CHARACTERIZATION OF ACID PHOSPHATASE ISOZYMES FROM HUMAN ERYTHROCYTES

DISSERTATION

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By

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Approved by

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PUBLICATIONS

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TABLE OF CONTENTS

Table of Contents (Continued) Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

STATEMENT OF THE PROBLEM

Acid Phosphatases, or orthophosphoric monoester phosphorylases (3. 1. 3. 2), catalyze the hydrolysis of phosphate esters under acid conditions. In human erythrocytes these enzymes are an example of genetic polymorphism. Six phenotypes have been described by Hopkinson and co-workers (1) which can be explained by the presence of three allelic genes at an autosomal locus. This hypothesis is based on genetic studies of a large number of families.

Upon investigation of the three homozygous phenotypes AA, BB, and CC, four electrophoretically distinguishable areas which hydrolyze phenolphthalein diphosphate are observed. Each homozygous type shows two electrophoretically different isozymes. The electrophoretic patterns in phenotypes BB and CC cannot be distinguished on the basis of electrophoretic mobility and apparently differ only in the quantity of the isozymes present.

The major aim of this study was to determine as unambiguously as possible the number of isozymes present in human erythrocytes, and to determine whether isozymes which were indistinguishable by electrophoresis were the same on the basis of kinetic and physical properties.

Furthermore, could the isozymes which differed in electrophoretic mobility be differentiated by these same properties.

Erythrocytic acid phosphatases have been implicated in congenital non-spherocytic hemolytic anemia (CNSHA) in Caucasians because of low levels of acid phosphatase activity in these individuals (2). By determining some physical and kinetic parameters of the isozymes found in normal blood, values could be obtained to compare with the isozymes of Caucasian patients with CNSHA, to determine whether the reported deficiency in acid phosphatase levels in these patients is due to a quantitative or qualitative lack of one or more of the isozymes. Some of the parameters which were studied and could be used for comparison were molecular weights, determined by gel filtration and polyacrylamide disc electrophoresis, Km for para-nitrophenylphosphate, stability in the presence of phosphate, Ki for phosphate, and substrate specificity towards phenylphosphate and phenolphthalein diphosphate.

The results of this study could be significant from several standpoints. It may help to clarify the role of acid phosphatases in erythrocytes, and their role, if any, in red cell hemolysis. It may also lead to a clearer understanding of the genetic nature of the isozymes and significance of enzyme polymorphism.

CHAPTER II

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 (6). His results showed the presence of one pH optimum and activation of the enzyme by magnesium. 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 phenylphosphate 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 activitated 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 (9). 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 2×10^{-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 (10-13). 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°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 (11). The enzyme had a broad pH 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 phenylphosphate was found to be 9×10^{-4}M and that for alpha-glycerophosphate 7×10^{-3}M (13). The purified enzyme was found to be resistant to inactivation by

fluoride or L-tartrate and was shown to be unstable to surface forces (12). 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 (14). Using p-nitrophenylphosphate as substrate, the first peak had an optimum pH near 4.5, and the second and third peaks both had an optimum pH near 5.5. The enzyme of the first peak showed about 40 per cent inhibition with 20mM 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, Spencer, and Harris (1). 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' (figure 1). 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 3×10^{-2} M D (+)-tartrate had no apparent effect on the activity. This reported effect of tartrate is questionable since 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 were determined by three allelic autosomal genes P^a, P^b, and P^c. In a later study of 216 families, Harris (15) supported this hypothesis of genetic polymorphism. The mean activity for five of the phenotypes was also presented (A<BA<B<AC<CB). This hypothesis predicted the occurrence of a sixth phenotype. The presence of this phenotype has been confirmed (16).

In a study of 134 unrelated French families with 465 children, Van Cong and Moullec (17) tested the inheritance of the acid phosphatase types as recognized by starch gel electrophoresis of human red cell hemolysates. No exception was found to the three alleles rule established by Hopkinson. An example of the rare homozygous type C

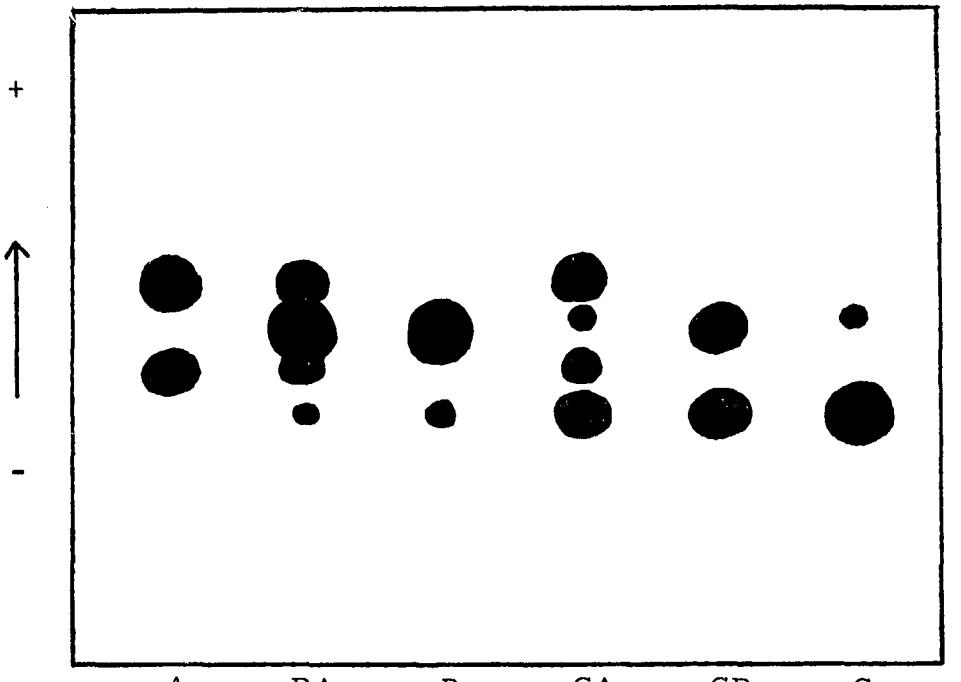

Figure 1. Diagram of isozyme components seen in the various red cell acid phosphatase phenotypes after electrophoresis at pH 6.0. Source: Harris, Harry, Proc. Roy. Soc. (London), Ser. B, 164, 298 (1966).

was found in the offspring of a AC x BC family.

A seventh phenotype of low frequency has also been reported when a formate buffer system was used in the electrophoretic procedure (18). Karp and Sutton (19) confirmed the presence of R bands in electrophoretic patterns. Support for the presence of a P^r gene came from family studies of known phenotypes. Chemical support for the hypothesis of a P^r allele came from failure to observe strong R bands in combination with a band pattern characteristic of heterozygosity for two established alleles. Karp and Sutton also proposed a P^d allele. However, no family studies were done.

Georgatsos (20) has prepared an acetone powder from human erythrocytes which has yielded two acid phosphatase peaks when fractionated by column chromatography using Sephadex G-75. 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 was inhibited by 6.6×10^{-3} M formaldehyde, 6.6×10^{-3} M oxalate, and 1.0×10^{-2} M ethanol; while E_1 was inhibited by 6.6×10^{-3} M formaldehyde, but to a lesser extent than E_2. Enzyme E_1 was also inhibited by 1.0×10^{-2} M fluoride which did not effect E_2. A very slight activation was 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.

Three isozymes were resolved by DEAE-Sephadex column chromatography (21). There was a direct relationship between the isolated isozymes and crude hemolysates as shown by starch gel electrophoresis. The isozymes were designated E_s, E_i, and E_f in accordance with their electrophoretic mobility. The pH optima for these isozymes were found to be 4.75, 5.25, and 5.75, respectively. All isozymes were inhibited by formaldehyde. Magnesium chloride inhibited E_f at 1.3×10^{-2} M but activated E_f at a concentration of 6.6×10^{-3} M. Enzyme E_s was inhibited by 6.6×10^{-3} M magnesium chloride at pH 5.5, but was unaffected at pH 4.75. Oxalate strongly inhibited E_s. Sodium fluoride inhibited only E_s and E_f.

Scott (22) purified two acid phosphatases from homozygous, phenotypically different human red cells to see whether their kinetic properties varied enough to explain the marked difference in total activity (0.66:1.00) found in homozygous AA and BB type human red cells respectively. He did not attempt to resolve the isozymes of the individual types. The Km using phenylphosphate as substrate was 8.7×10^{-4}M for AA and 7.5×10^{-4} M for BB. Phosphate was shown to be a competitive inhibitor of both isozymes. The Ki for AA was 9.2 x

10^{-4} M and for BB was 10.1 x10^{-4}M. The kinetic differences did not appear sufficient to explain the marked difference in total activity of crude hemolysates.

Hopkinson and Harris (23) have also examined human red cell acid phosphatase by column chromatography on DEAE-cellulose at pH 8.0. Their results indicated that the two main isozymes present in each of the postulated homozygous phenotypes differ from each other in net charge, but have similar molecular weights.

Luffman and Harris (24) compared some of the properties of the acid phosphatases in different phenotypes. This work was done on crude hemolysates and showed that in the crude state, types CA and CB are more thermostable than B, BA, and A. Type B is more stable than type A and type BA is intermediate between the two. No significant differences were found between the types in denaturation by guanidine or urea. These workers also found no marked differences between the phenotypes with respect to substrate specificity. The isozymes were greatly retarded on Biogel P-60 and led the authors to speculate that the isozymes had a low molecular weight.

Shinoda (25) also studied the isozymes in crude hemolysates. Phenotypes AC and BC were found to be less sensitive to heating at 50° C than were types A, BA, and B. Shinoda also found no differences in stability when the isozymes were treated with guanidine and sug-

gested that they have the same subunit structure or conformation.

Erythrocytic acid phosphatases have been implicated in some cases of chronic non-spherocytic hemolytic anemia. Caucasian patients with a deficiency of glucose-6-phosphate dehydrogenase (G-6-PD) also have a deficiency in erythrocytic acid phosphatase (2). This deficiency is not evident in Negro patients with glucose-6-phosphate dehydrogenase deficiency. It has been suggested that the lower levels of red cell acid phosphatase reported in carriers of the G-6-PD deficiency may be due to the excess of oxidized glutathione known to be present in the erythrocytes of these individuals (26). In later studies Bottini and coworkers prepared hemolysates from A, B, or CB individuals (27, 34). These hemolysates incubated with oxidized glutathione showed a duplication of the electrophoretic pattern as if each component had been split in two and the new ones were faster than the old ones. The pattern doubled after ten minutes; the fast component of the original pattern and its duplication faded after the first two hours of incubation. The slow component of the original pattern and its duplication faded at a slower rate. The treatment with oxidized glutathione also caused a loss of activity. Similar results were obtained when hemolysates were incubated with acetylphenylhydrazine. A statistical analysis of G-6-PD deficient Greeks also showed that the acid phosphatase activity was significantly lower when compared to normals (28). Increases in the level of acid phosphatase have been observed in the

macrocytes of megablastic anemias, sickle cell disease, paroxysmal nocturnal hemoglobinurias, and other hemolytic anemias (33). 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.

CHAPTER III

MATERIALS AND METHODS

Enzyme Source

Outdated blood containing acid-citrate-dextrose as an anticoagulant was obtained from the Ohio State University Blood Bank. The blood was centrifuged at 10, 000 RPM for 10 minutes and the plasma removed by suction. The cells were washed with physiological saline, centrifuged at 10, 000 RPM 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 17, 000 RPM 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 and 0. 025 per cent Triton X-100. This preparation will be referred to as the crude hemolysate.

Enzyme Assay

The enzyme was assayed by measuring the release of p-nitrophenylphosphate by a modification of the procedure for alkaline

phosphatases (29), or by measuring the release of inorganic phosphate by the method of Fiske and SubbaRow (30). 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 5.0 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 1.0 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. Activity is expressed as umoles of p-nitrophenol released per minute per ml of enzyme.

Protein Determination

Protein was estimated by the method of Warburg and Christian (31).

Electrophoresis

Starch gel electrophoresis was done according to the method of Hopkinson, Spencer, and Harris (1), except for a few minor modifications. The bridge buffer was used at pH 6.2 instead of pH 6.0. The gel buffer was diluted 1:1 before preparation of the gel. Hemolysates for electrophoresis were prepared by diluting the washed cells in 1 volume of cold, distilled water.

Preparation of DEAE-Sephadex

DEAE-Sephadex A-50 was allowed to swell in 0.005 M Trisphosphate buffer, pH 6.0. The fines were removed by decantation and the Sephadex was equilibrated for 24 hours by changing the buffer several times.

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 fraction. 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 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 glutathione per 100 ml.

Vacuum Ultrafiltration

Enzyme solutions from the columns were concentrated by vacuum ultrafiltration. 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 (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 (32). All other reagents were obtained commercially and were of reagent grade.

CHAPTER IV

RESULTS

Isozyme Resolution

Pints of outdated blood were classified according to phenotype as established by Hopkinson et. al. using starch gel electrophoresis (1). Each of the homozygous phenotypes AA, BB, and CC were resolved into two peaks by column chromatography which had phosphatase activity using p-nitrophenylphosphate as substrate. Fractions from each peak with activity greater than 0.2 O. D. reading per ml were pooled and separately concentrated by ultrafiltration. After ultrafiltration each concentrated peak gave a single zone of phosphatase activity on electrophoresis which corresponded to one of the original zones found in the crude hemolysates. Isozymes were designated as seen in figure 2. This figure also shows the four electrophoretically distinguishable areas of acid phosphatase activity.

The faster moving isozyme component from each phenotype was completely resolved from the slower moving isozyme component based on starch gel electrophoresis. The isozymes were not homogeneous with respect to other proteins lacking acid phosphatase activity. Since several preparations of individual isozymes were used

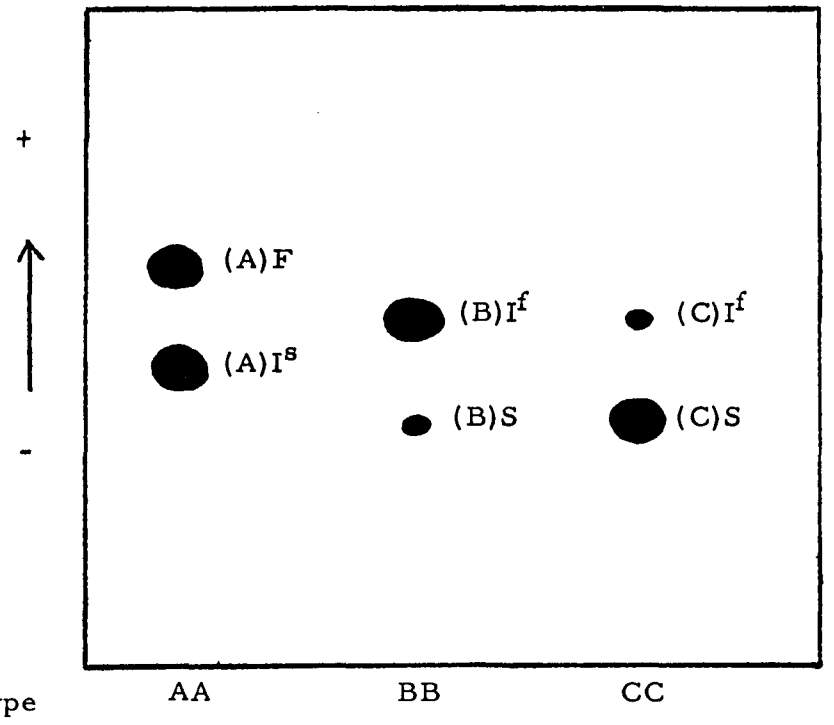

Figure 2. Electrophoretic pattern and nomenclature for acid phosphatase isozymes from homozygous phenotypes. Electrophoresis performed on starch gel as described in Methods.

throughout this study, Table 1 lists the range of specific activity for each isozyme.

pH Optima

The optimum pH using p-nitrophenylphosphate as substrate was determined for the six isozymes obtained from the three homozygous phenotypes. All of the isozymes exhibited a broad range of activity as shown in figures 3, 4, and 5. The isozymes (A)I^S, (B)S, and (C)S showed maximum activity between pH 4.6 and pH 5.3. Isozymes (A)F, (B)I^f, and (C)I^f had maximum activity between pH 5.5 and 5.8.

Stabilization by Added Phosphate

Phosphate, a competitive inhibitor of erythrocytic acid phosphatase (22), also tends to act as a stabilizing agent. Phosphate (NaH_2PO_4) was added to concentrated isozyme preparations so that the final concentration of added phosphate was 0.5M. Samples with and without phosphate were immediately assayed for activity. These were zero time values. Both samples were stored at 4°C and then assayed at various time periods. Figures 6, 7, and 8 show the inhibition of activity upon addition of phosphate. All of the isozymes were stabilized in the presence of phosphate. However, the extent of stabilization differed for some of the isozymes. Isozymes (B)S, (B)I^f, (A)F, and (C)I^f showed no significant loss of activity in the presence of phosphate for at least 240 hours. Isozyme

TABLE 1

Specific Activity of Isozyme Preparations

Isozyme	Range of Specific Activity	Minimum Fold Purification
(B)S	0.043-0.052	30
(B)I^f	0.150-0.237	70
(A)I^s	0.023-0.067	25
(A)F	0.043-0.130	60
(C)S	0.031-0.175	20
(C)I^f	0.002-0.117	50

Specific activity is expressed in umoles of p-nitrophenol released per minute per mg of protein.

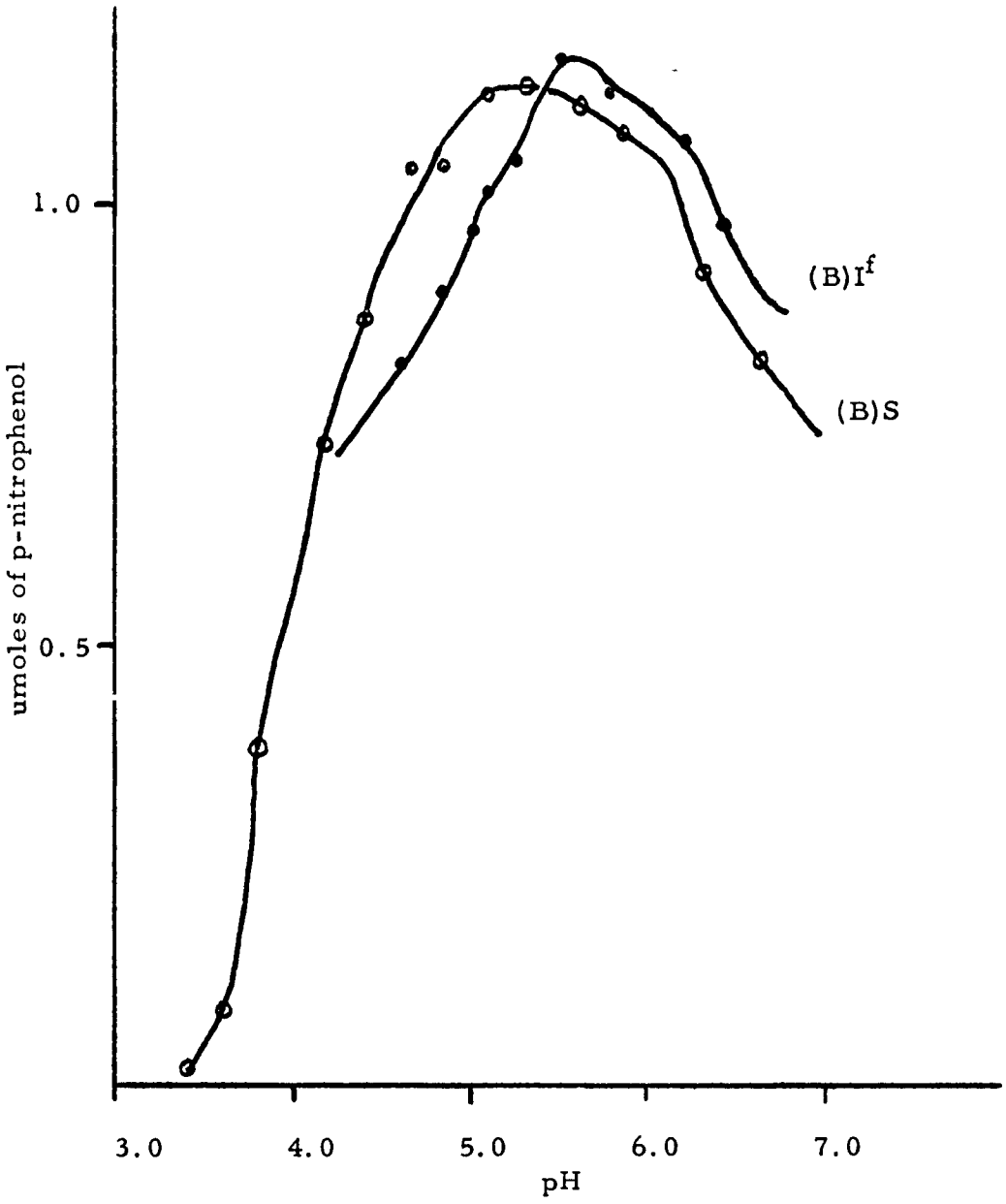

Figure 3. pH hydrolysis curves for phenotype BB isozymes. Assays were performed as described in Methods except that citrate buffers ranging in pH from 3.42-6.68 were used. Specific activity of (B)S was 0.052; specific activity of $(B)I^f$ was 0.173.

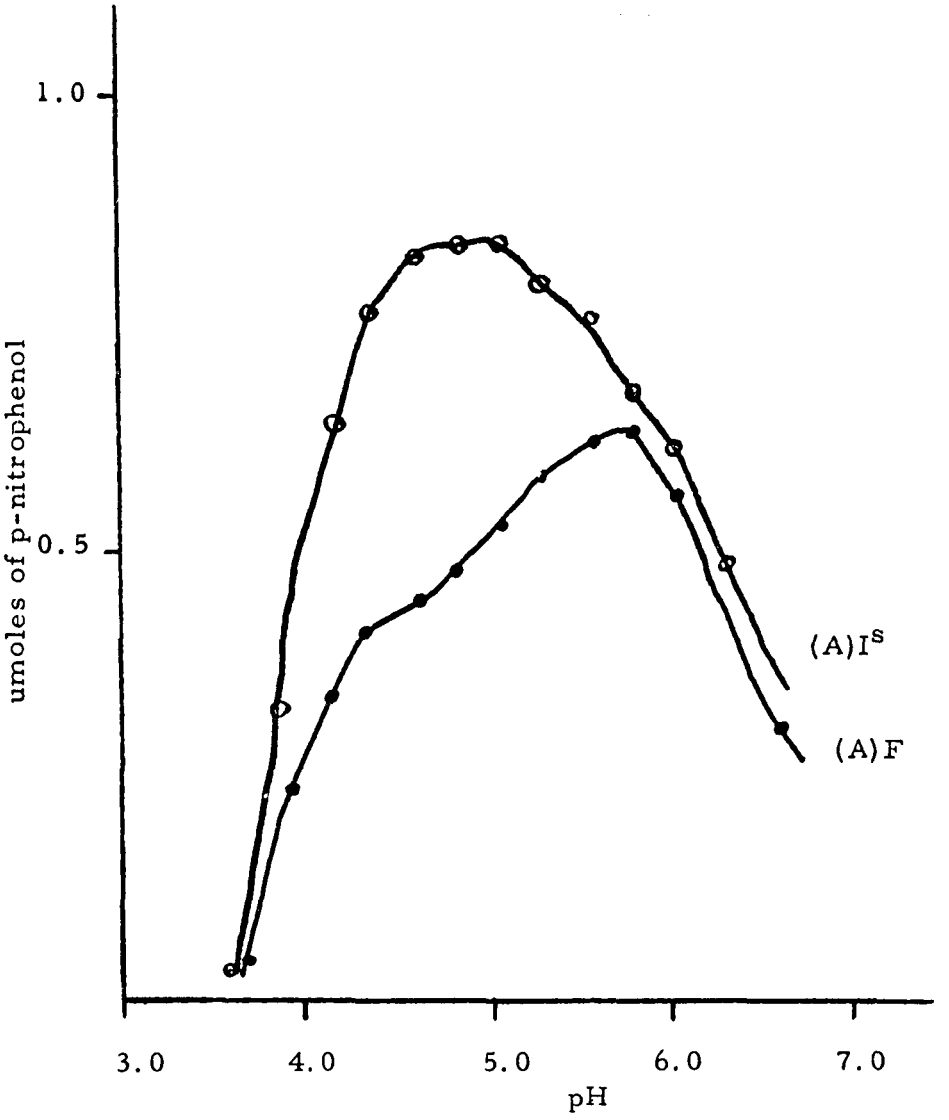

Figure 4. pH hydrolysis curves for phenotype AA isozymes. Assays were performed as described in Methods except that citrate buffers ranging in pH from 3.42-6.68 were used. Specific activity of (A)I^S was 0.03; specific activity of (A)F was 0.08.

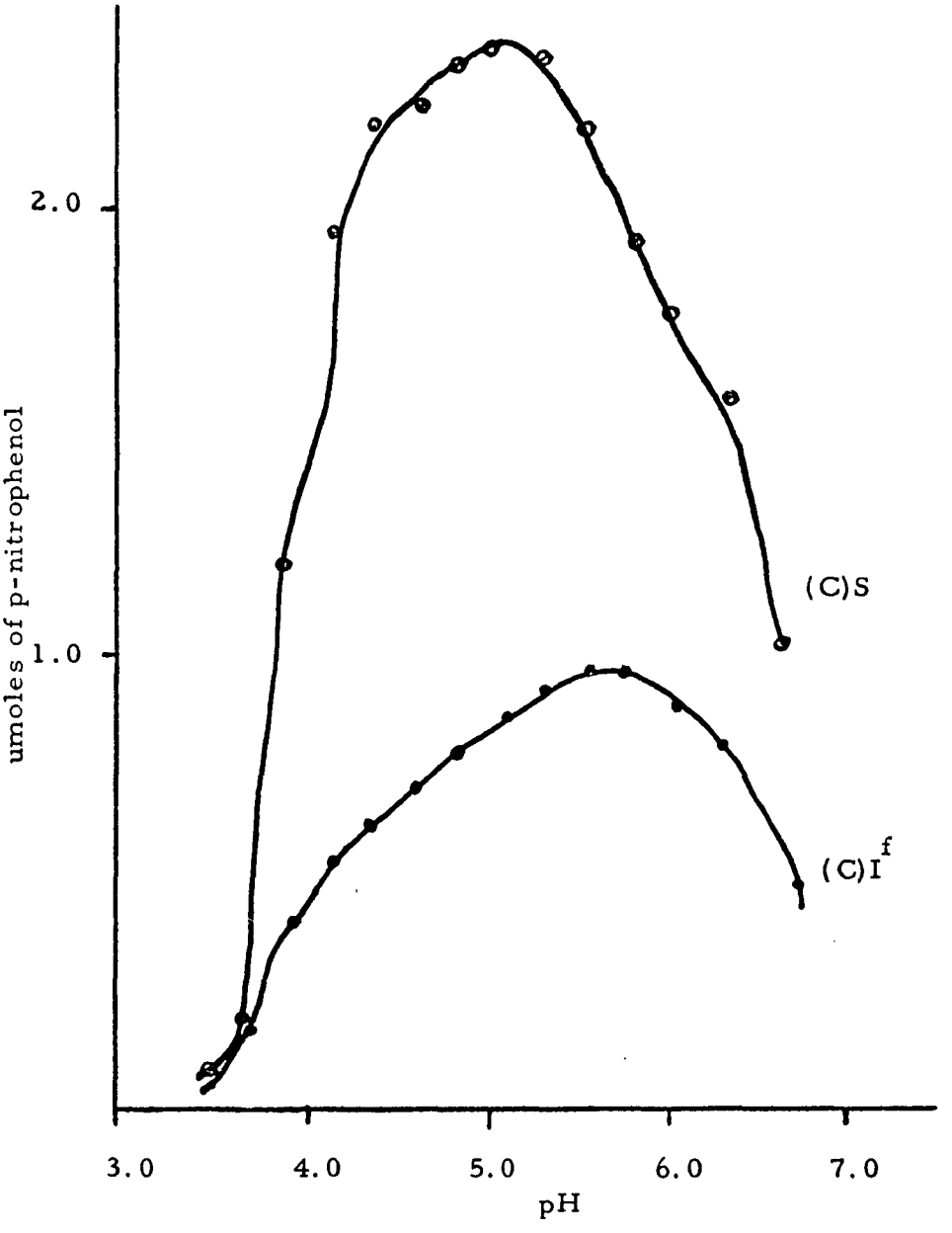

Figure 5. pH hydrolysis curves for phenotype CC isozymes. Assays were performed as described in Methods except that citrate buffers ranging in pH from 3.42-6.68 were used. Specific activity of (C)S was 0.05; specific activity of (C)I^f was 0.12.

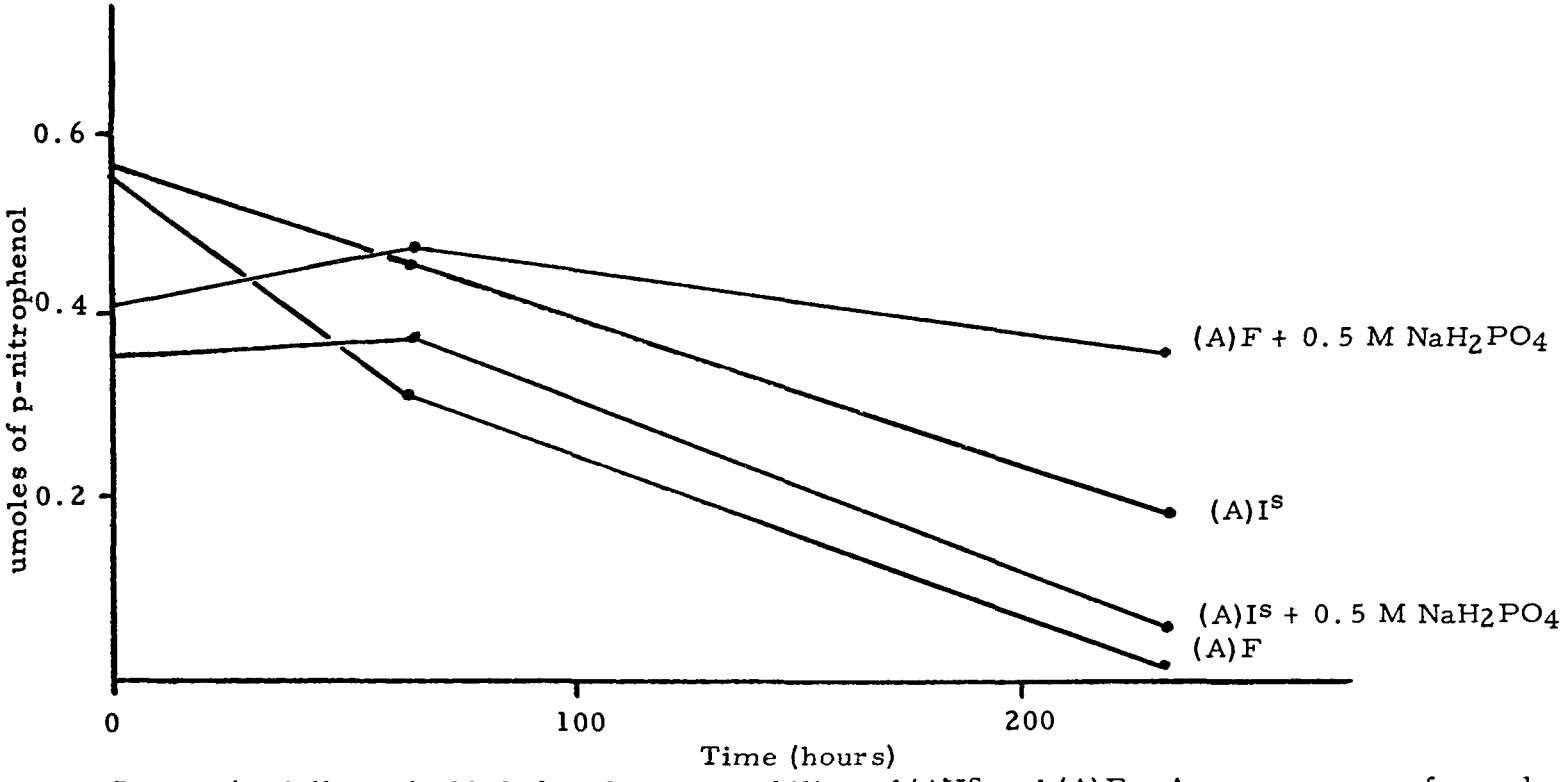

Figure 6. Effect of added phosphate on stability of (A)I^S and (A)F. Assays were performed as described in Methods. Specific activity of (A)I^S was 0.07; specific activity of (A)F was 0.04.

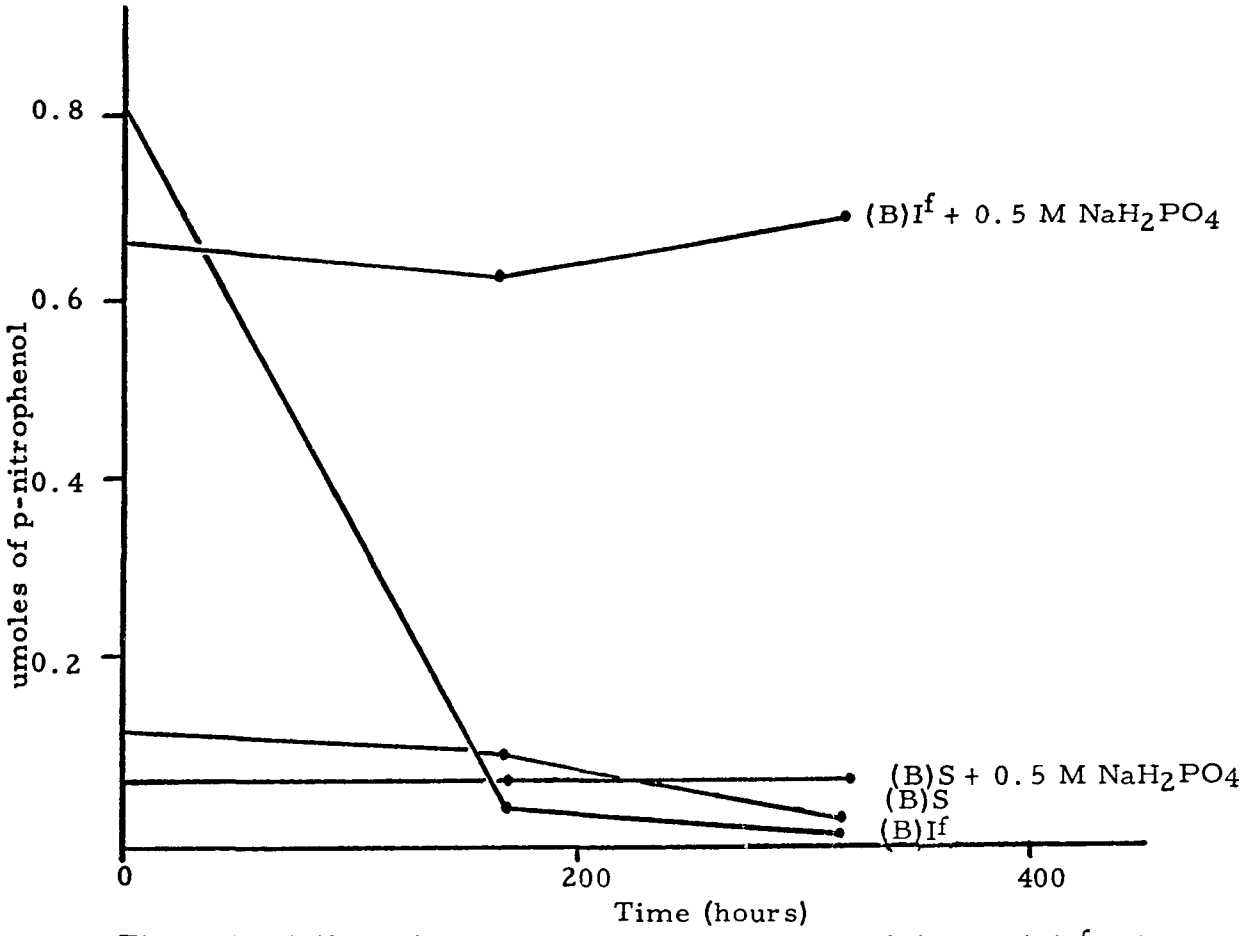

Figure 7. Effect of added phosphate on isozymes (B)S and (B)I^f. Assays were performed as described in Methods. Specific activity of (B)S was 0.04; specific activity of (B)I^f was 0.24.

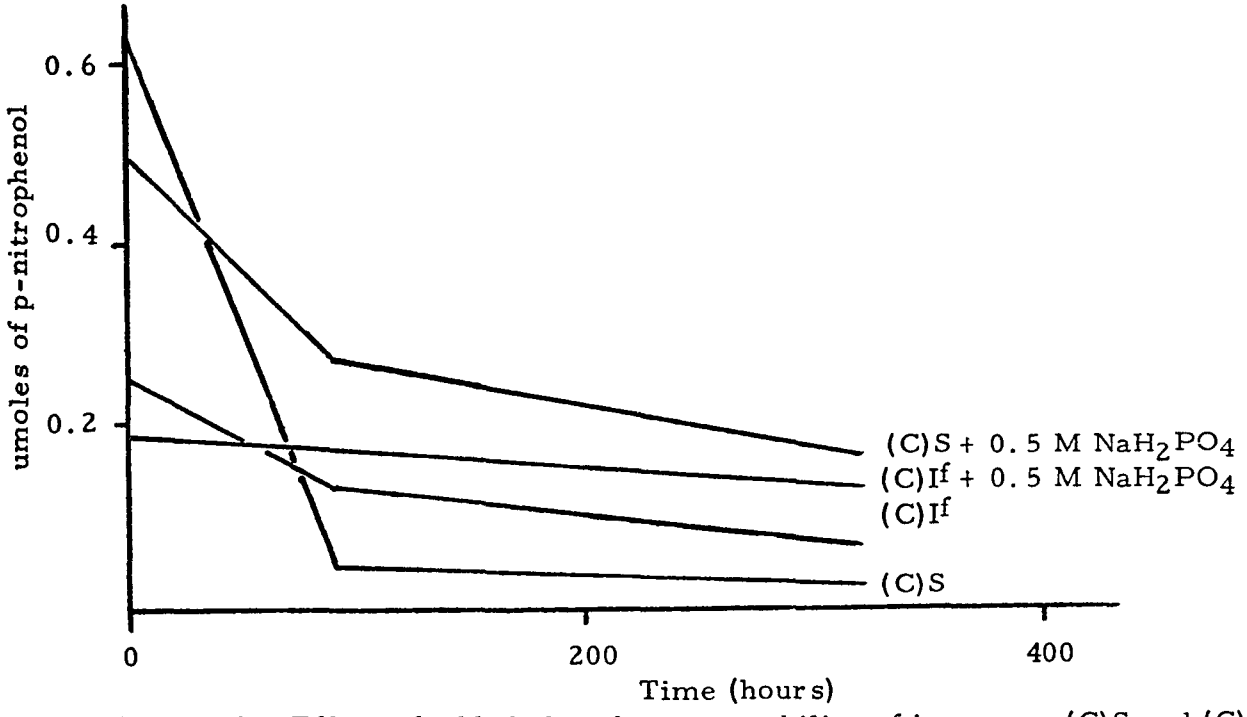

Figure 8. Effect of added phosphate on stability of isozymes (C)S and (C)I^f. Assays were performed as described in Methods. Specific activity of (C)S was 0.03; specific activity of (C)I^f was 0.002.

(A)I^S was stable for approximately 60 hours. Enzyme (C)S appeared to be the least stable in the presence of phosphate with a 50% loss of activity in one week. Without phosphate (C)S lost about 90 per cent of the original activity during the one week period.

The isozymes also appear to differ in their stability without added phosphate. Figures 6, 7, and 8 show that enzyme (C)I^f is more stable than (C)S; (B)S is more stable than (B)I^f; (A)I^S is more stable than (A)F. Isozyme (B)S showed the greatest stability in the absence of phosphate while (C)S the least stability. Concentrations of phosphate less than 0.5 M had little stabilizing effect on the concentrated isozyme preparations.

Determination of the Inhibition Constant for Phosphate

Phosphate has been reported to be a competitive inhibitor for acid phosphatases from erythrocytes (22). The inhibitor constant (Ki) was determined for each of the isozymes by plotting the reciprocal velocity versus phosphate concentration for at least four concentrations of p-nitrophenyl phosphate. All the reactions were linear during the 30 minute incubation period. The phosphate concentration given included that present in the enzyme and substrate as well as added phosphate. The results in figures 9-14 show that phosphate is a competitive inhibitor for all of the isozymes. The values of Ki fell into two groups (Table 2). The Ki values for isozymes (C)S, (B)S, and (A)I^S

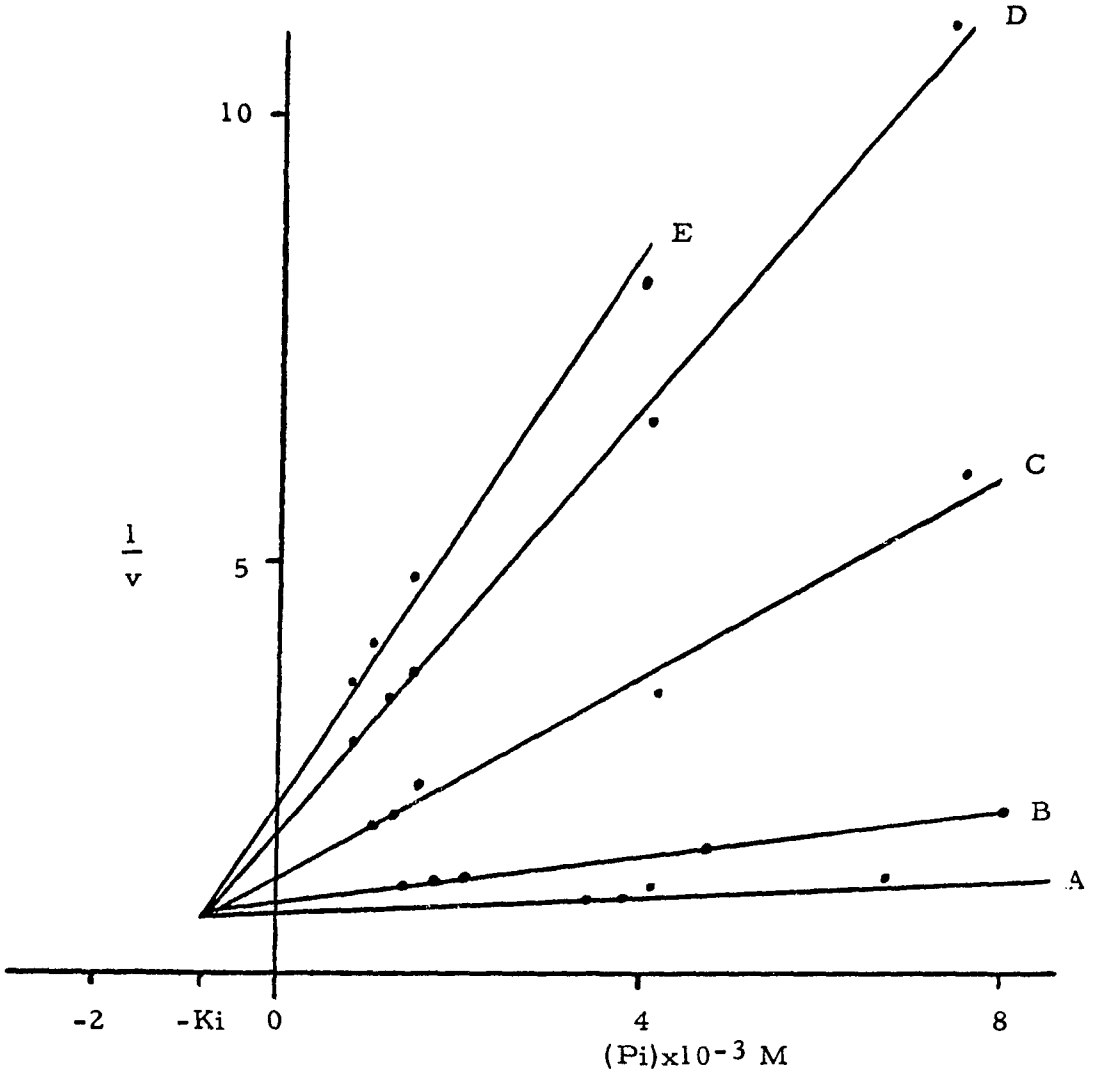

Figure 9. Ki determination for isozyme (A)I^s. Reciprocal velocity vs. phosphate concentration at various constant levels of p-nitrophenylphosphate. The concentrations of p-nitrophenylphosphate used were: (A)2.00×10^{-2}M, (B)5.00×10^{-3}M, (C)1.40×10^{-3}M, (D)6.60×10^{-4}M, (E)5.00×10^{-4}M. The assays were performed as described in Methods by measuring the release of p-nitrophenol at 415 mu. Enzyme of specific activity 0.03 was used.

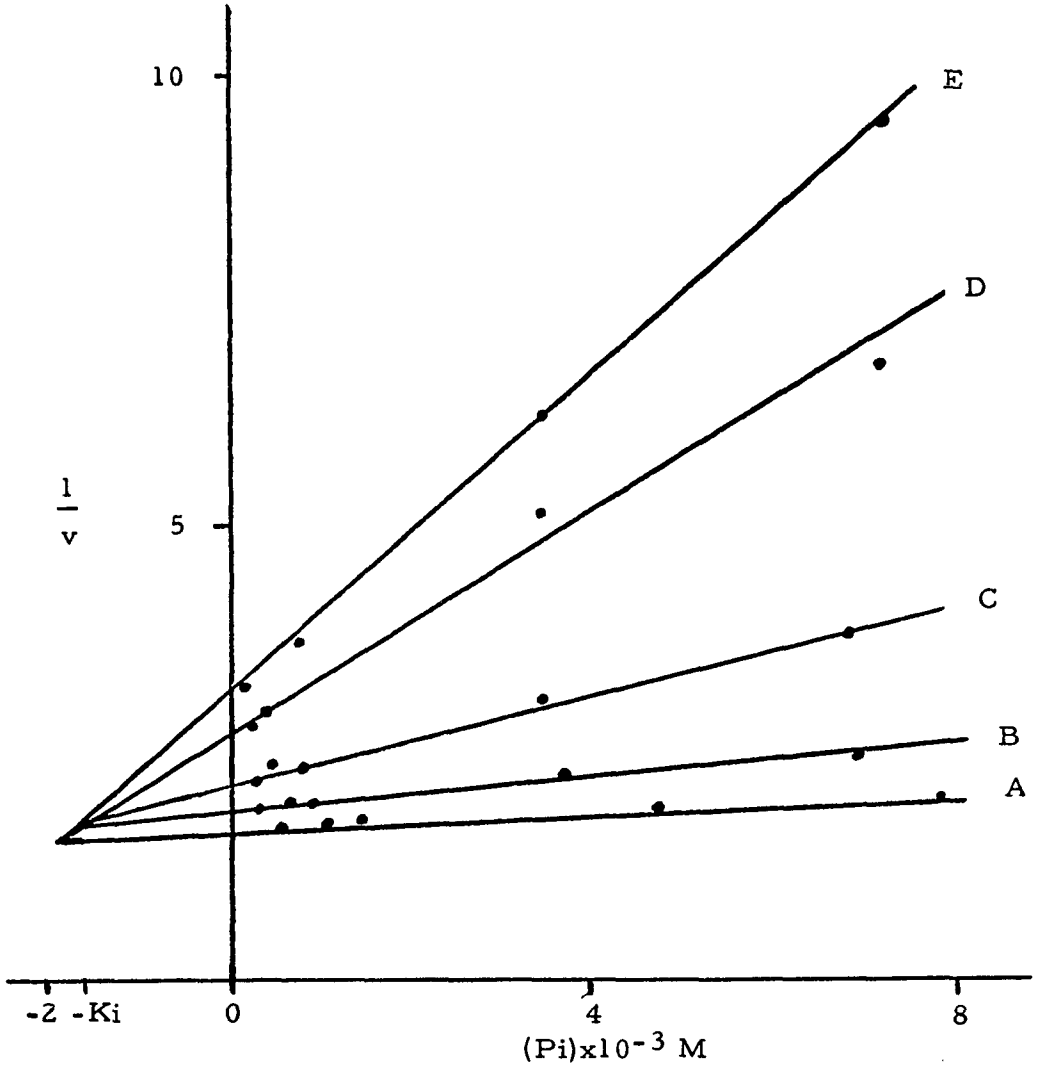

Figure 10. Ki determination for isozyme (A)F. Reciprocal velocity vs. phosphate concentration at various constant levels of p-nitrophenylphosphate. The concentrations of p-nitrophenylphosphate used were: (A)2.00×10^{-2}M, (B)5.00×10^{-3}M, (C)1.40×10^{-3}M, (D)6.60×10^{-4}M, (E)5.00×10^{-4}M. The assays were performed as described in Methods. Enzyme of specific activity 0.08 was used.

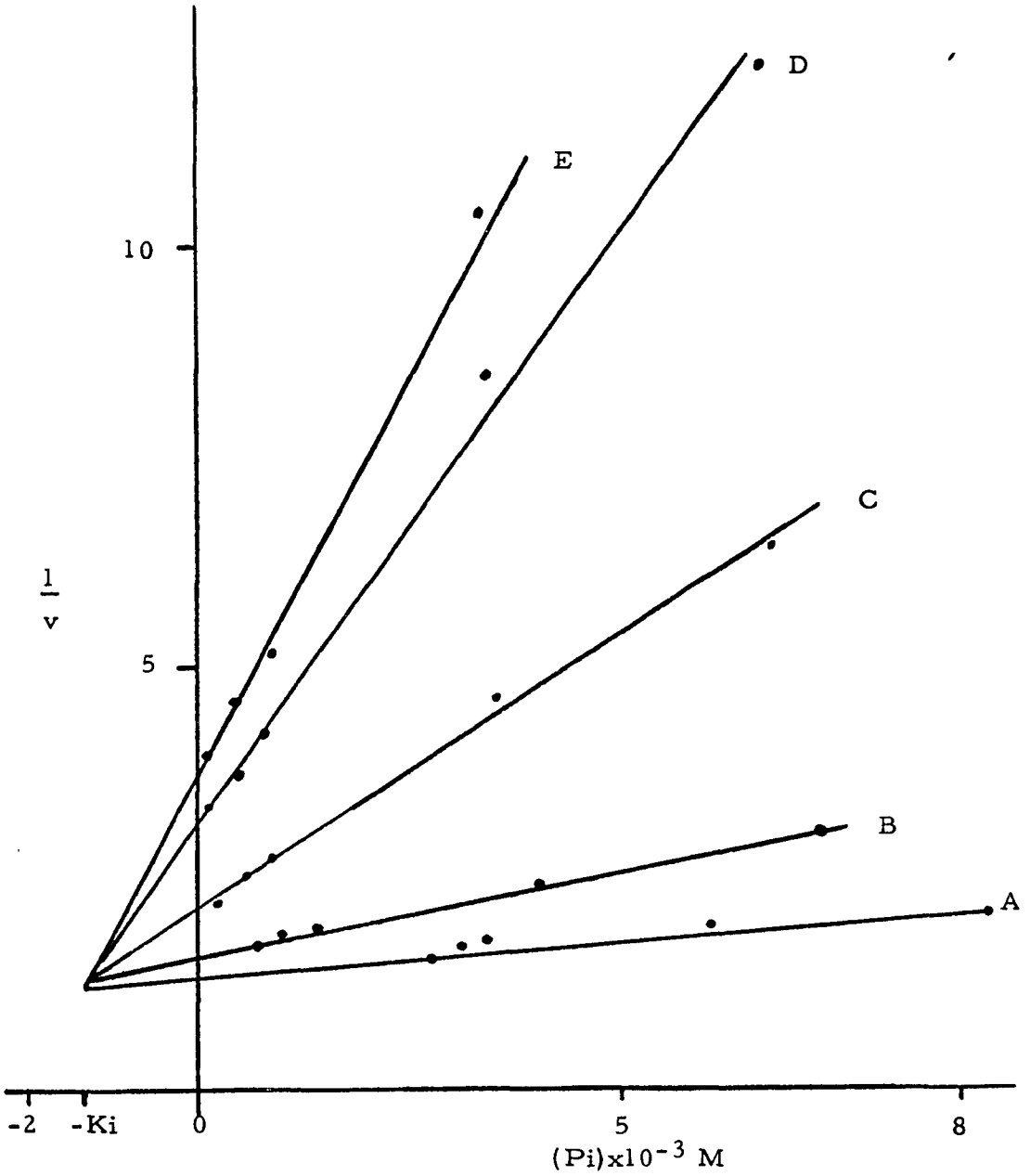

Figure 11. Ki determination for isozyme (B)S. Reciprocal velocity vs. phosphate concentration at various constant levels of p-nitrophenylphosphate. The concentrations of p-nitrophenylphosphate used were: (A)2.00×10^{-2}M, (B)5.00×10^{-3}M, (C)1.40×10^{-3}M, (D)6.60×10^{-4}M, (E)5.00×10^{-4}M. Assays were performed as described in Methods. Enzyme of specific activity 0.04 was used.

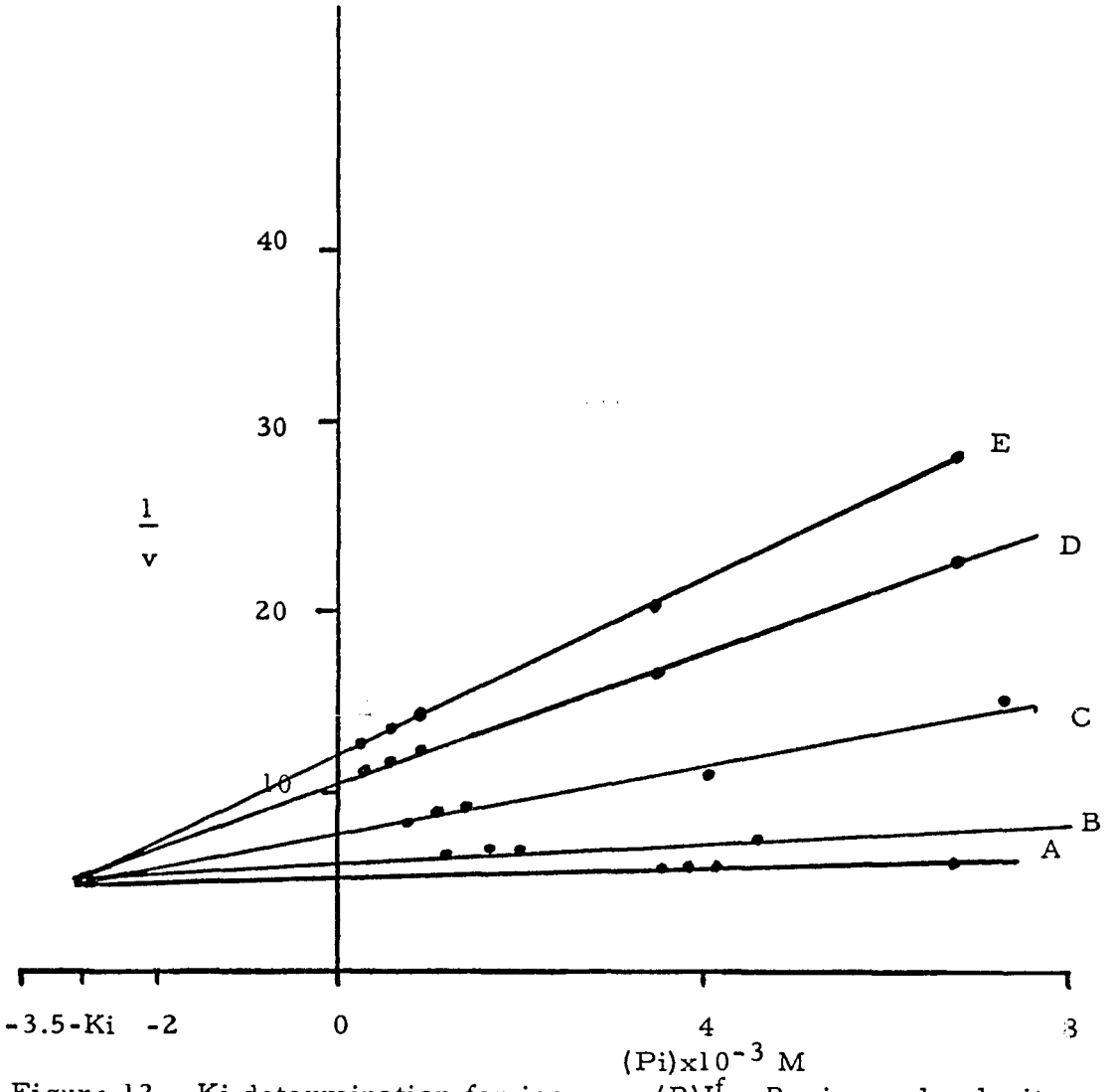

Figure 12. Ki determination for isozyme (B)I[f]. Reciprocal velocity vs. phosphate concentration at various constant levels of p-nitrophenylphosphate. The concentrations of p-nitrophenylphosphate used were: (A)2.00×10^{-2}M, (B)5.00×10^{-3}M, (C)1.40×10^{-3}M, (D)6.60×10^{-4}M, (E)5.00×10^{-4}M. The assays were performed as described in Methods. Enzyme of specific activity 0.15 was used.

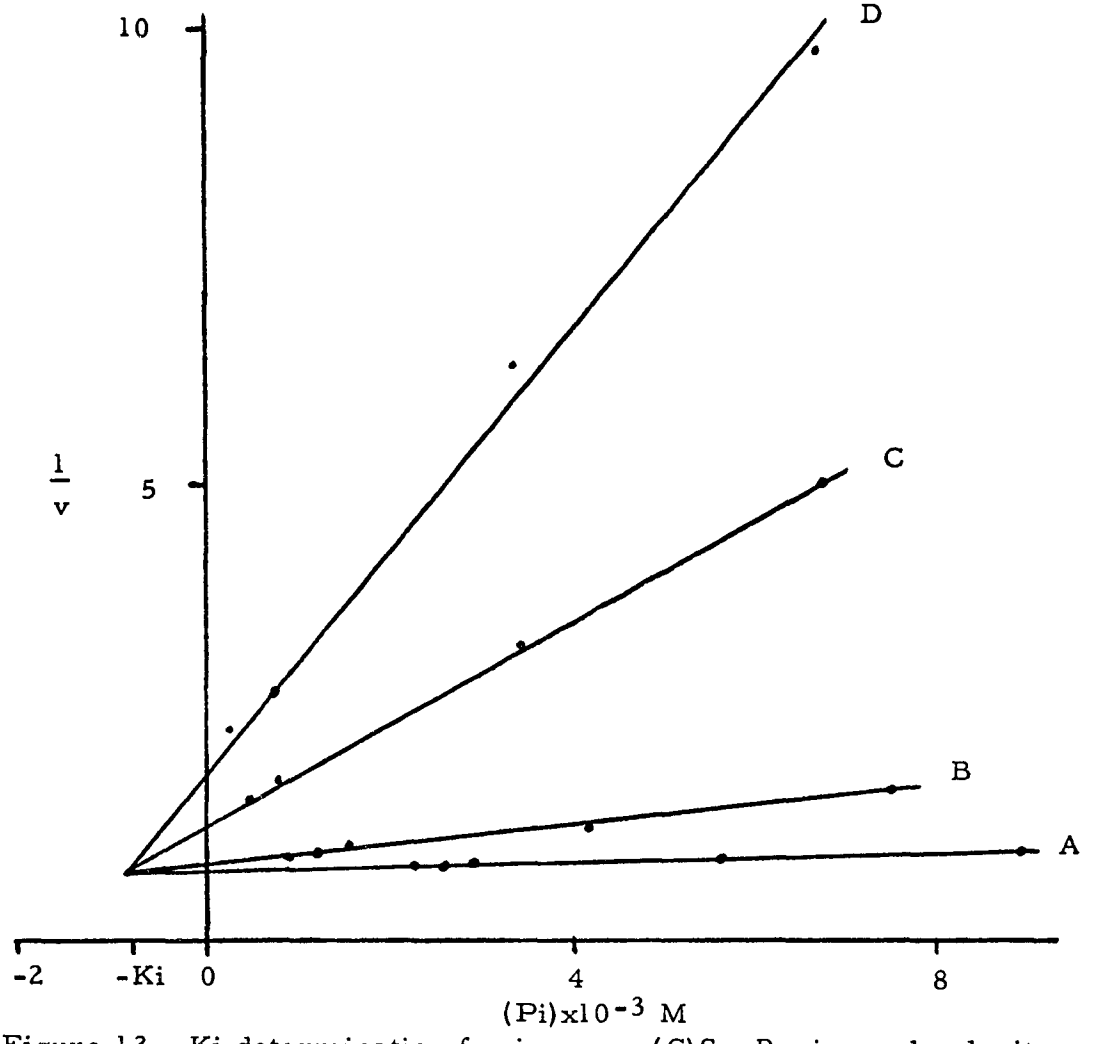

Figure 13. Ki determination for isozyme (C)S. Reciprocal velocity vs. phosphate concentration at various constant levels of p-nitrophenylphosphate. The concentrations of p-nitrophenylphosphate used were: (A)2.00×10^{-2}M, (B)5.00×10^{-3}M, (C)1.00×10^{-3}M, (D)5.00×10^{-4}M. Assays were performed as described in Methods. Enzyme of specific activity 0.05 was used.

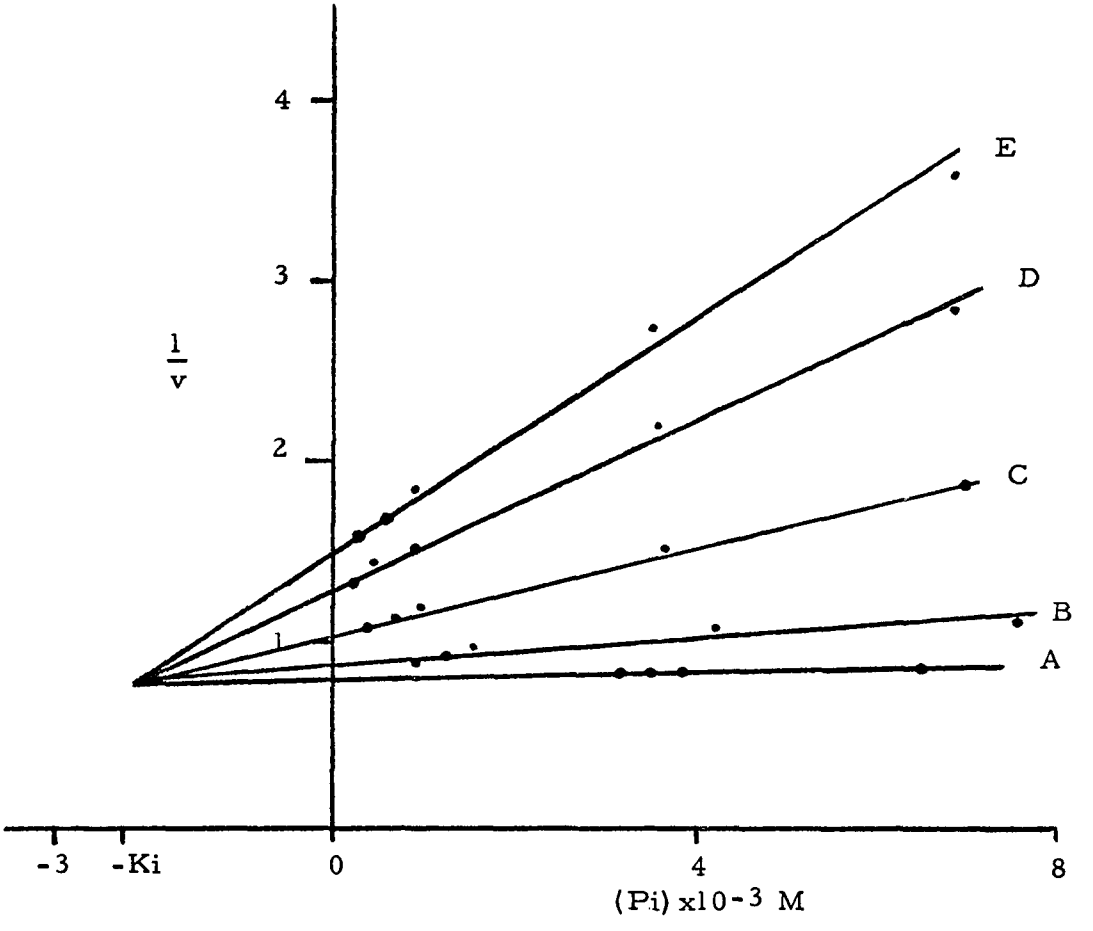

Figure 14. Ki determination for isozyme (C)I[f]. Reciprocal velocity vs. phosphate concentration at various constant levels of p-nitrophenylphosphate. The concentrations of p-nitrophenylphosphate used were: (A)2.00×10^{-2}M, (B)5.00×10^{-3}M, (C)1.40×10^{-3}M, (D)6.60×10^{-4}M, (E)5.00×10^{-4}M. Assays were performed as described in Methods. Enzyme of specific activity 0.12 was used.

TABLE 2

Inhibitor Constants for Phosphate

Isozyme	Ki (M)
(A)I^s	9.0×10^{-4}
(A)F	2.2×10^{-3}
(B)S	1.4×10^{-3}
(B)I^f	2.8×10^{-3}
(C)S	9.0×10^{-4}
(C)I^f	2.2×10^{-3}

were between 8 x 10^{-4} M and 1 x 10^{-3} M. The values for (C)I^f, (B)I^f, and (A)F ranged from 2 x 10^{-3}M to 3 x 10^{-3}M.

Determination of the Michaelis Constant

To determine the Michaelis Constant (Km) for the isozymes, it was necessary to remove or correct for the inorganic phosphate present in the enzyme preparation and also that present in the substrate due to hydrolysis on storage. The presence of a competitive inhibitor would alter the value of Km. Since the isozymes are more stable in the presence of phosphate, the effect of phosphate on the value of Km was corrected for by using the data obtained from the Ki determinations. The lines obtained by plotting reciprocal velocity versus phosphate concentration were extrapolated to zero phosphate concentration. The value of $\frac{1}{v}$ at zero phosphate concentration was then plotted against the reciprocal substrate concentration (figures 15-17). The actual substrate concentration was determined by calculating the purity of the substrate preparation (32). The Km values also fell into two groups (Table 3). The values for the isozymes of slower electrophoretic mobility from each phenotype were between 8.1 and 8.8 x 10^{-4} M. The Km values for the isozymes of greater mobility were between 4.3 and 5.8 x 10^{-4}M.

Molecular Weight Determination

The molecular weight of the isozymes was determined by gel

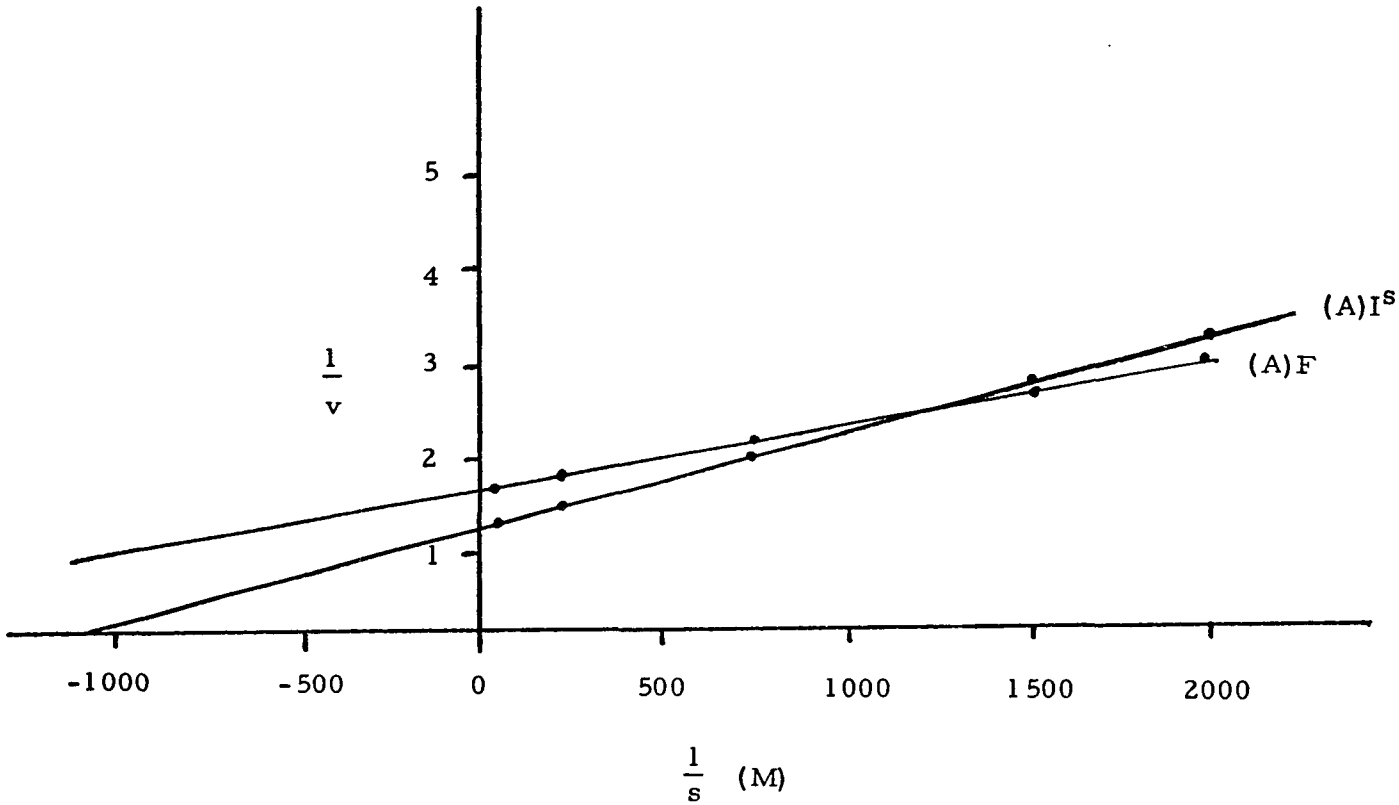

Figure 15. Km determination of the isozymes from phenotype AA using p-nitrophenylphosphate as substrate. Double reciprocal plots of initial velocity vs. p-nitrophenylphosphate concentration. Specific activity of (A)I^S was 0.03; specific activity of (A)F was 0.08.

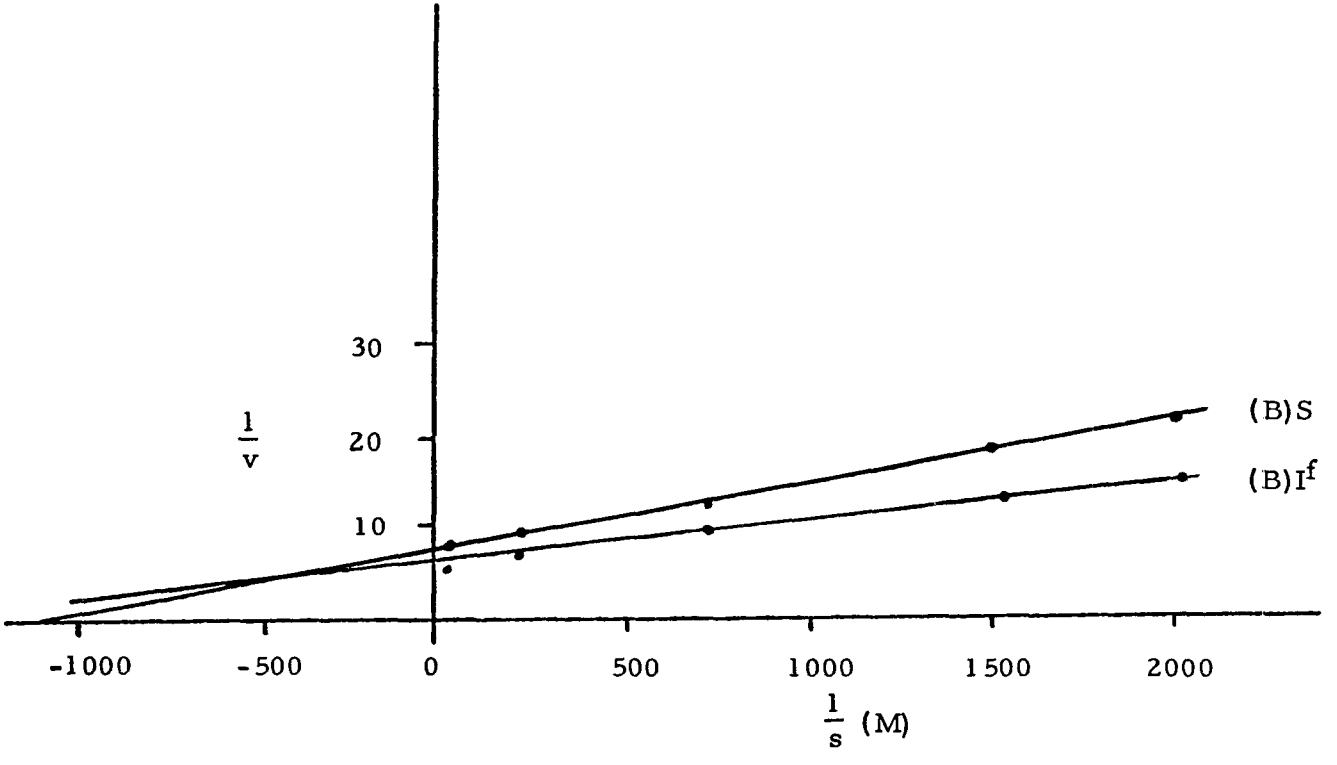

Figure 16. Km determination of the isozymes from phenotype BB using p-nitrophenylphosphate as substrate. Double reciprocal plots of initial velocity vs. p-nitrophenylphosphate concentration. Specific activity of (B)S was 0.04; specific activity of (B)I^f was 0.15.

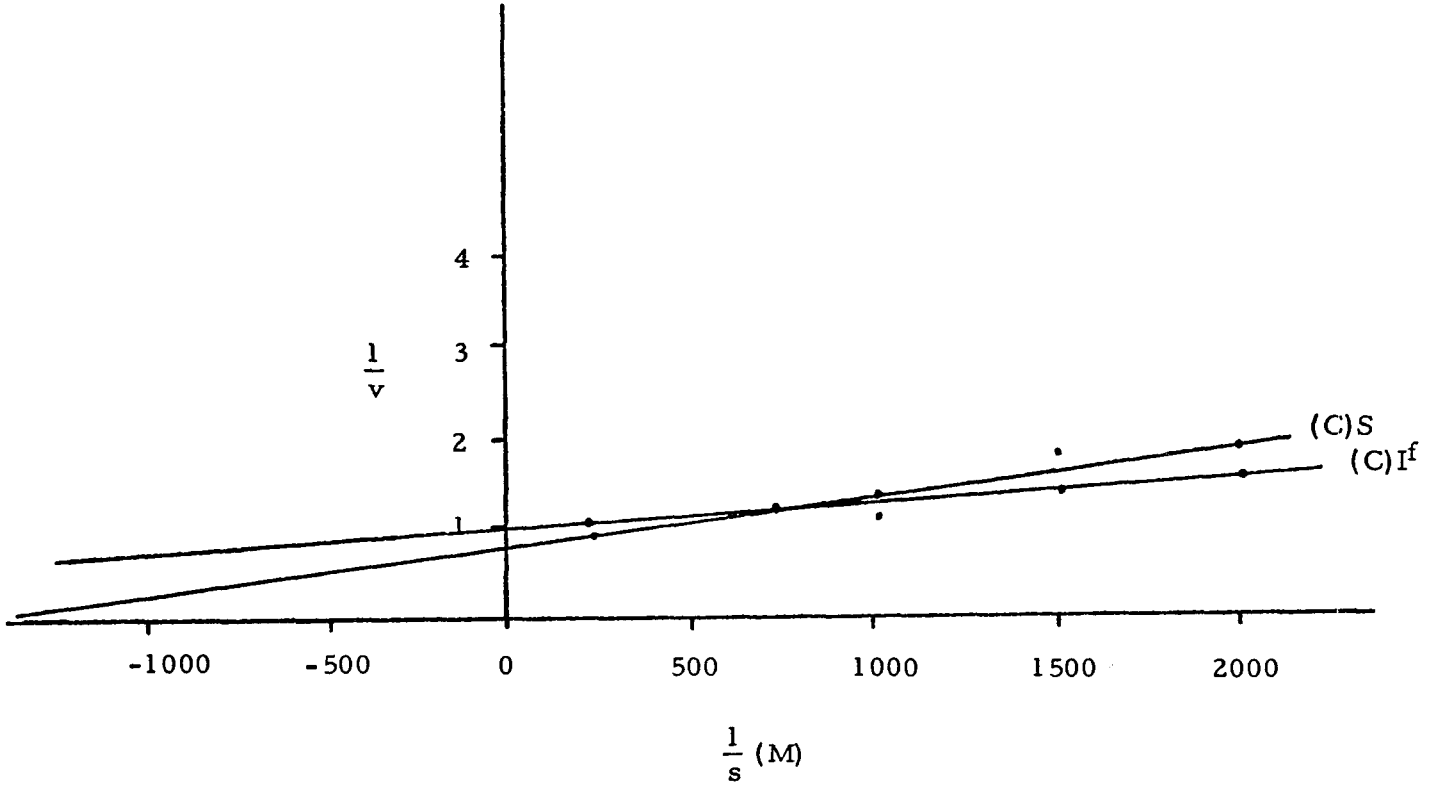

Figure 17. Km determination of the isozymes from phenotype CC using p-nitrophenylphosphate as substrate. Double reciprocal plots of initial velocity vs. p-nitrophenylphosphate concentration. Specific activity of (C)S was 0.05; specific activity of (C)I^f was 0.12.

TABLE 3

Michaelis Constants for p-Nitrophenylphosphate

Isozyme	Km (M)
(A)I^s	8.3×10^{-4}
(A)F	5.0×10^{-4}
(B)S	8.7×10^{-4}
(B)I^f	5.0×10^{-4}
(C)S	8.6×10^{-4}
(C)I^f	5.0×10^{-4}

filtration on Sephadex G-150 according to Determann (35). Sephadex G-150 was equilibrated in 0.1 M NaH_2PO_4 pH 6.0, containing 12 mg/ml penicillin (Bristol Staphcillin) to retard bacterial growth, by heating in a boiling water bath for three hours (36). Fines were removed by decantation. A water jacketed column maintained at 4° C was packed (2.5x85 cm), fitted with reverse flow adaptors, and washed with the equilibration buffer for 48 hours. Three ml of blue dextran (2mg/ml) dissolved in the equilibration buffer was used to determine the void volume (V_o). The equilibration buffer was used to elute the proteins. Five ml fractions were collected using an ISCO model 270 fraction collector with a volumetric siphon. Standard proteins were also dissolved in the phosphate buffer (2mg/ml)and three ml applied to the column. Elution volumes (V_e) for these proteins were determined by measuring absorbance at 280 mu except for cytochrome C (412 mu) and ovalbumin (230 mu). A standard curve was prepared by plotting $\frac{V_e}{V_o}$ versus the log of the molecular weight for each standard protein.

After ultrafiltration isozyme preparations were removed from dialysis tubing with 0.1 M NaH_2PO_4 pH 6.0. Enough of each isozyme to cause the release of 1.5 umoles of p-nitrophenol/min was placed on the column and the elution volume for the isozymes was determined by assaying each fraction for activity with p-nitrophenylphosphate.

The results in Table 4 indicate that the isozymes have a very low molecular weight. The weights range from 14, 000 to 22, 000 using this method. With the possible exception of (A)I^S the isozymes cannot be distinguished by molecular weight. The molecular weight for isozyme (C)I^f was not determined by this procedure because of difficulty in obtaining sufficient quantities of this isozyme.

Molecular weights were also determined by polyacrylamide disc electrophoresis according to the method of Hedrick and Smith (37). The actual electrophoresis was carried out according to Reisfield (38) except that solution C contained 40 grams of acrylamide and 1.333 grams of BIS. This was made up to 100 ml with water. The small pore gel contained 1 ml/tube and the large pore gel 0.2 ml/tube of solution C. Ten, seven, and four per cent gels were used. Enough enzyme to hydrolyze 0.1 umole of p-nitrophenylphosphate/min in 0.1 ml of 40% sucrose containing 0.04% methyl green was layered on the large pore gel. The current was set at 2 m.a./gel until the front reached the small pore gel, then the current was set a 3 m.a./gel.

The enzyme activity following electrophoresis was detected by incubation of the gels for one hour in para-rosanaline-Naphthol ASBI Phosphate at 37°C according to Barka (39). Standard protein solu-

TABLE 4

Molecular Weights of Acid Phosphatase Isozymes

Isozyme	Sephadex G-150	Polyacrylamide	Sephadex G-75
(A)I^s	22,000	20,000	15,400
(A)F	15,100	20,000	17,800
(B)S	14,200	19,000	15,200
(B)I^f	15,800	19,000	15,800
(C)S	14,200	17,000	--
(C)I^f	--	20,400	--

tions (20 ug/gel in 0.1ml 40% sucrose) of cytochrome C, myoglobin, trypsin, and carbonic anhydrase were detected by staining with Coomassie blue. The migration of the protein and dye from the small pore gel--spacer gel junction was measured. The log of the ratio of protein migration to dye migration (Rm) was plotted against gel concentration. The slope of each line was then plotted against the molecular weight of the corresponding standard protein. The values obtained by this method were slightly higher than those obtained on Sephadex. The values ranged from 17,000 to 20,000.

The molecular weights of the isozymes from phenotypes AA and BB were also determined by thin-layer chromatography on Sephadex G-75 (40). Sephadex G-75 superfine was allowed to swell in 0.25 M NaH_2PO_4 pH 6.0 containing 12 mg/ml of Bristol Staphcillin and equilibrated by standing at 25°C for 24 hours. Glass plates (20x20cm) were covered with Sephadex using a Desaga applicator at a thickness of 0.5 mm. The plates were stored in a humid chamber.

Standard proteins, cytochrome C, myoglobin, trypsin, and carbonic anhydrase, were dissolved in 0.25 M NaH_2PO_4 pH 6.0 (4mg/ml). Plates were equilibrated at 4°C by a 12 hr prerun with 0.25 M NaH_2PO_4 pH 6.0 which was also used as the elution buffer. The plate was spotted with the standard proteins and concentrated isozyme preparations. The length of the run was from 8-10 hours.

Whatman 3MM filter paper (20x20cm) was saturated with 30 ml phenolphthalein diphosphate (0.042 gm/15ml) and dried. The dried paper was carefully placed over the glass plate to absorb proteins and buffer. The paper was incubated for 3 hours at 37°C and then placed in an ammoniacal atmosphere to allow color development of the released phenolphthalein. The spots indicating the position of the isozymes were marked with pencil and the paper was again dried and sprayed with 0.3% ninhydrin solution in 95% ethanol to indicate the position of the standard proteins. The ratio of migration (Rm) of each standard to the distance of migration of cytochrome C was determined. The value of Rm vs the log of the molecular weight of the corresponding protein was plotted. From the resulting standard curve, the molecular weight of each isozyme was determined. The values obtained ranged from 15,000-18,000 which was in close agreement with the values obtained by column chromatography.

Inhibitor Studies

The effects of various inhibitors on the isozymes are shown in Table 5. At pH 5.75 only formaldehyde inhibited the isozymes. The isozymes (B)I^f and (A)F were inhibited more readily by formaldehyde than (B)S and (A)I^s. This may be a reflection of the higher specific activity of the isozymes of greater mobility from each phenotype. These compounds could not be used to differentiate the isozymes of

TABLE 5

Effect of Various Compounds on the Activity of Acid Phosphatase Isozymes

Addition	Concentration M	% Original Activity				
		Acid Phosphatase Isozymes				
		(B)S	(B)I^f	(A)I^s	(A)F	(C)S
------	------	100	100	100	100	100
EDTA	6.6×10^{-3}	104	108	106	113	--
	3.3×10^{-3}	110	103	--	--	--
$MgCl_2$	1.3×10^{-2}	92	103	108	107	98
	6.6×10^{-3}	111	106	100	106	96
$MgSO_4$	1.3×10^{-2}	110	103	--	--	--
	6.6×10^{-3}	110	114	--	--	--
NaF	2.0×10^{-2}	102	104	100	100	97
	1.3×10^{-2}	105	104	--	--	97
Formal-dehyde	1.3×10^{-2}	84	57	81	50	--
	6.6×10^{-3}	98	75	91	80	--
Na Oxalate	1.3×10^{-2}	97	100	101	102	--
	6.6×10^{-3}	103	101	--	--	93
L-Tartrate	1.3×10^{-2}	107	107	108	100	--
	6.6×10^{-3}	110	107	--	--	--
p-nitro-phenol	1.0×10^{-4}	111	114	105	109	--
	1.0×10^{-5}	109	100	--	--	--

Assays were performed as described in Methods with p-nitrophenylphosphate as substrate. Dashes indicate that the assay was not performed.

one phenotype from another. The effects of p-nitrophenol were examined to insure that no inhibition occurred at the concentration that was inherently present in the substrate. The studies done on phenotype CC were limited because of the infrequent occurrence of the phenotype.

Association-Dissociation

The effects of repeated freezing and thawing were examined to see if this would cause aggregation of the isozymes which would be reflected by a change in the molecular weight and a change in the electrophoretic pattern. A concentrated preparation containing a mixture of isozymes was assayed, a sample was run on electrophoresis, and a portion placed on the Sephadex G-150 column to determine the molecular weight. The preparation was then frozen and thawed every day for five days. At the end of this time, there was no significant change in activity, the electrophoretic pattern was unchanged, and the molecular weight was 18, 200 which was the same as the original weight. Repeated freezing and thawing did not cause aggregation under the conditions employed in this experiment.

Concentrated enzyme samples showed a change in mobility on Sephadex G-75 thin-layer chromatography after several days of storage at 4°C. The mobility was greater than that of carbonic anhydrase which has a molecular weight of 30, 000. The mobility change tends

to indicate aggregation of the isozymes. These aggregates still retained some enzyme activity. Attempts were made to reverse the association by adding 1M urea or Cleland's reagent (20 mg/ml) to the samples. After 1 hr at 4°C the samples were again run on thin-layer. No change was observed in mobility. These same samples were stored at 4°C for 24 hr and run on thin-layer; again no change in mobility was observed. The aggregates were not dissociated under these conditions.

Temperature Stability

Erythrocytic acid phosphatases are reportedly unstable to heat (24, 25). The concentrated isozymes were found to be unstable at 50°C even during short incubation periods. Therefore, a temperature stability study was done at 37°C. Isozymes in the concentrated state were incubated at 37°C in the absence of substrate or any other additions. At the end of the incubation period, samples were immediately transferred to an ice bath. A portion of each sample was assayed. Another portion was examined by starch gel electrophoresis. Isozyme (B)I^f was very unstable upon incubation at 37°C. This is shown in Table 6 by a 67 per cent loss in activity in 30 minutes. The high loss of activity could be due to the higher purity of this preparation (Sp. Act. -0.24). Isozymes (C)S and (C)I^f showed a loss in activity which was not as great as (B)I^f. These isozymes lost 20-30 per cent of the original activity in 30 minutes. Isozymes (B)S,

TABLE 6

Effect of Incubation at 37°C on Acid Phosphatase Isozymes

Time (minutes)	umoles p-nitrophenol released /min					
	Acid Phosphatase Isozymes					
	(A)I^s	(A)F	(B)S	(B)I^f	(C)S	(C)I^f
0	0.35	0.25	1.29	1.57	0.55	0.20
10	0.32	0.24	1.24	0.70	0.47	0.17
20	0.30	0.20	1.22	0.74	0.45	0.13
30	0.32	0.22	1.26	0.54	0.39	0.16
60	0.32	--	1.16	--	0.36	0.09

Assays were performed as described in Methods using p-nitrophenylphosphate as substrate.

(A)I^s, and (A)F were quite stable for 30 minutes. The electrophoresis showed no change in mobility of the samples; it merely reflected the change in activity seen in the assays.

Sulfhydryl Groups and Enzyme Activity

Tsuboi and Hudson reported the stabilization of acid phosphatase preparations in the presence of the sulfhydryl reagent cysteine (12). They further showed that the preparations were inactivated by p-chloromercuribenzoate (PCMB) and heavy metals. Other reports have indicated that reduced glutathione is a possible cofactor for acid phosphatases (2) and that treatment with oxidized glutathione caused a loss in enzyme activity (26, 27). Several studies were done to determine the effects of PCMB, mercury, and oxidized glutathione on the isozymes from phenotypes AA and BB. The ability of Cleland's reagent to prevent inactivation by PCMB, as well as the inactivation caused by incubation at 37°C, was also examined.

The loss of activity upon preincubation at 37°C for 15 minutes is shown in Table 7. The addition of Cleland's reagent (2mg/ml) to the sample during the preincubation prevented this loss in activity. Both mercury and PCMB at a concentration of 6.6×10^{-4} M inactivated the isozymes. The presence of Cleland's reagent in the reaction mixture prevented this inactivation. The effect of oxidized glutathione on (B)S and (B)I^f is shown in Table 8. Concentrated isozyme samples were preincubated with oxidized glutathione (2mg/ml enzyme) for various

TABLE 7

Stabilization by Cleland's Reagent

Treatment	umoles p-nitrophenol released/ min			
	Acid Phosphatase Isozymes			
	(A)I^s	(A)F	(B)S	(B)I^f
A. ----	2.01	2.99	0.71	3.39
B. 15 minute preincubation at 37°C	0.45	0	0.32	0.11
C. 15 minute preincubation at 37°C with 2mg/ml Cleland's reagent added	2.18	2.47	[illegible]	3.80
D. 6.6×10^{-4} M $HgCl_2$	0	0.03	0	0
E. 6.6×10^{-4} M PCMB	0.09	0.06	0	0
F. 6.6×10^{-4} M $HgCl_2$ and 6.7 mg Cleland's reagent/ml reaction mixture	2.24	3.31	0.86	3.85
G. 6.6×10^{-4} M PCMB and 6.7 mg Cleland's reagent/ml reaction mixture	2.36	3.16	0.79	3.91

Assays were performed as described in Methods using p-nitrophenylphosphate as substrate.

TABLE 8

Effect of Oxidized Glutathione on Isozymes (B)S and $(B)I^f$

Preincubation Time (minutes)	umoles p-nitrophenol released/min			
	Isozyme (B)S		Isozyme $(B)I^f$	
	Addition ----	2mg/ml oxidized glutathione	Addition ---	2mg/ml oxidized glutathione
0	0.41	0.39	0.33	0.27
15	0.40	0.21	0.30	0.03
30	0.40	0.11	0.29	0.03
60	0.41	0.01	0.30	0.11

Assays were performed as described in Methods using p-nitrophenylphosphate as substrate.

time periods. At the end of the preincubation, samples were immediately assayed. Both isozymes showed a loss of activity in the presence of oxidized glutathione.

CHAPTER V

DISCUSSION

The electrophoretic patterns of the homozygous phenotypes of erythrocytic acid phosphatases reveal four electrophoretically distinct isozymes. The isozymes from phenotype AA migrate slightly ahead of the corresponding isozymes from phenotypes BB and CC. The isozymes from phenotypes BB and CC are indistinguishable on the basis of electrophoretic mobility. Although there are four isozymes on the basis of charge, values of Km for p-nitrophenylphosphate, pH optima, and Ki for phosphate indicate that there are only two kinetically distinguishable groups of acid phosphatase isozymes (Table 9). The slower moving components from each homozygous phenotype exhibit very similar kinetic properties. Likewise, the faster moving components are also indistinguishable from one another on the basis of the kinetic studies completed to date.

The isozymes have nearly the same molecular weight which ranges from 15, 000 to 20, 000 depending on the method used for the determination. Luffman and Harris (24) in some preliminary work on the molecular weight of erythrocytic acid phosphatases also indicated low values for the molecular weight (7, 000-10, 000) of these isozymes.

TABLE 9

Summary of Properties of Acid Phosphatase Isozymes from Human Erythrocytes

Isozyme	pH optimum	Molecular Weight			Ki Phosphate M	Km p-Nitrophenyl-phosphate M
		Sephadex G-150	Sephadex G-75	Polyacrylamide		
(A)I^s	4.62-5.10	22,400	15,400	20,000	9.0×10^{-4}	8.2×10^{-4}
(A)F	5.85	15,000	17,800	20,000	2.2×10^{-3}	4.6×10^{-4}
(B)S	5.10-5.34	14,200	15,200	19,000	1.4×10^{-3}	8.8×10^{-4}
(B)I^f	5.50-5.70	15,800	15,800	19,000	2.8×10^{-3}	5.4×10^{-4}
(C)S	5.10-5.34	14,200	------	17,000	9.0×10^{-4}	8.0×10^{-4}
(C)I^f	5.63	-----	------	20,400	2.2×10^{-3}	5.0×10^{-4}

These authors further stated that the low molecular weight could be a reflection of an interaction of the enzyme with the Biogel adsorbant used in the determination and not a true measure of the molecular weight. The molecular weight determinations by polyacrylamide disc electrophoresis gave only slightly higher results than those obtained by gel filtration and tends to lend support to the small size of these isozymes. A value of 79,000 has been reported for human erythrocytic acid phosphatase (41). This value was based on the sedimentation and diffusion coefficients experimentally determined for the enzyme preparation. Several pints of blood were pooled to prepare this sample and it is difficult to relate it to one on the six isozymes from the homozygous phenotypes.

The similarity in molecular weight of the isozymes leads to some speculation about the structure of the isozymes. The genetic evidence that the AA, BB, and CC phenotypes are determined by alleles of the same gene is quite well documented (1, 15). These isozymes would be under the control of a codominant allelic gene. Such isozymes could not be distinguished from one another immunochemically (42) and would have small differences in primary sequence (43). The differences in the AA isozymes would be such that they would migrate faster than the BB and CC isozymes. It is likely that the only way the BB isozymes could be distinguished from CC isozymes would be by determining the complete primary structure for the isozymes.

The next question that arises is how are the two isozymes from a single phenotype related to one another. As stated previously these isozymes have the same molecular weight but are readily differentiated on the basis of Km, Ki, and pH optima. These isozymes are under the control of a single gene. Therefore, a single polypeptide chain is initially produced but gives rise to two different molecular species by polymer formation, conformational change, or some other modification. The possibility of polymer formation does not seem likely since the isozymes have the same or nearly the same molecular weight. Two types of conformational varients must be considered. One type, called ligand-induced conformational isozymes, arises from the interaction of one or more small molecules with an enzyme. The second type results from the ability of the polypeptide chain to spontaneously assume more than one stable conformation. These isozymes would be true conformational isozymes or "conformers" (44).

The binding of small molecules to enzymes may give rise to ligand-induced isozymes in several ways. The molecule may combine with or affect a functional group on the protein to produce a change in charge or ionization. The ligand itself may possess an electrical charge and therefore change the effective charge of the enzyme. If the acid phosphatases from a single phenotype were the result of ligand binding, this binding must in some way affect not only the

charge of the molecule but also the active site in order to produce the kinetic differences which are readily apparent. Similarly, if the isozymes are true conformers the only difference would be in the tertiary structure of the proteins. This change in tertiary structure would also have to affect the active site to account for the kinetic differences. At present the data is insufficient to determine whether the isozymes from a single phenotype are ligand-induced variations or whether the isozymes are true conformers.

The isozymes from a single phenotype could also arise from covalent differences introduced after translation. Examples of this type of change could be deamination, phosphorylation, sulfation, or even attachment of carbohydrate. Kidney alkaline phosphatase has been shown to be a mixture of isozymes (46). Differences in these isozymes have been shown to be due to the amount of bound sialic acid which can be removed by the action of neuraminidase. The erythrocytic acid phosphatases could possibly be related to one another in a similar manner.

In a previous publication (21) the presence of an isozyme was reported that was activated by magnesium. This isozyme was labelled E_f. This isozyme appears to be a minor component of the BB phenotype and is often not observed in the electrophoresis of crude hemolysates. In the present work only the major components from each of the homozygous phenotypes were studied, those which are readily

observed on electrophoresis of the crude hemolysates. Hopkinson and Harris (23) have also reported the presence of a third isozyme from homozygous BB individuals. Rapidly moving zones have been examined electrophoretically and also appear to be genetically determined (45). Other differences could be due to the differences in pH employed in the two studies. In the present work all inhibitor effects were examined at pH 5.75 and only formaldehyde was shown to inhibit the isozymes.

Scott (22) has reported that the rate of the BB reaction was less dependent on substrate concentration than the AA reaction. However, the Km values for the individual isozymes using p-nitrophenylphosphate under the conditions employed in this study do not reflect this difference. He further reported that AA is inhibited more by phosphate than BB. Isozymes (A)I^s and (B)S showed no detectable difference in Ki; however, the Ki value for (B)I^f was slightly greater than the Ki for (A)F.

Luffman and Harris (24) have indicated the possibility of interconversion of the isozymes upon heating crude hemolysates for various time periods. Shinoda (25) and Bottini and Modiano (26) reported the appearance of new zones of electrophoretic mobility upon treatment of crude preparations with oxidized glutathione. Studies of the effects of heating and the effects of oxidized glutathione on the individual isozymes showed a decrease in activity. These changes in activity

were not however reflected by changes in electrophoretic mobility of the isozymes. The isozymes appeared to be stable and under the conditions employed and showed no indication of interconversion. There is some indication that concentrated samples may aggregate on storage into a protein of higher molecular weight which still retains enzymic activity.

There seems to be some difference between the isozymes of phenotypes BB and CC with respect to stability in the presence of added phosphate and the effect of incubation at 37°C. Isozyme (B)I^f is much less stable than (C)I^f in the absence of added phosphate and also upon incubation at 37°C, whereas (B)S was more stable than (C)S in these two studies. These results could be due to slight differences in phosphate concentration during preparation, differences in ionic strength, or even differences in the specific activity of the preparations.

Scott reported that the rate of inactivation of AA and BB were indistinguishable (22). Shinoda reported that AC and BC are less sensitive to heat exposure at 50°C than A, AB, and B. (25). Similar results have been reported by Luffman and Harris (24). Both of these latter studies were done on crude hemolysates which makes the results difficult to compare with results found with more pure preparations of the individual isozymes.

This study of erythrocytic acid phosphatases, far from complete, has merely opened the door for further investigation and made several

new directions of study apparent. The low molecular weight of the isozymes makes them reasonable candidates for primary sequence determinations. This would of course necessitate the preparation of homogeneous isozymes. Work along this line has been unsuccessful to date due to the instability of the isozymes. The determination of the primary sequence would of course clear up the relationship between the various isozymes with respect to structure. The problem as to whether the isozymes from an individual phenotype are true conformers seems to be more easily approached. If two isozymes are true conformers, then unfolding in the presence of guanidine hydrochloride or urea should eliminate any differences in the two. Upon removal of the guanidine or urea, the preparations should yield an isozyme or isozymes which are indistinguishable on electrophoresis.

Finally, there still remains the unanswered question as to how these isozymes are related to G-6-PD deficiency in Caucasians. The isozymes from homozygous individuals with G-6-PD deficiency can now be resolved and compared with respect to pH optima, Km for p-nitrophenylphosphate, Ki for phosphate, and molecular weight to values that have been obtained from isozymes of normal blood. Studies such as these should reveal whether the reported deficiency involves a qualitative difference in one or more of the isozymes.

CHAPTER VI

SUMMARY

The homozygous phenotypes AA, BB, and CC of erythrocytic acid phosphatase have each been resolved into their two major acid phosphatase components. The isozymes have been designated (A)I^s, (A)F, (B)I^f, (B)S, (C)S, (C)I^f in accordance with their electrophoretic mobilities. Each of these isozymes can be stabilized by the addition of phosphate and Cleland's reagent (dithiothreitol). Phosphate has been shown to be a competitive inhibitor of the isozymes.

The isozymes appear to fall into four electrophoretic types, but into two groups on the basis of pH optima, inhibitor constant (Ki) for phosphate, and Michaelis constant (Km) using p-nitrophenylphosphate. The optimum pH for (A)I^s, (B)S, and (C)S was 4.62-5.10, 5.10-5.34, and 5.10-5.34 respectively. The optimum pH for (A)F, (B)I^f, and (C)I^f was 5.85, 5.50-5.70, and 5.63 respectively. The Ki values for (A)F, (B)I^f, and (C)I^f were 2.2×10^{-3} M, 2.8×10^{-3} M, and 2.2×10^{-3} M. The values for (A)I^s, (B)S, and (C)S were found to be 9.0×10^{-4} M, 1.4×10^{-3} M, and 9.0×10^{-4} M. The Km values were 8.2×10^{-4} M, 8.8×10^{-4} M, and 8.0×10^{-4} M for (A)I^s, (B)S, and (C)S respectively. The values determined for (A)F, (B)I^f, and (C)I^f were

4.6×10^{-4}M, 5.4×10^{-4}M and 5.0×10^{-4}M.

The molecular weight for each of the isozymes was determined by gel-filtration employing a Sephadex G-150 column, by disc electrophoresis, and also by thin-layer chromatography using Sephadex G-75. The molecular weight of the isozymes by all three methods ranged from 15,000 to 20,000. The isozymes appear to be indistinguishable on the basis of molecular weight.

Concentrated samples of the isozymes are unstable to heat and also appear to form aggregates upon standing at 4°C for several days. These aggregates still retain some enzymic activity. No evidence was found for interconversion of the isozymes. Stabilization of the isozymes by sulfhydryl reagents such as reduced glutathione and Cleland's reagent and inhibition by oxidized glutathione, PCMB, and mercury lead to the hypothesis that sulfhydryl groups are essential for enzymic activity.

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