purified enzyme. An earlier purification[22] was reported in which a 1500 fold purification was achieved.

This preparation was stable for at least three months when refrigerated at -15C.

Table I

Summary of Erythrocyte Acid Phosphatase Purification

Stage of Purification	Vol. ml	Units per ml	Total Activity	Protein mg/ml	S.A.	Fold Purification
Lysed cells	960	120.0	115,200	123.00	0.894	1
After DEAE	1300	75.4	98,020	1.80	41.900	47
First $(NH_4)_2SO_4$ ppt.	107	890.0	95,230	15.40	57.200	64
Dialysis	131	720.0	94,330	11.60	62.000	70
Second $(NH_4)_2SO_4$ ppt.	25	3480.0	87,000	14.00	248.000	278
Dialysis	36	2374.0	84,444	7.80	304.400	340
Calcium Phosphate gel	90	795.0	71,550	0.63	1262.000	1412
Third $(NH_4)_2SO_4$ ppt.	25	2142.0	53,550	0.94	2279.000	2550
Dialysis	31	1675.0	51,925	0.69	2428.000	2716

pH Optimum of the Partially Purified Enzyme

A study of the pH optimum at 37C in o.1 M citrate buffer of the 2700 fold purified enzyme showed an optimum at pH 5.75 (Figure 7). This corresponded to the pH optimum of the crude hemolysate after incubation at room temperature for three hours. Para-nitrophenyl-phosphate was the substrate.

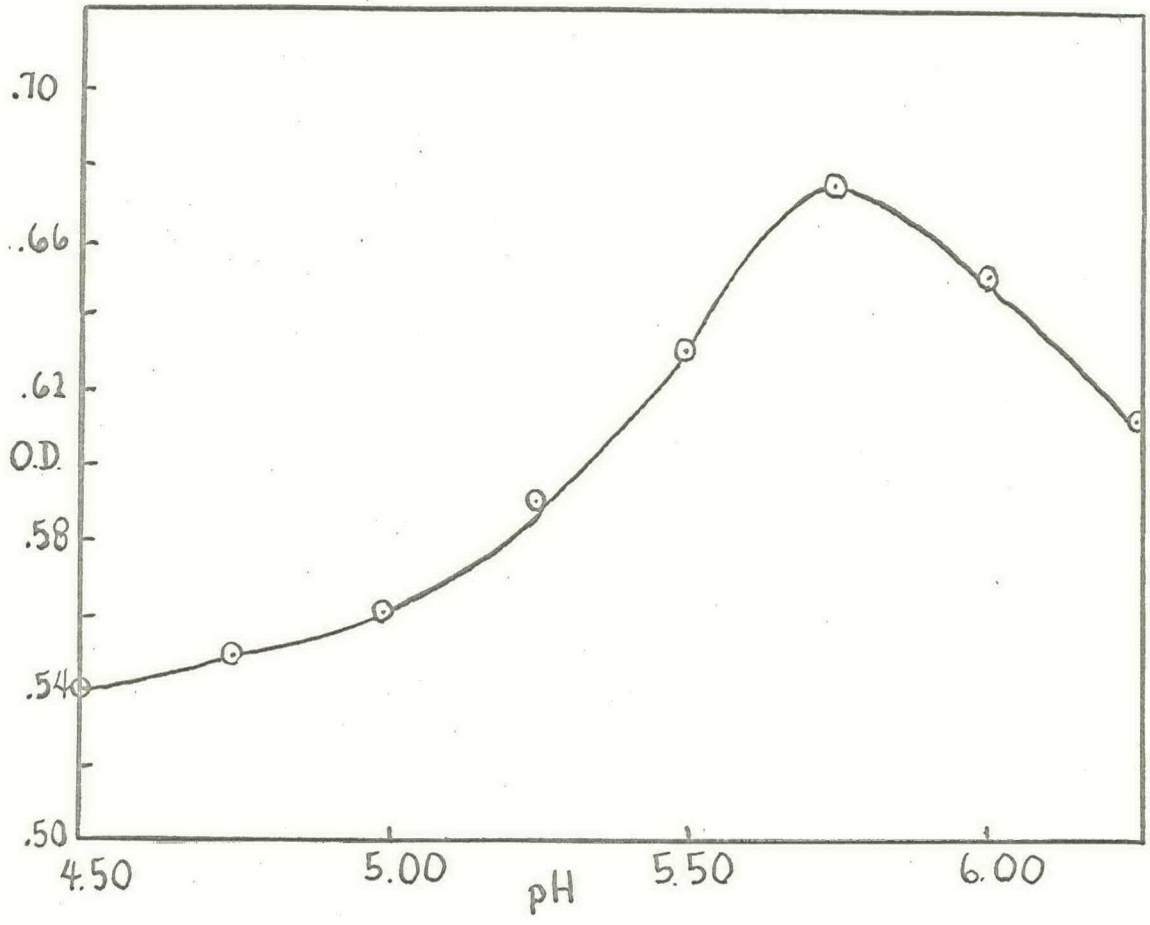
Determination of the Michaelis Constant

The Michaelis constant, K_m, which has a characteristic value for each enzyme under rigidly specified conditions, was determined by plotting the reciprocal of the velocity of the reaction versus the reciprocal of the substrate concentration. The X-intercept has the value $-1/K_m$. The K_m represents the substrate concentration at half-maximum velocity.

The Michaelis constant was determined at 37° with para-nitrophenylphosphate (Table II, Figure 8) and phenylphosphate (Table III, Figure 9). The determinations were performed in 0.1 M acetate buffer at pH 5.75 or pH 5.5. The K_m was determined at two different enzyme levels.

At pH 5.5 the enzyme isolated in this study had a K_m of 7.7×10^{-4}M with phenylphosphate as the substrate. At pH 5.75 the K_m with phenylphosphate was 2.7×10^{-3}M. Tsuboi and Hudson[22] have isolated phosphatase from human erythrocytes for which they reported a K_m of 9×10^{-4}M at

FIGURE 7



pH Optimum Curve for 2700 Fold Purified Enzyme

Phosphatase activity against para-nitrophenylphosphate at 37°C in 0.1 M citrate buffer was measured spectrophotometrically at 415 mu. The optimum pH was 5.75.

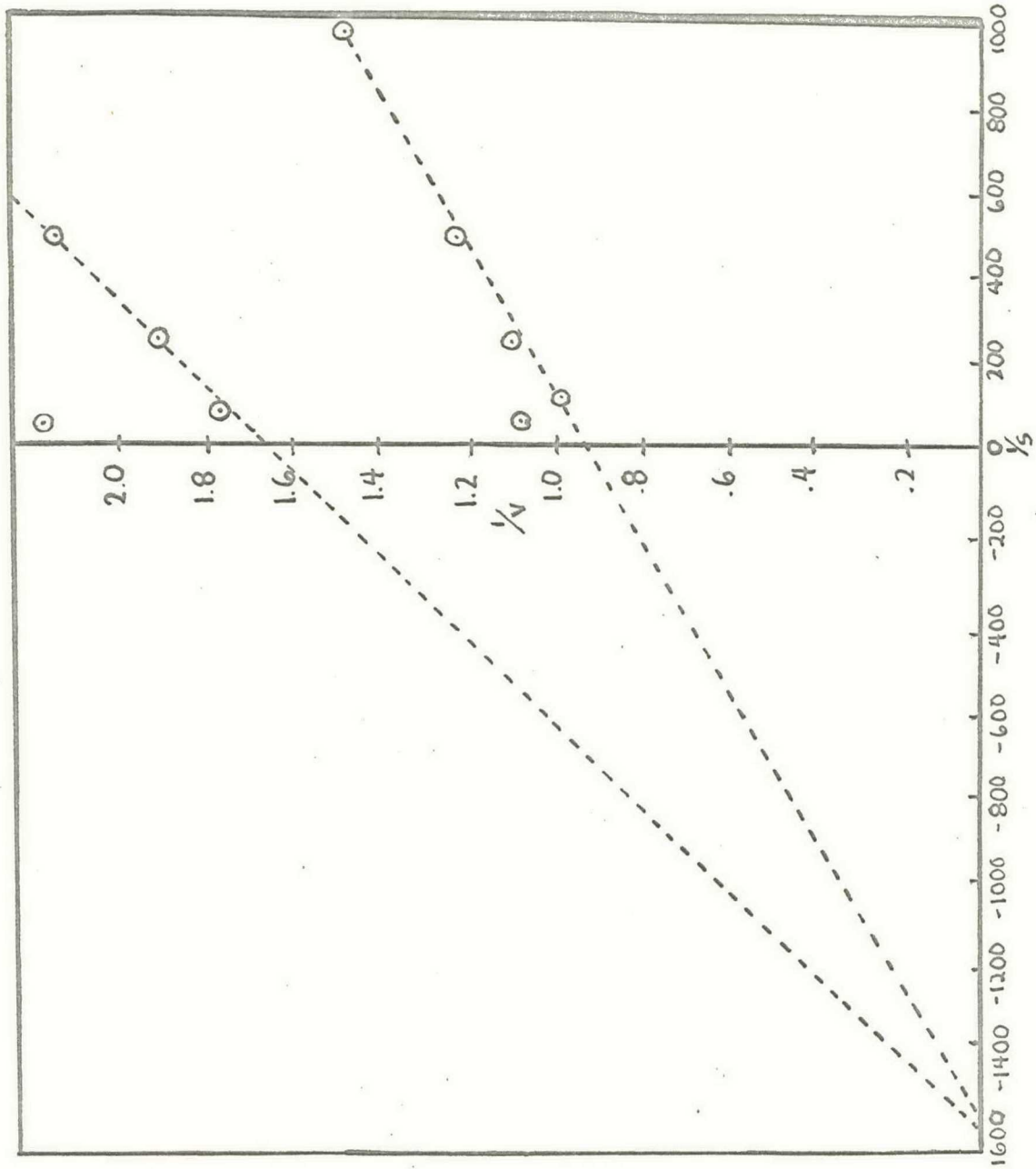
Table II

Effect of Substrate Concentration on Reaction Velocity

S (M)	1/S	O.D.(415 mu)	1/O.D.
0.03330	30	0.660	1.52
0.01670	60	0.940	1.06
0.00833	120	1.020	0.98
0.00416	240	0.920	1.09
0.00208	480	0.825	1.21
0.00156	960	0.680	1.47
0.00078	1920	0.450	2.06
0.03330	30	0.370	2.70
0.01670	60	0.460	2.17
0.00833	120	0.480	2.09
0.00416	240	0.520	1.90
0.00208	480	0.470	2.13
0.00156	960	0.380	2.63
0.00078	1920	0.275	3.64

The effect of substrate concentration on the reaction velocity was studied at 37°C and pH 5.75. The substrate was para-nitrophenylphosphate. The optical density at 415 mu was a measure of the velocity.

FIGURE 8



K_m with p-NO_2 Phenyl Phosphate

The Michaelis constant, K_m, was determined with para-nitrophenylphosphate as the substrate at 37°C and pH 5.75. The optical density at 415 mu was a measure of the velocity. The reciprocal of the relative velocity was plotted versus the reciprocal of the substrate concentration, and the X-intercept represents $-1/K_m$. $K_m = 6.4 \times 10^{-4}$

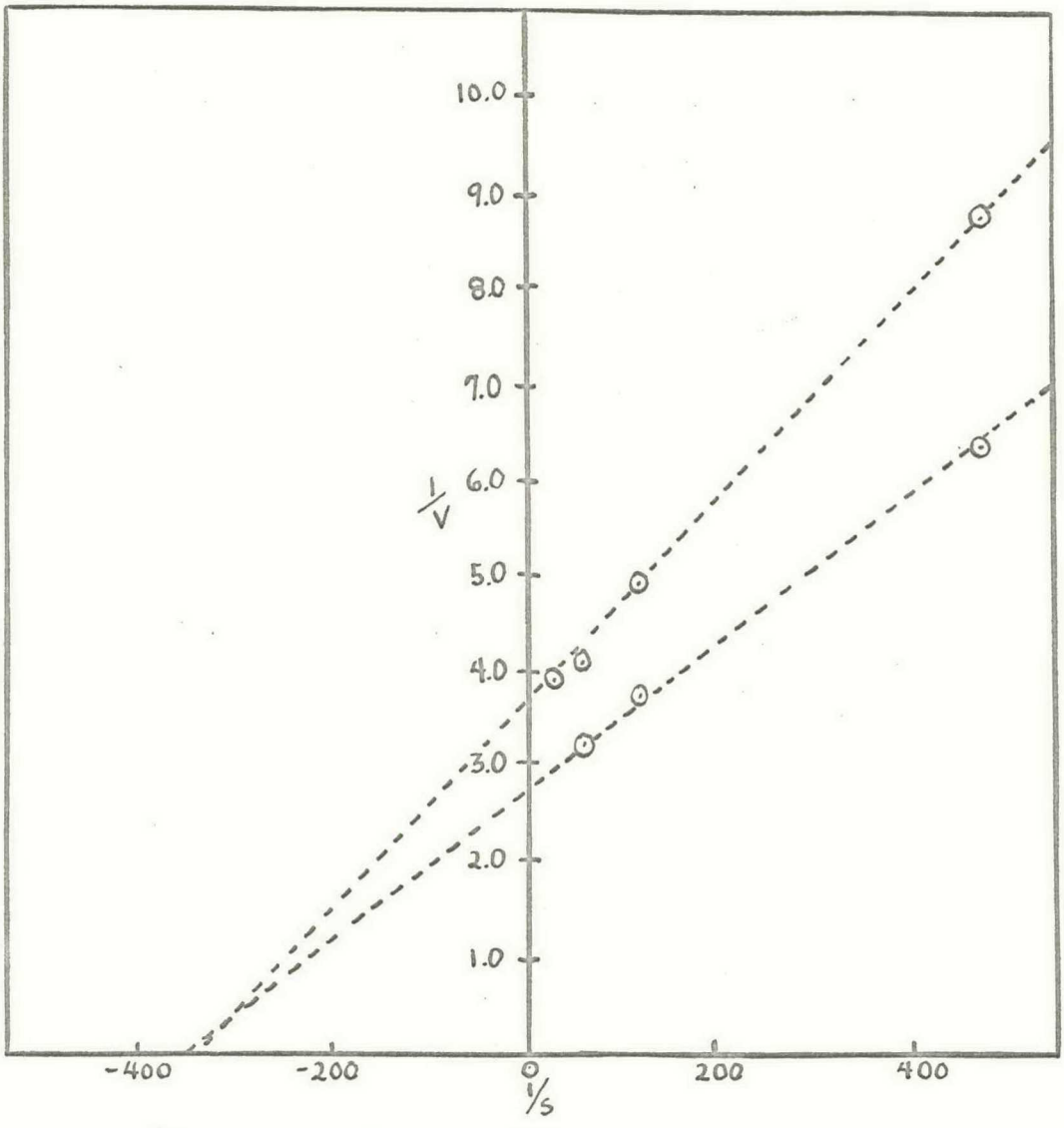
Table III

Effect of Substrate Concentration on Reaction Velocity

S (M)	1/S	O.D. (660 mu)	1/O.D.
0.03330	30	0.260	3.85
0.01670	60	0.250	4.00
0.00830	120	0.205	4.88
0.00416	240	0.240	4.17
0.00208	480	0.115	8.70
0.00104	960	0.070	14.30
0.03330	30	0.280	3.58
0.01670	60	0.320	3.13
0.00830	120	0.270	3.70
0.00416	240	0.170	5.89
0.00208	480	0.160	6.25
0.00104	960	0.100	10.00

The effect of substrate concentration on the reaction velocity was measured at 37° and pH 5.75. The substrate was phenylphosphate. The optical density at 660 mu was a measure of the velocity.

FIGURE 9



K_m with Phenyl Phosphate

The Michaelis constant, K_m, for the enzyme with phenyl-phosphate was determined at 37° C and pH 5.75. The optical density at 660 mu was a measure of the velocity. The reciprocal of the relative velocity was plotted versus the reciprocal of the substrate concentration, and the X-intercept represents $-1/K_m$. $K_m = 2.7 \times 10^{-3}$

pH 5.5. The procedure of Tsuboi and Hudson was employed and the enzyme isolated had a K_m of 2.9×10^{-3} at pH 5.75 with phenylphosphate.

Effect of Temperature on the Reaction Velocity

An increase in temperature leads to a corresponding increase in reaction velocity for chemical and enzymatic reactions. Special caution must be taken with enzymes because an excessive increase in temperature will also lead to heat inactivation.

Determinations were made in 0.1 M citrate buffer at pH 5.75 over a temperature range of 25°C to 45°C. Para-nitrophenylphosphate was the substrate.

An activation energy of 11.9 kcal/mole was observed over the entire range of temperatures employed (Table IV, Figure 10). The acid phosphatase of Tsuboi and Hudson showed an activation energy of 8.8 kcal/mole for alpha glycerophosphate over the same temperature range (0.1 M acetate buffer, pH 6.0).

Inhibition

The 2700 fold purified enzyme was studied with respect to inhibition by various reported inhibitors of phosphatase enzymes. The concentration of the inhibitors was 0.01 M, the only exception being the p-chloromercuribenzoate (PCMB). In this case a saturated solution was used since PCMB is partially insoluble at a concentration of 0.01 M.

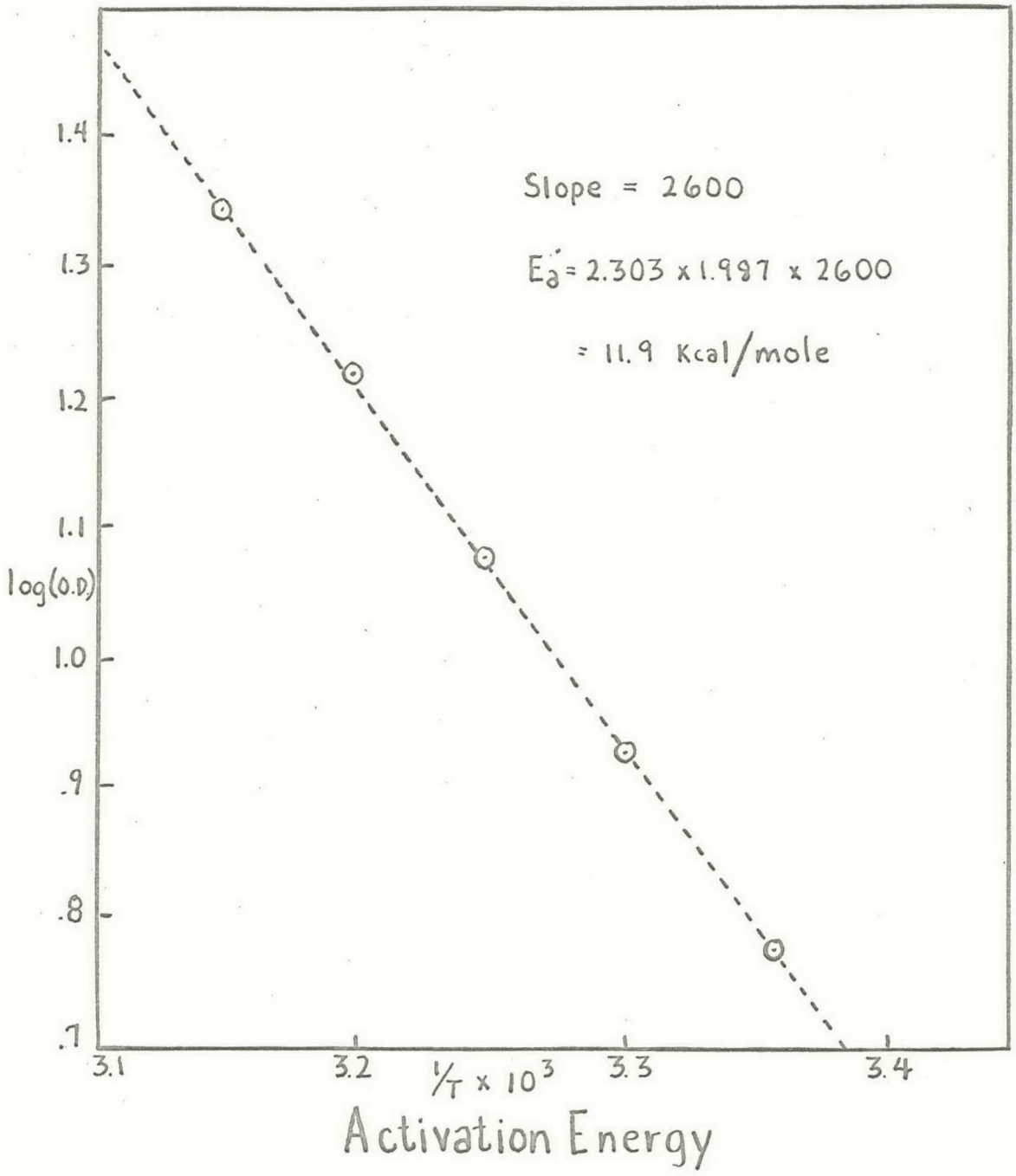
Table IV

Effect of Temperature on the Reaction Velocity

Temperature (°C)	O.D.	(log O.D.)+ 1	(1/T) x 10^3
25	0.60	0.7782	3.352
30	0.85	0.9294	3.300
35	1.20	1.0792	3.247
40	1.65	1.2175	3.195
45	2.20	1.3424	3.145

The effect of temperature on the reaction velocity was measured at pH 5.75 with 0.015 M para-nitrophenyl-phosphate as substrate.

FIGURE 10



The activation energy, E_a, was determined at pH 5.75 with para-nitrophenylphosphate. The optical density at 415 mu was a measure of the velocity. The log of the relative velocity was plotted versus the reciprocal of the absolute temperature. E_a = 11.9 kcal/mole.

Complete inhibition was obtained with PCMB and cyanide (Table V). Tartrate, formaldehyde, and arsenate produced only partial inhibition. Fluoride had no effect. The inactivation caused by PCMB was eliminated by the simultaneous addition of beta mercaptoethanol. This would suggest the presence of a sulfhydryl group at the active site of the enzyme.

Tsuboi and Hudson[21] have reported no inhibition with tartrate or formaldehyde, but complete inactivation was observed by PCMB.

Effect of Metal Cations

The effect of various metal cations on the activity of the enzyme was studied at five different cation concentrations. The studies were made using both citrate (Table VI) and acetate buffers (Table VII). Citrate buffer had a chelating effect which was not exhibited by the acetate buffer.

All of the 0.1 M cation solutions inhibited the enzyme to some extent which was probably due to the effect of high ion concentration. Outside of this initial inhibition, no effect was apparent using sodium, nickel, calcium or manganese. Copper and zinc showed marked inhibition. Cobalt, magnesium and potassium inhibited at higher concentrations but have a slight activating effect at lower concentrations.

Tsuboi and Hudson[25] have reported slight activation

Table V

Inhibition

Inhibitor	% Inhibition
Tartrate	29
Formaldehyde	48
Arsenate	83
PCMB	100
Cyanide	100
Fluoride	0

The effect of inhibitors on the activity of the substrate was measured at 37°C and pH 5.75 with 0.015 M para-nitrophenylphosphate as substrate. The concentration of the inhibitor was 0.01 M, the only exception being PCMB with which a saturated solution was used.

with 0.001 M nickel, 0.001 M manganese, and 0.001 M cobalt. They obtained marked inhibition with 0.0001 M copper. Abul-Fadl and King[20] have reported almost complete inhibition with 0.0002 M copper.

Substrate Specificity

Enzymes exhibit different degrees of substrate specificity. Some act only on one specific substrate, i.e. glucokinase. Others such as hexokinase act on a number of different substrates.[37]

The substrate specificities of the 2700 fold purified enzyme and the crude hemolysate were determined (Table IX). One ml of the 0.1 M phosphate compound was incubated with one ml of enzyme for 30 minutes in 0.1 M citrate buffer at pH 5.75. After termination of the reaction by TCA, the free phosphate was determined by the Fiske-Subbarow method.

Several substrates, 3-phosphoglyceric acid,carboxyl-1-phosphate, pyridoxine phosphate, flavin mononucleotide, inositol monophosphate, para-nitrophenylphosphate, and phenylphosphate were acted on by the crude hemolysate. Only para-nitrophenylphosphate and phenylphosphate were acted on by the partially purified enzyme.

Substrate specificity studies have indicated that there are at least two phosphatases in human erythrocytes which have an optimum pH of 5.5 to 5.75. One of them is highly specific and acts only on para-nitrophenyl-phosphate and phenylphosphate. If only one other

Table VI

Metal Cation Effects in Citrate Buffer

Metal	Concentration of the cation(M)				
	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}
Mg	-28%	0%	0%	0%	0%
Ca	-48%	- 8%	0%	0%	0%
K	-40%	-11%	0%	0%	0%
Na	-12%	0%	0%	0%	0%
Co	-70%	0%	+ 8%	0%	0%
Mn	-21%	-11%	- 9%	-7%	0%
Cu	-95%	-65%	-34%	0%	0%
Zn	-98%	-17%	- 8%	0%	0%
Ni	-92%	0%	0%	0%	0%

The effect of metal cations was measured at 37°C and pH 5.75 with 0.015 M para-nitrophenylphosphate as substrate. The results are expressed in per cent inhibition or activation.

Table VII

Metal Cation Effects in Acetate Buffer

Metal	Concentration of the cation (M)				
	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}
Mg	-25%	+ 4%	+ 9%	+ 9%	+ 9%
Ca	-74%	-18%	0%	0%	0%
K	-44%	- 9%	+11%	+10%	+ 6%
Na	-11%	0%	0%	0%	0%
Co	-94%	-41%	+ 7%	+ 9%	+16%
Mn	-86%	0%	0%	0%	0%
Cu	-99%	-97%	-97%	-16%	- 6%
Zn	-98%	-92%	-73%	0%	0%
Ni	-98%	-75%	0%	0%	0%

The effect of metal cations was measured at 37°C and pH 5.75 with 0.015 M para-nitrophenylphosphate as substrate. The results are expressed in per cent inhibition or activation.

Table IX

Substrate Specificity of the Enzyme

Substrate	O.D. Crude	O.D. Purified
Triose phosphate esters	0.080	0.00
3-phosphoglyceric acid	0.300	0.00
Carboxyl-1-phosphate	0.250	0.00
Cytidine-5-triphosphate	0.095	0.00
Propanediol phosphate	0.160	0.00
Pyridoxal phosphate	0.145	0.00
Glucose-1-phosphate	0.050	0.00
Adenosine monophosphate	0.125	0.00
Adenosine triphosphate	0.100	0.00
1-phosphoserine	0.100	0.00
Uridine diphosphate	0.110	0.00
Guanine diphosphate	0.080	0.00
Inositol monophosphate	0.185	0.00
Thiamine pyrophosphate	0.085	0.00
Glycolaldehyde phosphate	0.120	0.00
Glucose-6-phosphate	0.120	0.00
Fructose-6-phosphate	0.160	0.00
UDP-glucose	0.095	0.00
Uridine triphosphate	0.080	0.00
Cytidine monophosphate	0.120	0.00
2-deoxy glucose-6-phosphate	0.120	0.00
Pyridoxine phosphate	0.500	0.00
Phosphoryl choline	0.075	0.00
Cytidine diphosphate	0.090	0.00
Creatine phosphate	0.200	0.00
Adenosine diphosphate	0.090	0.00
Uridine monophosphate	0.105	0.00
Flavin mononucleotide	0.590	0.00
Alpha-beta glycerophosphates	0.105	0.00
Beta glycerophosphate	0.110	0.00
Phenyl phosphate	0.850	1.20
Para-nitrophenylphosphate	0.850	1.50

Substrate specificity studies were done at 37° and pH 5.75 with 0.02M substrate. All O.D.'s were read at 660 mu except for para-nitrophenylphosphate, which was read at 415 mu.

phosphatase which is active at pH 5.75 is present, it is relatively non-specific.

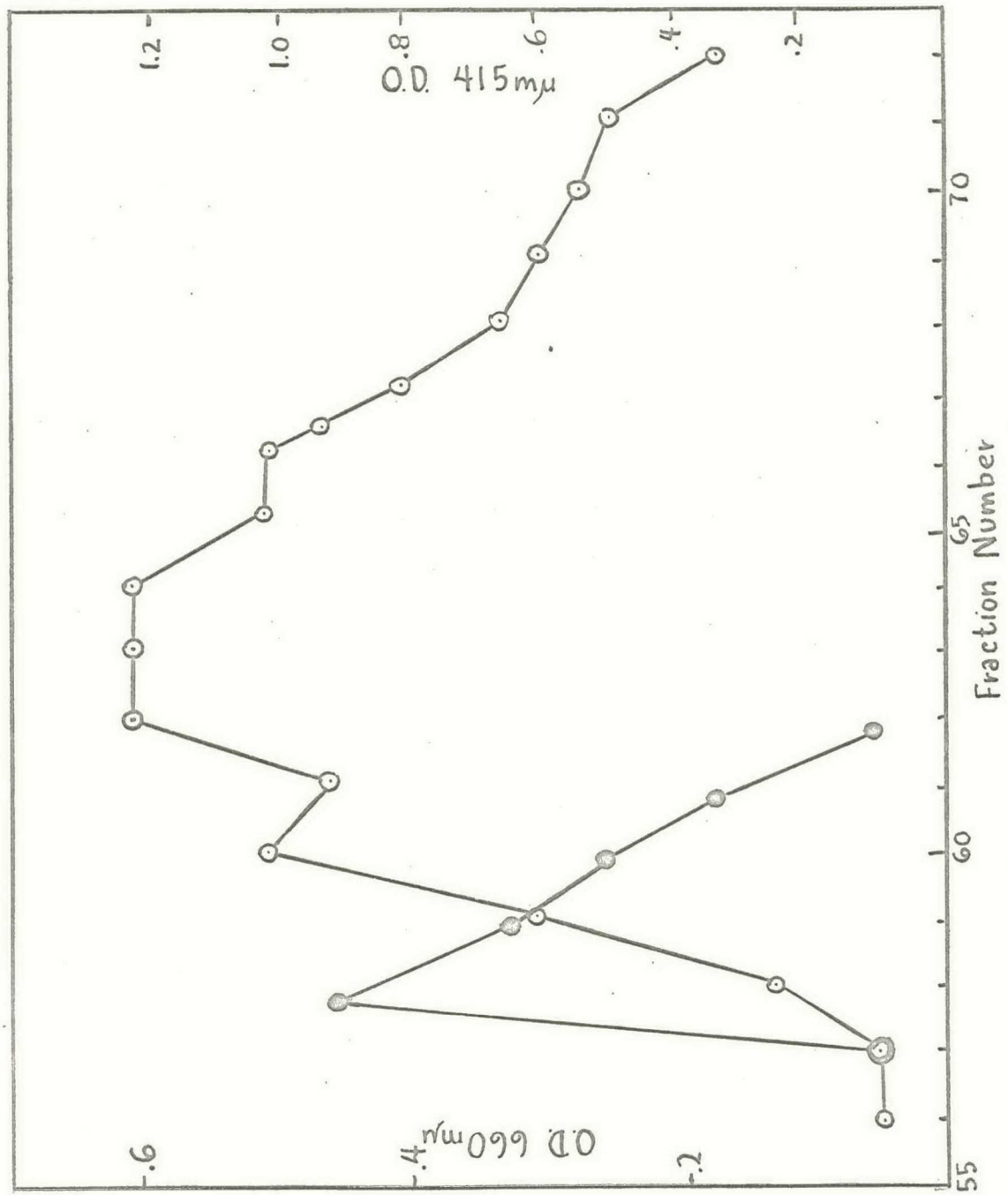
Elution Pattern from DEAE

The dialyzed hemolysate was applied to the DEAE column and eluted as given in the purification of the enzyme. Each 15 ml fraction was tested for its activity on para-nitrophenylphosphate and FMN at pH 5.75 (Figure 11).

Fractions 57 through 77 hydrolyzed para-nitrophenyl-phosphate, but only fractions 57 through 62 hydrolyzed FMN. This indicated the presence of two enzyme species, one of which acts on FMN while the other does not.

These enzymes could not be separated using a linear gradient from 0 to 0.5 M NaCl.

FIGURE 11



Phosphatase activity against para-nitrophenylphosphate and FMN was measured in each 15 ml. fraction at 37° C in 0.1 M citrate bufferm pH 5.75. ●——● FMN activity; ○——○ para-nitrophenylphosphate activity.

Discussion

Comparison of the Enzyme with Tsuboi and Hudson's Enzyme

Tsuboi and Hudson have reported a K_m of 9×10^{-4} M for their enzyme with phenylphosphate. The determination was made in 0.1 M acetate buffer, pH 5.5. The enzyme isolated in this study has a K_m of 7.7×10^{-4} M under the same conditions. With phenylphosphate at pH5.75 Tsuboi and Hudson's enzyme had a K_m of 2.7×10^{-3} M, and the enzyme isolated in this study had a K_m of 2.9×10^{-3} M.

Studies on the effect of temperature on the reaction velocity of the enzyme isolated in this study have given an activation energy of 11.9 kcal/mole in 0.1 M acetate buffer, pH 5.75. The substrate was para-nitrophenyl-phosphate. Tsuboi and Hudson determined the activation energy of their preparation to be 8.8 kcal/mole. They used alpha glycerophosphate in 0.1 M acetate buffer, pH 6.0. Since different substrates were used, no conclusions can be drawn.

Tsuboi and Hudson have reported that the enzyme which they isolated was resistant to tartrate inhibition over a wide range of concentration. The enzyme isolated in this study was inhibited 29% by 0.1 M tartrate.

The enzyme isolated by Tsuboi and Hudson was relatively non-specific and acted on phenylphosphate, alpha glycerophosphate, propanediol phsophate, and riboflavin-5-phosphate. The enzyme isolated in this study hydrolyzed only para-nitrophenylphosphate.

The two enzyme preparations appear to contain the same enzyme, although it would seem that Tsuboi and Hudson's enzyme is contaminated with a second phosphatase which also has an optimum of pH 5.75 and is relatively non-specific.

Number of Acid Phosphatases and Possible Clinical Applications

There are at least three acid phosphatases in human erythrocytes. The relative amounts of these three enzymes could be of possible clinical significance. It appears that a relatively simple assay procedure could be set up to determine the relative activities of these enzymes. The assay would employ equipment easily obtainable in any hospital laboratory, and the chemicals involved are relatively inexpensive.

One of the enzymes is active at pH 4.75 and could be assayed at this pH using alpha beta glycerophosphate. It is unstable and cannot be detected after incubation at room temperature for three hours. A second enzyme acts at pH 5.75 on a number of different substrates, among them FMN which is a good indication of its

activity. Also active at pH 5.75 is a very specific phosphatase which acts on para-nitrophenylphosphate.

The assay would be as follows:

(1) Assay the crude hemolysate with alpha beta glycerophosphate at pH 4.75.

(2) Allow the hemolysate to stand at room temperature for three hours.

(3) Assay with FMN at pH 5.75.

(4) Assay with para-nitrophenylphosphate at pH 5.75.

Normal ranges of activity using para-nitrophenylphosphate at pH 4.75 and pH 5.75, as well as the activity on FMN at pH 5.75, should be determined using the assay procedure outlined above. Any deviation from the normal could easily be detected.

It has been reported that Caucasian patients with chronic non-spherocytic hemolytic anemia have a phosphatase deficiency in addition to the glucose-6-phosphate-dehydrogenase deficiency. It is not known if the phosphatase deficiency involves an over-all decrease in phosphatase activity or if it is a complete absence or at least a decrease in only one of the phosphatases. The clinical assay proposed in this study would establish which of the possibilities exists.

Summary

There are at least three acid phosphatases in human erythrocytes. One (I) has a pH optimum of 4.5 to 4.75 and is very labile. It cannot be detected in blood hemolysates which have been incubated at room temperature for three hours. A second (II) has a pH optimum of 5.5 to 5.75 and is relatively non-specific with respect to substrates. A third (III) also has a pH optimum of 5.5 to 5.75 and, of the substrates employed, acted only on phenylphosphate and para-nitrophenylphosphate.

Phosphatase III has been partially purified and characterized. The enzyme was purified 2700 fold and represented a specific activity of 2400. At pH 5.75 and 37°C, the K_m with para-nitrophenylphosphate was 6.45×10^{-4} M. Under the same conditions the K_m with phenylphosphate was 2.7×10^{-3} M. The activation energy was 11.9 kcal/mole using para-nitrophenylphosphate as substrate . Marked inhibition was observed with zinc and copper. The enzyme was completely inhibited by PCMB and cyanide. It appears that this enzyme is the same enzyme reported by Tsuboi and Hudson[22] although their

enzyme preparation was apparantly contaminated with phosphatase II, which is relatively non- specific.

A clinical assay for the three acid phosphatases in the crude hemolysates has been proposed.

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