nucleoside diphosphate kinase. Apparently, the pppA-mediated effect is indirectly correlated to the decay reaction. A more direct role for pppA in the decay reaction may be implied by our finding with more purified enzyme preparations: the conversion of ppGpp to ppG is enhanced by pppA, whereas no pppG is formed (unpublished data). That breakdown of ppGpp is accompanied by a pppA-P $\sim$ P_i exchange reaction may also indicate that pppA has a central function in the decay reaction.

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Phosphate Content of *Escherichia coli* Alkaline Phosphatase Isozymes. The Effect of Phosphate and Zinc on the Separation of Isozymes†

James McManaman‡ and Irwin B. Wilson*

ABSTRACT: Alkaline phosphatase from *Escherichia coli* was isolated as two major isoenzyme forms that were separated by DEAE-cellulose chromatography. Each form contained 2 equiv of endogenous phosphate. The endogenous phosphate, although difficult to remove, readily exchanges with phosphate. The forms also were separable by polyacrylamide gel electrophoresis. Apoenzyme prepared from native enzyme by the removal of zinc (and phosphate) also contains electrophoretically distinct enzyme forms which are indistinguishable from the native forms on gel electrophoresis. The isozymes were also found to have similar affinities for inorganic phosphate and susceptibilities to inactivation by EDTA. These results are not consistent with the notion that the formation or separation of isoenzyme forms is dependent upon different amounts of bound phosphate. They are consistent with the suggestion that a difference in amino acid composition is the basis for the occurrence and separation of these isoenzymes.

Alkaline phosphatase from *Escherichia coli* is composed mainly of three isoenzyme forms which are separable by ion-exchange chromatography or electrophoresis (Lazdunski & Lazdunski, 1967; Levinthal et al., 1962). It is concluded that the isozymes are the result of epigenetic modifications since a single cistron codes for both subunits of the enzyme (Singer, 1961).

The nature of the differences between isozymes of alkaline phosphatase has been investigated in a number of laboratories (Lazdunski & Lazdunski, 1967; Schlesinger & Anderson, 1968; Bosron & Vallee, 1975). In general there has been a lack of agreement about the basis of the isozyme differences. On one hand, evidence has been presented which supports differences in the N terminus of the isozymes as the basis for the isozymes (Kelley et al., 1973; Schlesinger et al., 1975). On the other hand, there is evidence that suggests that differences in the amount of inorganic phosphate bound to the isozymes is the basis for the isozymes (Bosron & Vallee, 1975). Neither of these interpretations excludes the other, as it is possible that differences in the N terminus of the two subunits could be re-

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sponsible for differences in the amount of phosphate bound by the isozymes. Because of these different conclusions about the difference in isozymes, we decided to investigate whether isozymes can be separated even if they contain no phosphate or contain the maximum amount of phosphate. We also studied whether zinc is required for the separation of isozymes and measured the relative affinities of the two isozymes for phosphate.

Materials and Methods

Escherichia coli strain C-90 was obtained from Dr. Ray Fall of this department. Tris and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co., St. Louis, Mo. Bactopeptone was purchased from Difco Laboratories, Detroit, Mich. $Na_2H^{32}PO_4$ was purchased from New England Nuclear, Boston, Mass. All chemicals used in the preparation of polyacrylamide gels were electrophoresis quality purchased from Bio-Rad Laboratories, Richmond, Calif. Chelex was also purchased from Bio-Rad Laboratories. DEAE-cellulose was Whatman DE-52 purchased from Whatman Inc., Clifton, N.J. All other chemicals were reagent grade.

Growth Conditions and Enzyme Isolation. Escherichia coli strain C-90 was grown in a 10-L fermentation flask under constant aeration at 37 °C to an OD_{540} of 4.54. The growth medium was prepared according to Levinthal et al. (1962) with the modification of 10% bactopeptone instead of 5% bactopeptone. The cells were harvested in the cold by centrifugation and washed three times with cold 0.01 M Tris, pH 8. The enzyme was isolated from the cells by osmotic shock according to the procedure of Neu & Hepple (1965). After the shock step, the enzyme was pumped onto a 1.5 × 30 cm column of DEAE-cellulose equilibrated with 0.01 M Tris, 10^{-3} M $MgCl_2$, and 10^{-5} M $ZnSO_4$, pH 7.5 (TMZ buffer). The bound enzyme was washed with 1 L of TMZ buffer and eluted with a solution of 0.1 M NaCl in TMZ buffer. The enzyme was then concentrated and dialyzed using an Amicon ultrafiltration system equipped with a PM-30 membrane. The enzyme was equilibrated with 0.01 M imidazole, 10^{-3} M $MgCl_2$, 10^{-5} M $ZnSO_4$, pH 7.2 (IMZ buffer), using the Amicon system. After concentration and equilibration part of the enzyme solution was used for the separation of isozymes by DEAE-cellulose chromatography.

Separation of Isozymes by DEAE-Cellulose Chromatography. Partially purified enzyme (~30 mg) was pumped onto a 1.5 × 22 cm column of DEAE-cellulose equilibrated with IMZ buffer, pH 7.2. After the enzyme was applied to the column, it was washed with 50 mL of IMZ buffer. The isozymes were eluted with a 1-L linear NaCl gradient (0–0.07 M) in the IMZ buffer. Five-milliliter fractions were collected. The linearity of the NaCl gradient was verified using a Radiometer type CDC114 conductance cell.

Protein Determination. Protein was determined by absorbance at 278 nm according to Malamy & Horecker (1964).

Enzyme Activity. Enzyme activity was assayed according to Malamy & Horecker (1964).

Scintillation Counting. The sample to be counted was added to 0.5 mL of Beckman Biosolve 3 and 10 mL of scintillant.

The scintillant was prepared by dissolving 26.5 g of diphenyloxazole (PPO) and 1.6 g of 1,4-bis(4-methyl-5-phenyloxazolyl)benzene (POPOP) in 3.8 L of scintillation grade toluene. The samples were counted in a Beckman LS 250 liquid scintillation counter.

Phosphate Content of the Isozymes. The amount of inorganic phosphate bound to the isozymes was determined using the method of Chen et al. (1956) as modified by Chappelet-Tordo et al. (1974).

DEAE Chromatography. Columns (0.9 × 15 cm) of DEAE-cellulose were used to examine the effect of inorganic phosphate on the isozyme distribution. Small (~1.5 to 3.0 mL) samples were applied to the column and eluted with a 1-L linear 0–0.07 M gradient of NaCl, in the 0.01 M IMZ buffer, pH 7.2.

Binding Experiments. The binding of inorganic phosphate by alkaline phosphatase isozymes was examined by using the technique of Hummel & Dryer (1962); a 0.6 × 15 cm column of Sephadex G-50 fine was equilibrated with IMZ buffer containing either 10^{-5} or 10^{-6} M $Na_2H^{32}PO_4$ (equilibration buffer). A sample of enzyme (~25 μL) in the equilibration buffer, and containing $Na_2H^{32}PO_4$ at the same concentration as the equilibration buffer was applied and eluted at a constant rate, using a Razel syringe pump, with the equilibration buffer. Fractions were assayed for enzyme activity and radioactivity.

Electrophoresis. Slab gel electrophoresis system was performed in the following manner. The running gel was prepared by mixing 5 parts of 30% acrylamide–0.8% bisacrylamide, 5 parts of 0.1 M citric acid, pH 7.2, 0.05 M Temed,[1] 9 parts of distilled water, 1 part of 0.5% ammonium persulfate. The electrode buffer was 0.1 M boric acid, pH 7.2. The sample was mixed 1:1 with 20% glycerol which contained the tracking dye (bromophenol blue) and applied to the sample wells. Electrophoresis was carried out at room temperature, for 1.5 h with a constant current of 15 mA. There was no significant ohmic heating of the gel during electrophoresis.

Electrophoresis of apoenzyme was performed using the same system with the following modifications to eliminate zinc: the running gel contained 10^{-4} M EDTA, and the 20% glycerol contained 10^{-4} M EDTA. The electrode buffer was stirred with 1 g of Chelex 100 for 2 h before use. The electrode buffer was analyzed for Zn^{2+} using spark ionization atomic adsorption. The Zn^{2+} concentration in the electrode buffer after Chelex treatment was negligible.

After electrophoresis the gels were stained for activity by adding equal volumes of 8.8 mM α-naphthyl phosphate in 1 M 3-amino-2-methyl-1,3-propanediol and 4 mg/mL of 4-aminodiphenylamine diazonium sulfate in 1 M 3-amino-2-methyl-1,3-propanediol. Gels containing apoenzyme were stained using the activity stain described above, with the addition of 10^{-4} M EDTA to eliminate possible reactivation by zinc in the activity stain buffer. This concentration of EDTA does not significantly affect the activity of native enzyme during the staining interval.

Protein Stain. After electrophoresis gels were stained with a solution of 50% ethanol, 10% acetic acid, and 0.1% of Coomassie Blue R-250. The maximum sensitivity of this staining procedure was estimated to be ~0.04 μg of alkaline phosphatase. This was determined by staining a gel which ranged from 0.016 μg of alkaline phosphatase to 0.16 μg of alkaline phosphatase.

Exchange Experiments. Enzyme containing endogenous phosphate was prepared by adding radioactive phosphate (2 mCi) to *E. coli* C-90 cells in stationary phase. The enzyme was isolated and purified as described above. The amount of phosphate in each peak was determined analytically as described above. The radioactivity associated with the peaks was determined by counting 0.2-mL samples.

The exchange experiment was performed by adding a sample (1.2 mL) of the enzyme containing radioactive en-

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Temed, *N,N,N',N'*-tetramethylethylenediamine.

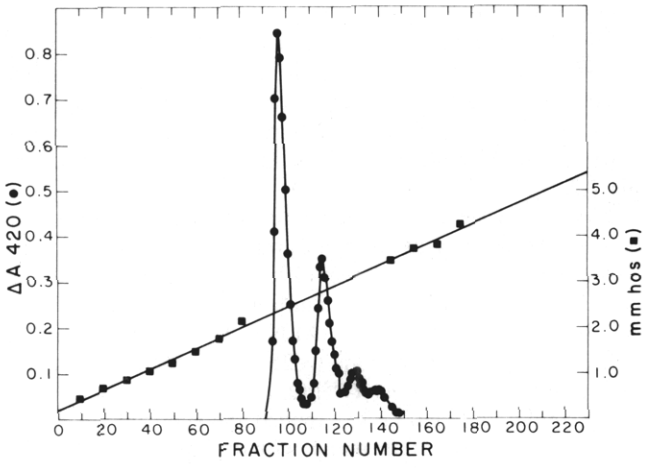

FIGURE 1: Separation of isozymes by DEAE-cellulose chromatography. Isozymes 1 and 2 were separated using a linear NaCl gradient (0–0.07 M) in 0.01 M imidazole, pH 7.2, containing 10^{-3} M $MgCl_2$ and 10^{-5} M $ZnSO_4$.

dogenous phosphate to a 3.0-mL Millipore ultrafiltration cell equipped with a PTGC membrane (10 000 mW cut-off). The system was then pressurized and tested for enzyme leakage, and the concentrations of radioactive label in the filtrate and in the cell were determined. Buffer was added to the cell to restore the volume to the initial volume and a small volume (1 μL) of 10^{-2} M unlabeled Na_2HPO_4 was added to the cell. The cell was pressurized and the concentration of labeled phosphate was determined in the filtrate and in the cell. The concentration of labeled endogenous phosphate which was not exchanged was determined by subtracting the concentration of the labeled phosphate in the filtrate from the concentration of labeled phosphate in the cell.

Dissociation–Reassociation. Isozymes 1 and 2 (~2 mg/mL) were each diluted 1:100 into 1.0 mL of 0.01 M citric acid, pH 3.0, solution and incubated for 1 h at 0 °C. At the end of the incubation, the samples were tested for activity. Enough Tris base was added to each solution to bring the pH up to 8. The samples were then placed in a 25 °C water bath and 10 μL of 0.01 M ZnCl were added. The samples were incubated for 30 min at 25 °C and then tested for activity. Each sample was examined by electrophoresis and for enzyme activity as described above.

Inactivation by EDTA. Enzyme (3 mg/mL) was diluted 1:99 into a solution of 0.02 M EDTA, in 0.1 M Tris–1.0 M NaCl, pH 8.0. At various times 0.01 mL were removed for assay in 1 mM *p*-nitrophenyl phosphate in 1.0 M Tris, pH 8.0, which contained 10^{-5} M EDTA to prevent reactivation of the enzyme during the assay.

Results

Number of Isozymes and Purity of Isozyme Preparations. DEAE-cellulose chromatography of partially purified alkaline phosphatase resulted in the separation of two major peaks of enzyme activity and two minor peaks, Figure 1. (The peak eluting first will be referred to as isozyme 1, the peak eluting second as isozyme 2, etc.) Electrophoresis of fractions from each of the two major peaks and fractions between the two major peaks showed that each chromatographic peak is composed of a single distinct electrophoretic species, while the fractions between the peaks are composed of two electrophoretically distinct species, Figure 2.

Isozymes 1 and 2 were judged to be pure and at least 95% separated on the basis that we obtained only a single and different electrophoretic band for each isozyme when stained either for enzyme activity or for protein.

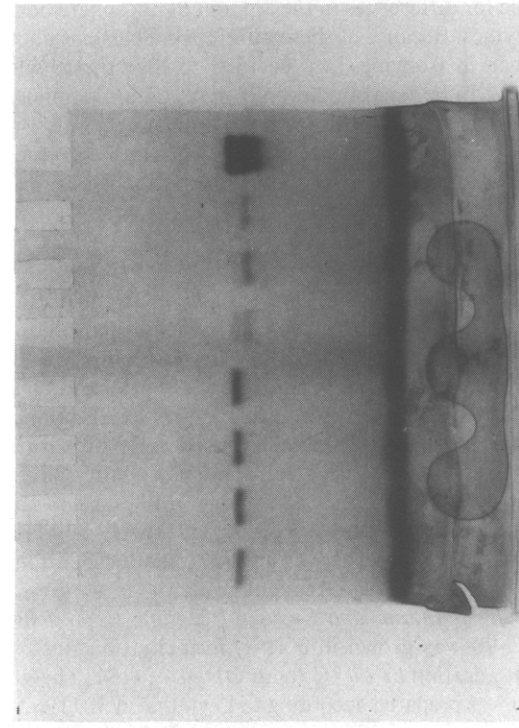

FIGURE 2: Gel electrophoresis of fractions across the peaks of isozymes 1 and 2, a fraction which was between the two peaks and a mixture of the isozymes. Alkaline phosphatase activity was located using an activity stain (see Materials and Methods). The different staining intensity is due to different amounts of protein in the samples.

The specific activity of pure crystalline alkaline phosphatase has been reported to be 48 units (μmol min^{-1} mg^{-1}; Malamy & Horecker, 1964). However, our preparation of isozymes was found to have specific activities for isozymes 1 and 2 of 73 and 58 units, respectively.

Alkaline phosphatase occurs as three major isozyme forms but the number and identity of the forms that are obtained depend upon the growth conditions of the bacterium. Since we obtained only two isozymes in large quantity, we could not be certain whether we had isozymes 1 and 2 or isozymes 2 and 3. In order to determine the identity of our isozymes, a sample of each isozyme was dissociated into its subunits by incubation at pH 3, and then reassociated by bringing the pH back to 8 (Lazdunski & Lazdunski, 1967). We recovered ~80% of the activity for each isozyme. Reassociated isozyme 1 gave rise to only the original electrophoretic band, while reassociated isozyme 2 gave rise to three bands in the approximate ratio of 1:2:1 corresponding to isozymes 1, 2, and 3.

This experiment confirms the finding of Lazdunski & Lazdunski (1967) that isozymes 1 and 3 do not give rise to new electrophoretic bands when subject to dissociation and reassociation, while isozyme 2 does, and identifies our isozymes as isozymes 1 and 2.

Phosphate Stoichiometry of Isozymes and the Nature of Endogenous Phosphate. Phosphate analysis of peak fractions of isozymes 1 and 2 showed that both isozymes had the same molar ratio of phosphate to enzyme (2:1). Rechromatography of each isozyme on DEAE-cellulose resulted in a single peak of enzyme activity. These results show that the two isozymes are not readily interconvertible and that separation of isozymes by DEAE-cellulose chromatography does not depend upon differences in bound phosphate.

Purified alkaline phosphatase contains tightly bound "endogenous" phosphate (Bloch & Schlesinger, 1973). Our results

TABLE I: Exchange between Endogenous Phosphate and Free Inorganic Phosphate.[a]

[enz] (M)	$[P_i]^b$ (M)	$[^{32}P_i]_{total}{}^c$ (M)	$[^{32}P_i]_{bound}{}^d$ (M)	$[^{32}P_i]_{free}{}^e$ (M)	$[^{32}P_i]/[enz]$
3.9×10^{-6}	0	7.4×10^{-6} (±7%)	7.4×10^{-6} (±7%)	0	1.9 ± 0.1
3.9×10^{-6}	8.3×10^{-6}	7.4×10^{-6} (±7%)	3.8×10^{-6} (±7%)	3.6×10^{-6} (±7%)	0.95
3.9×10^{-6}	8×10^{-5}	6.5×10^{-6} (±7%)	0.5×10^{-6} (±7%)	6.0×10^{-6} (±7%)	0.13

[a] Unlabeled inorganic phosphate is added to a solution of enzyme containing labeled endogenous phosphate. A small volume of filtrate was collected (see Materials and Methods) to determine the percentage of the label released by the addition of unlabeled inorganic phosphate. [b] The concentration of added unlabeled inorganic phosphate in the cell. [c] The concentration of labeled inorganic phosphate in the cell. [d] The concentration of labeled inorganic phosphate that did not exchange. [e] The concentration of labeled inorganic phosphate that did exchange.

confirm this finding, and extend it by showing that each isozyme can be isolated containing two equivalents of "endogenous" phosphate. It was not known whether endogenous phosphate is the same as normally bound phosphate. Since this question bears on the nature of endogenous phosphate and on the validity of equilibrium binding studies, we tested the ability of endogenous phosphate to exchange quickly with free phosphate. Table I shows that all of the "endogenous" phosphate is readily exchangeable with unbound phosphate.

The Effect of Phosphate on the Separation of Isozymes 1 and 2 by DEAE-Cellulose Chromatography. The presence of 4×10^{-4} M inorganic phosphate in the DEAE-cellulose column did not change the relative quantities of the two major isozymes or their elution position relative to free inorganic phosphate. Isozyme 1 had an elution position relative to free inorganic phosphate of 1.42 when eluted from a column containing no added inorganic phosphate and 1.49 when eluted from a column containing 4.0×10^{-4} M inorganic phosphate, while isozyme 2 had an elution position of 1.19 relative to free inorganic phosphate in the absence of any added phosphate in the DEAE-cellulose column and 1.23 in the presence of 4×10^{-4} M inorganic phosphate in the DEAE-cellulose column.

Electrophoresis of Native and Apoalkaline Phosphatase Isoenzymes. The electrophoretic separation of isozymes does not require the presence of metal ions. A solution of partially purified alkaline phosphatase isozymes, as yet unseparated into isozymes, was dialyzed against 0.01 M 8-hydroxyquinolin-5-sulfonic acid to produce a solution of apoalkaline phosphatase isozymes (Simpson & Vallee, 1968). Analysis of this enzyme solution showed that 90% of the zinc and all of the phosphate had been removed.

Duplicate samples of both native and apoalkaline phosphatase were subject to electrophoresis on a 7.5% polyacrylamide gel which had been carefully prepared to exclude zinc and magnesium (see Materials and Methods). After electrophoresis, half of the gel was stained using an activity stain which contained 10^{-3} M $MgCl_2$ and 10^{-5} M $ZnSO_4$. The other half was stained using an activity stain which contained no added zinc or magnesium but contained 10^{-4} M EDTA to prevent accidental reactivation of the apoalkaline phosphatase isozymes. Figure 3 shows the result of the electrophoresis. As expected, both the native and the apoalkaline phosphatase samples stained rapidly in the staining solution containing zinc and magnesium. The apoalkaline phosphatase sample contained the sample number of electrophoretic bands as the native sample. The mobilities of the bands in both samples, relative to the tracking dye, were nearly the same. In contrast, only the native enzyme sample stained rapidly in the activity stain containing EDTA. The apoenzyme sample did stain slightly but only after prolonged incubation in the activity stain, and even then distinct bands were not obtained. However, when zinc and magnesium were added to the staining solution in excess of the EDTA, the apoalkaline phosphatase bands darkened rapidly and definite bands became visible. This experiment clearly shows that neither zinc nor phosphate are necessary for the separation of the isozymes.

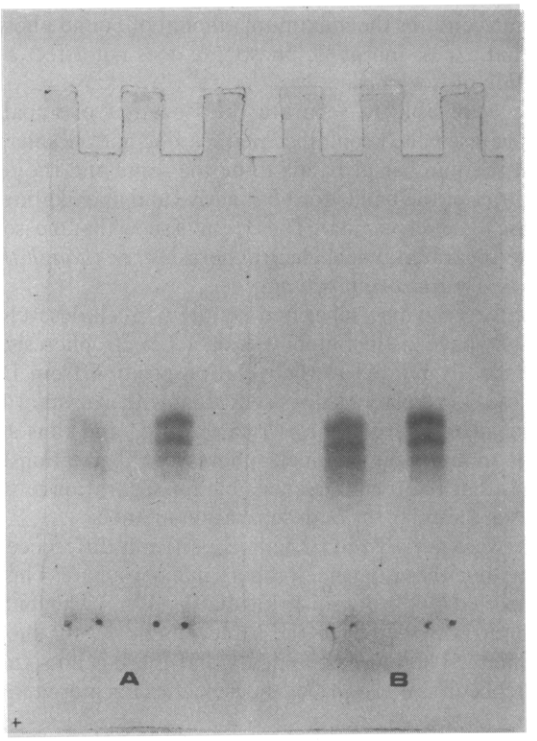

FIGURE 3: Electrophoresis of native and apoalkaline phosphatase isozymes. Panel A shows the apoalkaline phosphatase isozymes (left) and the native alkaline phosphatase isozymes (right) when stained in the presence of 10^{-4} M EDTA to prevent reactivation of apoalkaline phosphatase. Panel B shows apoalkaline phosphatase isozymes (left) and native alkaline phosphatase isozymes (right) when stained in the presence of 10^{-5} M $ZnSO_4$ and 10^{-3} M $MgCl_2$.

Comparison of the Affinity of Isozymes 1 and 2 for Inorganic Phosphate. The affinity of isozymes 1 and 2 for inorganic phosphate was estimated by determining the average number of phosphates bound to each isozyme at two different equilibrium concentrations of free inorganic phosphate using the chromatographic technique of Hummel & Dryer (1962). Isozyme 1 bound 1.94 ± 0.15 and 1.30 ± 0.07 mol of phosphate per mol of enzyme at free inorganic phosphate concentrations of 10^{-5} and 10^{-6} M, respectively. Isozyme 2 bound 1.69 ± 0.14 and 1.43 ± 0.07 mol of phosphate per mol of enzyme at free inorganic phosphate concentrations of 10^{-5} and 10^{-6} M, respectively.

Inactivation of Isozymes 1 and 2 by EDTA. Isozymes 1 and 2 were inhibited by 10^{-3} M EDTA. The rate of inhibition was found to be the same for both isozymes. Both isozymes were inactivated with a pseudo-first-order rate constant of 0.061 min^{-1}. This experiment indicates that the isozymes bind zinc

similarly and that the expression of isozymes is not caused by differences in zinc binding.

Discussion

Our results show that isozymes can be separated by DEAE-cellulose chromatography, although they each have 2 equiv of "endogenous" phosphate. The isozymes also separated even after incubation with excess phosphate and applied to the column with excess phosphate and when the eluting salt gradient contained 4×10^{-4} M phosphate. These results clearly show that alkaline phosphatase isozymes can be separated by DEAE-cellulose chromatography even though each isozyme contains the maximum amount of bound phosphate, and that excess inorganic phosphate does not affect a redistribution of isozymes.

We were able to separate the isozymes of apoalkaline phosphatase which contained neither zinc nor phosphate. We found the number of bands to be the same and the relative mobilities of the bands for both native and apoalkaline phosphatase to be very similar. These results show that the isozymes can be separated by gel electrophoresis *even though they do not contain zinc or phosphate.*

Native isozymes separated by DEAE-cellulose chromatography have distinct mobilities on gel electrophoresis which are inversely related to their elution position from DEAE-cellulose. This relationship is consistent with isozyme 1 having less negative charge than isozyme 2, etc., and thus a lower anodic mobility on gel electrophoresis. This relationship indicates that the factors responsible for separation of the isozymes are similar for both separation methods.

We were not able to find any significant difference in the affinity of either isozyme, for inorganic phosphate. This result is supported by Lazdunski & Lazdunski (1967) who found that all three isozymes have the same kinetic K_i for inorganic phosphate. Therefore, it appears that there is no significant difference in the affinity of the isozymes for inorganic phosphate.

Since it is readily exchangeable with free phosphate, there is no reason to believe that there is anything special about "endogenous" phosphate. Dialysis is usually done with enzyme concentrations much higher than the dissociation constant for phosphate and therefore only a very small part of the phosphate will be free and able to diffuse through the dialysis tubing. Therefore, removal of phosphate will be extremely slow.

Our results are consistent with the notion that differences in amino acid composition of the isozymes are the basis for their separation by techniques based on charge.

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