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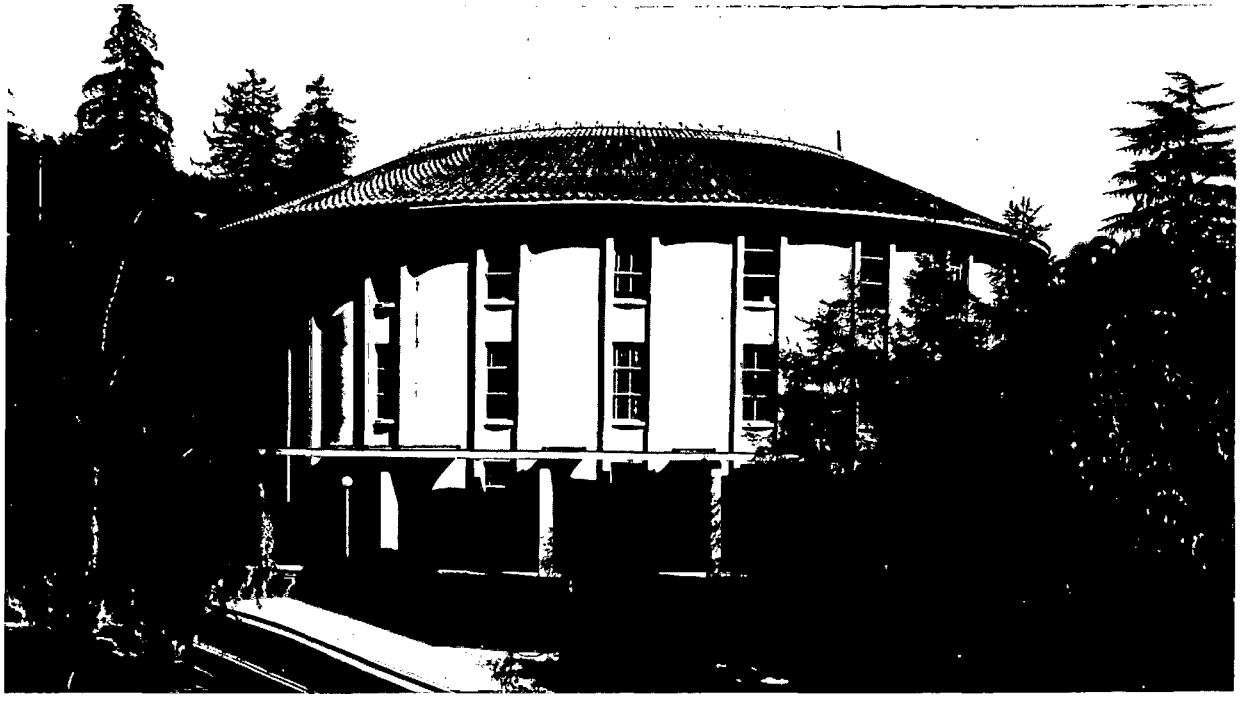

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Leaf Pyruvate Kinase

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Pyruvate kinase (pyruvate-ATP phosphotransferase, EC 2.7.1.40), is an important regulatory enzyme in the intermediary metabolism of many organisms (16). While the regulation of this enzyme from animal sources has been widely studied (22), there are relatively few reports on the regulation of the enzyme from plants, especially from photosynthetic tissue. Pyruvate kinase from cotton seeds (8) and from pea seeds and carrot tissue (31) was partially purified. These preparations showed normal kinetic dependence on ADP and phosphoenolpyruvate, were inhibited by ATP and citrate but activated by AMP (8), were unaffected by fructose 1,6-bisphosphate and some other metabolites, and had a broad pH dependence with maximum activity at about pH 7.

More recently, De Luca and Dennis (7) reported the presence of isoenzymes of pyruvate kinase from castor bean endosperm, with one isoenzyme located in the proplastids and requiring special treatment (mercaptoethanol and ethylene glycol) for stabilization. Ireland et al (12) found pyruvate kinase isoenzymes in green leaves of castor bean and in pea leaves. Isoenzymes of pyruvate kinase and other enzymes are found in a number of oilseed storage tissues (11), and in a more detailed study (13) of the cytosolic (PK_c) and plastid (PK_p) isoenzymes from developing castor bean endosperm tissue, it is stated that the properties of the these enzymes are similar to those of the respective cytosol and chloroplast isoenzymes found in the castor bean leaf. PK_p was found to have a sharp pH optimum at pH 8, whereas PK_c had a much broader pH dependence, with maximum activity at about pH 7.

A general stimulation by added ammonia of the flow of reduced carbon metabolites from photosynthetically-formed triose phosphates to the carbon skeletons of certain amino acids (including glutamine, glutamate, aspartate, asparagine, and alanine) and their acid precursors has been found in the unicellular algae, <u>Chlorella pyrenoidosa</u> (14,15) and later in

leaf discs of alfalfa (27), isolated poppy cells (25), and spinach cells (33,34,17,18). This increased amino acid formation has also been observed in the dark (10,15).

With Chlorella during both photosynthesis and respiration (14,15) and with poppy cells during photosynthesis (25), activation of pyruvate kinase in vivo upon ammonia addition was demonstrated by an increased ratio of the steady state levels of pyruvate to phosphoenolpyruvate. Increased anaplerotic flow of carbon into C_4 and C_5 ketoacid skeletons of the tricarboxylic acid cycle requires activation also of phosphoenolpyruvate carboxylase, and such activation was demonstrated with photosynthesizing (25) and respiring (10) poppy cells.

While some effects of ammonia addition to green cells such as the greatly accelerated formation of glutamine from glutamate are readily explainable, it is difficult to positively identify the mechanism whereby such addition stimulates pyruvate kinase activity. Monovalent cations such as K^+ and NH_4^+ are known to activate pyruvate kinase (21) but the levels required, compared with the likely physiological levels of ammonia and of K^+ in green cells, and the properties of the enzyme as described in this study appear to eliminate a direct effect of ammonia on the enzyme as the explanation of the observed in vivo activation. More plausible might be the proposal (3) that the rapid utilization of ATP for the ammonia-accelerated synthesis of glutamine could cause a decrease in the energy charge (2) leading to an activation of pyruvate kinase. The enzyme from cotton seeds exhibits maximum activity at an energy charge from about 0.2 to 0.6, with a substantial decline by 0.9 (8). Detailed studies of the kinetic and regulatory properties of purified puruvate kinase from photosynthetic tissue are lacking, possibly because of the difficulty in obtaining preparations free of phosphatases (8,12). The present study was carried out to provide information about the purified enzyme from leaves.

MATERIALS AND METHODS

Fresh spinach was obtained from a local market. Chemicals were obtained from the following sources: lactate dehydrogenase (beef heart)

and pyruvate kinase (rabbit muscle) from Miles Laboratories (Elkhart, Ind.); Sephacryl S-200 and cytochrome c from Pharmacia Fine Chemicals (Piscataway, N.J.); enzyme grade ammonium sulfate from Ballard-Schlesinger Chemical (Carle Place, New York); NADH (cyclohexylamine salt), phosphoenolpyruvate (cyclohexylamine salt), ADP (Tris salt), ATP (Tris salt), glutamic acid, glutamine, aspartic acid, alpha-ketoglutaric acid, asparagine, and imidazole from Sigma Chemical (St. Louis, MO); Cibacron Blue F3GA agarose (Affi-Gel Blue) from Bio-Rad Laboratories (Richmond, CA). All other chemicals were reagent grade. Membrane filters used for protein concentration were obtained from Amicon (Lexington, MA).

Enzyme Assay: Pyruvate kinase activity was determined spectrophotometrically by a modification of the method of Bucher and Pfleiderer (5), which couples pyruvate production to NADH oxidation using lactate dehydrogenase and follows the change in absorbance at 340 nm. The standard assay mixture contains 50 mM imidazole-HCl, pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 2 mM ADP, 0.1 mM NADH, and 10 units of lactate dehydrogenase in a final volume of 3 ml. The assay was initiated by the addition of pyruvate kinase and was run at 30°C on a Cary 118 spectrophotometer. In all experiments, reaction mixtures without ADP were included as controls in order to correct for phosphatase activity.

Kinetic studies were performed under these same conditions, but the concentrations of specified reagents were varied as indicated. The kinetic parameters were calculated using the linear forms of the Michalis-Menton equation derived by Lineweaver-burke (19) and Woolf (35). The data were fitted by a weighted least squares procedure contained in the Hewlett-Packard 38-E calculator. One unit of activity is defined as one umole of NADH oxidized per minute at 30°C.

Protein Determination: The amount of protein was estimated by the method of Lowry, et al (20), after precipitating the protein with 7% trichloroacetic acid. Protein in column eluates was monitored by measuring absorbance at 280 nm.

Molecular Weight Determination: Gel filtration for determining the molecular weight of pyruvate kinase was performed at $5^{o}C$ by the method described by Andrews (1). Molecular weight standards included rabbit muscle pyruvate kinase (237,000 daltons), beef heart lactate dehydrogenase (135,000 daltons), yeast hexokinase (105,000 daltons), bovine serum albumin monomer (66,200 daltons), and horse heart cytochrome c (12,400 daltons). A column (2.5 x 80 cm) of Sephacryl S-200 equilibrated with buffer A was calibrated with the molecular weight standards, which were eluted with the same buffer. An aliquot of the Sephacryl S-200 fraction from the spinach pyruvate kinase purification procedure was chromatographed on the same column under the same conditions. From a plot of elution volume versus log molecular weight of the standards, a molecular weight estimation of spinach pyruvate kinase is made.

Pyruvate kinase assays were performed using the standard assay outlined above. Lactate dehydrogenase was assayed using the procedure of Reeves and Fimognari (28), and hexokinase was assayed using the method of Beisenherz, et al. (4). Bovine serum albumin and cytochrome c were monitored by their absorbance at 340 nm and 415 nm, respectively.

Partial Purification Procedures: Pyruvate kinases from plant sources are unstable in dilute buffer (21,23,31). The crude extract of spinach pyruvate kinase loses approximately 20% of its initial activity when stored at $4^{o}C$ for five days. The crude extract of spinach pyruvate kinase was stabilized by the addition of 10 mM Mg^{2+} and glycerol or ethylene glycol to a final concentration of 20%. In order to conserve enzyme activity, the purification of spinach leaf pyruvate kinase was carried out in the presence of Mg^{2+} and ethylene glycol.

All purification operations took place in a cold room at $5^{o}C$. pH values of buffers used in the purification procedure outlined below were determined at room temperature.

Preparation of Crude Extract: Fresh spinach leaves were washed and carefully deveined. After excess water was removed and the spinach weighed, buffer consisting of 100 mM Tris - HCl, pH 7.4, 10 mM $MgSO_4$, 2mM

DTT, and 20% ethylene glycolwas added to the spinach in a weight to volume ration of 1:2. The spinach was homogenized using a Waring blender set at medium speed for four 15 second bursts. The pureed spinach was strained twice through six layers of cheese cloth. The resultant dark green solution was centrifuged twice at 24,000 x g for thirty minutes in order to remove cellular debris. The straw yellow supernatant is the crude extract.

With the crude extract, the pyruvate kinase enzyme assay blank (standard assay mixture minus ADP) gave a reading of about 30% that with the complete assay, possibly due to presence of phosphatases.

Ammonium Sulfate Fractionation: Solid ammonium sulfate (194 g/L) was added to the crude extract to give a 35% saturated solution (at $0^{o}c$). This solution was stirred overnight in an ice bath and the precipitate was removed by centrifugation at 18,000 x g for thirty minutes. Solid ammonium sulfate (87 g/L) was then added to the supernatant to give a 50% saturated solution (at $0^{o}C$). After being stirred for six hours in an ice bath, the suspension was centrifuged at 18,000 x g for thirty minutes. The supernatant was discarded. The precipitate was suspended in approximately 2% of the original crude extract volume in a 10mM $MgSO_4$, and 2 mM DTT, and 20% ethylene glycol (Buffer A). The suspension was allowed to dissolve in an ice bath for several hours (ammonium sulfate fraction).

G-25 Sephadex Chromatography: The dissolved ammonium sulfate fraction was applied to a 2.5 x 95 cm column of G-25 Sephadex, which had been previously equilibrated with two column volumes of Buffer A. The enzyme was eluted with Buffer A at a flow rate of about 1 ml/min. The fractions containing enzyme activity were pooled (G-25 fraction).

This step is included to remove yellow pigmented material, which may interfere in later purification steps. This material was retained in the G-25 Sephadex column and was removed only by washing the column with several column volumes of Buffer A.

Affi-Gel Blue Chromatography: The G-25 fraction was dialyzed against Buffer A solution until its conductivity was less than 7.5×10^3

umhos at $5^{o}C$ as measured on a conductivity meter using a Beckman electrode. The dialyzed preparation was centrifuged at 10,000 xg for 20 minutes to remove a fine white particulate suspension which appears during dialysis. No pyruvate kinase activity was lost in the pellet. The supernatant is applied to a 2.5 x 18cm column of Affi-Gel Blue, which has been previously washed with Buffer A until the conductivity of the eluant equals the conductivity of the starting buffer. The column is washed with about 600 ml of Buffer A at a flow rate of 0.75 ml/min.

Pyruvate kinase was eluted with a linear gradient made by adding 300 ml of Buffer A to one side of the gradient reservoir and 300 ml of the same buffer containing 500 mM KCl to the other side. The column was washed with 150 ml of Buffer A containing 500 mM KCl. Remaining protein adhering to the column was removed with 150 ml of Buffer A containing 1 M KCl. Over 90% of the phosphatase activity flows through the column in the initial wash. The peak in the pyruvate kinase activity elutes off the column at a salt concentration of about 0.26M. The fractions containing the enzyme activity peak were pooled (affi-Gel Blue fraction).

Sephacryl S-200 Chromatography: The Affi-Gel Blue fraction was concentrated ten to twenty fold by ultracentrifugation using an Amicon P-30 membrane filter and then dialyzed against Buffer A. A 2.5 x 90 cm column of Sephacryl S-200 was washed with three column volumes of Buffer A in a downward flow direction and then washed with two column volumes of the same buffer in an upward flow direction at a flow rate of 1 ml/min., which was maintained by a peristaltic pump (Gilson Minipuls). The concentrated and dialyzed Affi-Gel Blue fraction was applied to the column and eluted with buffer A at a flow rate of 1 ml/min.

The fractions containing the enzyme activity peak (see RESULTS below) are pooled (S-200 fraction) and stored at $-20^{o}C$ in Buffer A solution.This preparation is stable for at least six weeks.

RESULTS

Purification

Affi-Gel blue chromatography of the ammonium sulfate fraction is shown in Figure 1. As mentioned above, most of the phosphatase activity is well separated from the pyruvate kinase activity.

Sephacryl S-200 gel filtration of the Affi-Gel Blue fraction is shown in Figure 2. The bulk of the protein elutes off the column in a sharp peak at or near the void volume. The peak fraction of pyruvate kinase activity appears after the protein peak.

The protocol of the purification procedure together with the yields and specific activities at each step is summarized in Table I. The greatest purification is achieved in the Affi-Gel Blue chromatography step. This gel has been used in the purification of several kinases and pyridine nucleotide-dependent dehydrogenases (30). It has been hypothesized that the blue dye ligand binds to the nucleotide binding sites of the enzymes (32).

Molecular Weight Determination

The molecular weight of spinach pyruvate kinase, obtained by gel filtration on Sephacryl S-200, was estimated at 280,000 daltons (figure 3). Castor bean endosperm pyruvate kinase is presumed to have a molecular weight close to 200,000 (23). Molecular weights ranging from 160,000 to 265,000 daltons have been reported for pyruvate kinases from a variety of animal and bacterial sources (16).

pH Dependence

The activity of spinach pyruvate kinase at various pH's is shown in Figure 4. The optimal pH for enzyme activity is approximately pH 7.1. Activity falls rapidly as the medium becomes more acidic, but remains fairly stable in alkaline solution. The pH optimum for spinach pyruvate

kinase is similar to that of other pyruvate kinases derived from plant sources. (8,21,23,26,31).

ADP-PEP Kinetic Relationship

Pyruvate kinase showed typical hyperbolic dependence towards both ADP and PEP. A Lineweaver-Burke graph of 1/V against 1/[ADP] at different constant PEP concentrations is shown in Figure 5A. This plot yields a family of substrate dependence lines which have a common intersection just above the substrate axis. The apparent Michaelis constants for ADP at each fixed PEP concentration show that there is a slight dependence of the ADPkm's on PEP concentration (Table II).

A graph of 1/v against 1/[PEP] at different constant ADP concentrations is shown in Figure 5B. The lines on this graph discernably intersect at a point above the 1/[PEP] axis. The apparent km's for PEP at several fixed ADP concentration show a 23% decrease in the km's of PEP with a tenfold increase in the ADP concentration (Table III).

While these data are not sufficient to give any conclusions concerning enzyme mechanism, they are consistent with a single displacement reaction mechanism in which the substrates combine with the enzyme to form a ternary complex. The data also suggest that the enzyme's affinity for PEP is influenced by the level of ADP. This is in accord with addition of substrates to the enzyme in an ordered pattern, with PEP plus enzyme forming first a binary complex followed by the addition of ADP to form a ternary Enzyme-PEP-ADP complex.

Studies of pyruvate kinase's enzymatic mechanism have been made for rabbit muscle (29) and liver (5) enzymes. Kinetic measurements similar to those made in Figure 5 and inhibition studies lead to the conclusion that these pyruvate kinases use a sequential mechanism in which the substrates are added to the enzyme in a rapid random fashion. The data for cotton seed pyruvate kinase (9) are consistent with the random sequential mechanism; however, the data do not rule out an ordered reaction.

Recent studies with isozymes of pyruvate kinase from developing castor bean endosperm which included inhibition studies as well as substrate dependence kinetic studies concluded that both isoenzymes' enzymatic mechanisms are consistent with an ordered sequential mechanism and not a rapid equilibrium random addition mechanism (13).

Requirement for Monovalent Cation

Figure 6 shows that spinach leaf pyruvate kinase requires a monovalent cation for optimal activity. As has been observed with other pyruvate kinases (21) some enzyme activity was detectable in the absence of monovalent cation. This residual activity has been attributed to the presence of small quantities of monovalent cation present in the assay mixture in the absence of any added monovalent cation (21).

Relative activations by K^+, NH_4^+, Na^+, and Li^+ are similar to the activations obtained with pyruvate kinases from other plant sources (21,26,31) K^+ and NH_4^+ activations follow hyperbolic kinetics. Na^+ activation does not follow this pattern. These results are similar to the monovalent cation activation found with soybean root nodule pyruvate kinase (26). However, in cotton seed and pea seed pyruvate kinases Na^+ activation was observed to be hyperbolic (21).

Table III summarizes the kinetic parameters for NH_4^+ and K^+ activation obtained from a Hanes plot of the data presented in Figure 6. The Ka values for K^+ and NH_4^+ are similar while the Vmax for NH_4^4 is 65% of the Vmax for K^+. Rabbit muscle and pyruvate kinase from developing castor bean endosperm has similar Ka's for both K^+ and NH_4^+ (13). The data for soybean root nodule pyruvate kinase shows the Ka for NH_4^+ to be half that of K^+ (26).

Effect of NH_4^+ in the Presence of K^+

Experiments were conducted to determine the effect of NH_4^+ ion on pyruvate kinase activity in the presence of K^+ ion. Results

shown in Figure 7 reveal that NH_4^+ ion inhibits spinach pyruvate kinase when the KCl concentration was fixed at either 10mM or 50mM. From the slope of the curves the greatest inhibitory effect was observed at low concentrations of NH_4^+. The inhibitions at 10mM NH_4 were 25% and 12% for fixed KCl concentrations of 10mM and 50mM, respectively. At high concentrations of added NH_4^+ the pyruvate kinase activity in the presence of 50mM KCl approached the activity which would be expected if no K^+ were present (see Figure 6). With 10mM KCl the pyruvate kinase activity is below that observed with no K^+ present (see Figure 6). Control experiments in which varying concentrations of Li^+, a non-activating monovalent cation, were added , showed no activation at high added Li^+ concentrations. This indicates that high ionic strength cannot account for the levels of NH_4^+ inhibition observed. These results resemble those obtained from similar experiments conducted with soybean root nodule and rabbit muscle pyruvate kinase (26).

To examine further the interactive effects of monovalent cations and substrate concentrations, substrate kinetics for ADP (Fig 8) and for PEP (Fig 9) were determined in the presence of 50 mM KCl and 50 mM NH_4^+ (Figs 8a and 9a) and of 10 mM salts (Figs 8b and 9b). At all substrate levels and at both salt concentrations, KCl increases enzyme activity more that does the ammonium salt, and the enzyme activity with both cations is less than or equal to that with potassium alone.

Inhibition by ATP and by Citrate

Addition of ATP under standard assay conditions led to a small stimulation in enzyme activity at 0.5 mM and then a gradual inhibition of as much as 30 % at 10 mM ATP in the absence of added Mg^{+2} or slightly less with added Mg^{+2} (Fig 10). If physiological levels of ATP are less than 5 mM, as may often be the case, little or no inhibition could be expected from ATP.

Addition of citrate produced a slight inhibition of the enzyme when citrate levels were higher than 2 mM. Without added Mg^{+2}, 20 % inhibition was seen with 10 mM added citrate (Fig. 11).

Inhibition by Other Metabolites

Enzyme activities under standard assay conditions and with 0.1 mM PEP and 0.2 mM ADP were unaffected by the addition of any of the following metabolites at 1 mM concentrations: glutamine, glutamate, asparagine, aspartate, alpha ketoglutarate.

DISCUSSION

The kinetic properties of spinach leaf pyruvate kinase seen in these studies are generally consistent with properties of the enzyme from plant tissues as reported elsewhere (7,8,11-13,21,23,26,31). Considering the likely physiological levels of ammonium and potassium ions, the small increments in ammonium ion concentration resulting from addition of 1 mM NH_4^+ to the media, and the inhibitory effects of added ammonia found in this study when K^+ is already present, it seems clear that direct stimulation of the enzyme by ammonia is ruled out.

The levels of ATP required for inhibition of the enzyme activity (5 mM and above) are high compared to likely physiological levels of ATP in the cell. We have no direct information about the concentration of ATP in the cytosol, but it seems doubtful that stimulation of pyruvate kinase upon the addition of ammonia to the medium can be attributed to decreased ATP level, say from 10 mM to 5 mM. Much more likely would appear to be the possible stimulation of activity of the enzyme by an increased level of ADP in the 0.1 to 0.2 mM range. The Km value for ADP, around 0.165 mM, could easily be in the range of likely physiological concentration of ADP in the cytosol.

Steady state photosynthesis experiments with $^{14}CO_2$ and ^{32}P-labelled inorganic phosphate and photosynthesizing and respiring *Chlorella pyrenoidosa* (15) provide data that permit the estimation of ATP and ADP concentrations in those algae cells. In that report, it was established that for fully- labelled compounds such as PEP and 3-phosphoglycerate, one micromole of compound would have contained 300

microcuries of ^{32}P per phosphate position. There were about 200 microcuries of ^{32}P in ATP per cm^3 packed algae and this was located mainly in the terminal and middle phosphate groups. Thus the ^{32}P per phosphate position was 100 microcuries and the ATP level was 100/300 = 0.33 micromoles per cm^3. Similarly, the ~50 microcuries ^{32}P per cm^3 algae found in the single labelled phosphate position of ADP gives a calculated level of 0.17 micromoles per cm^3.

With spinach leaf cells, we do not have such estimates of ATP and ADP levels based on ^{32}P labelling, due to lack of rapid uptake of inorganic phosphate from the media by these isolated cells. From the labelling with ^{14}C following one hr photosynthesis with $^{14}CO_2$ (17), and from the chlorophyll content of these cells (~2.5 mg per cm^3) we estimate phosphoenolpyruvate at 0.02 micromoles cm^3 and phosphoglycerate at 0.25 micromoles cm^3, compared with 0.07 and 1.1 micromoles cm^3 respectively in Chlorella (15). The lower values for spinach leaves no doubt are due to the large part of the cell volume occupied by vacuole, but the similarity in ratios, and other levels of other metabolites suggest that comparable levels of these common metabolites may exist in the non-vacuolar parts of photosynthetic green cells. Thus we suspect that the levels of ATP and of ADP in the leaf cells may fall in about the same range as in the algae cells, that is 0.05 to 0.5 mM.

Given the Km for ADP of 0.17 mM (Table II, see also Fig 8a), enzyme activity would be almost linearly dependent on ADP concentration over most of this range. Whether or not substantial changes in ADP concentration in the cytosol of leaf cells would accompany ammonia-stimulated glutamine synthesis may depend on the rate of rebuilding of adenylate energy charge by transfer of ATP from the chloroplasts (in the light) or from mitochondria (in the dark). The rate of photosynthetic phosphorylation or oxidative phosphorylation would seem to be ample to negate any drop in energy charge due to glutamine synthesis in spinach cells (17), unless a local decline in energy charge (increase in ADP) occurs in the cytosol due to rate-limited transfer of ATP and ADP between cellular compartments.

In Chlorella (15), ammonia addition in the dark resulted in a decline

in steady state level of ATP of about 20% but in little or no increase in ADP level. At the same time PEP level declined more than 50% with ammonia addition at a level already below the Km PEP reported here for spinach. Thus, any increase in rate due to higher ADP would have been more than offset by lowered PEP concentration in those Chlorella. Evidence from studies with the procariotic organismm Anabaena (24) suggests that ammonia addition, which stimulates CO_2 fixation in the dark, does so via a decrease in energy charge resulting from increased glutamine synthesis. The change in energy charge and the stimulation of fixation of CO_2 could be abolished by addition of the inhibitor of glutamine synthesis, methionine sulfoximine. Thus, with micororganisms there seems to be a reasonable presumption that ammonia-stimulated regulatory effects are at least in part mediated by lowered energy charge resulting from increased glutamine synthesis.

Owing to the difficulties in determining levels of adenylates in subcellular compartments in spinach cells, it is not certain that such a mechanism is operating in these cells, but the kinetic properties of the enzyme described in this report, especially the Km value for the substrate ADP make this a possibility.

Acknowledgement

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FIGURE LEGENDS

Figure 1. Affi-Gel Blue Chromatography

The dialyzed G-25 fraction (94ml) was applied to a column of Affi-Gel Blue (2.5 x 18cm). Pyruvate kinase activity was eluted as described in the text. The initial wash fraction contained 8ml each. The gradient and final wash fractions contained 3ml each. The flow rate was 0.75ml/min.

Figure 2. Sephacryl S-200 Chromatography

The concentrated and dialyzed Affi-Gel Blue fraction (10ml) was applied to a column of Sephacryl S-200 (2.5 x 90cm). Pyruvate kinase activity was eluted as described in the text. Fractions of 4ml were collected at a flow rate of 1ml/min.

Figure 3. Molecular Weight Estimation

A column (2.5 x 80cm) of Sephacryl S-200 equilibrated with Buffer A was calibrated with molecular weight standards as described in Material and Methods. The same column was used for the rechromatography of a 1 ml aliquot of the S-200 fraction obtained from the pyruvate kinase purification procedure. Fractions of 3.1 ml each were collected at a flow rate of 0.75 ml/min.

Figure 4. Effect of pH on Spinach Pyruvate Kinase Activity

The standard reaction mixture as described in Material and Methods was used except that MES was employed to buffer the reaction solution between pH's 5.5 - 7.0 and Tris was used between pH's 8.0 and 9.0. The enzyme activity was measured using both MES and imidazole buffers at pH 7.0 and Tris and imidazole at pH 8.0. The enzyme activity was found not to be buffer dependent at those pH's.

Figure 5. Kinetics of Spinach Leaf Pyruvate Kinase with Respect to ADP and PEP.

Assays were conducted as described in material and Methods. In A, ADP was varied at various fixed PEP concentrations. In B, PEP was varied at various fixed ADP concentrations.

Figure 6. Dependence of Spinach Leaf Pyruvate Kinase on Concentration of Various Monovalent Cations

Enzyme assays were performed as described in Material and methods. The chloride salts were used in this experiment.

Figure 7. Inhibition of Spinach Pyruvate Kinase by Ammonium ion in the Presence of KCl

NH_4Cl and LiCl concentrations were varied in the presence of fixed levels of KCl. Assays were performed as described in Materials and Methods.

Figure 8. Kinetics of Spinach Pyruvate Kinase with Respect to ADP under varying Monovalent Cation Conditions

In A, substrate kinetics are measured in standard assay mixtures containing 50mM NH_4CL or 40mM KCl with and without added NH_4Cl. In B, substrate kinetics are measured in the standard assay mixture containing 10mM NH_4Cl or 10 mMKCl with or without added NH_4Cl.

Figure 9. Kinetics of Spinach Pyruvate Kinase with Respect to PEP under Varying Monovalent Cation Conditions.

In A, substrate kinetics are measured in standard assay mixtures containing 50mM NH_4Cl or 50mM KCl with and without added NH_4Cl. In B, substrate kinetics are measured in the standard assay mixture containing 10mM NH_4Cl or 10mM KCl with or without added NH_4Cl.

Figure 10. Effect of ATP on Spinach Pyruvate Kinase

Assays were performed as described in Material and Methods except that Tris ATP was added at varying concentrations with or without equimolar concentrations of Mg^{+2} ion.

Figure 11. Effect of Citrate on Spinach Pyruvte Kinase

Assays were performed as described in Material and Methods except that citrate was added to the reaction mixture at varying concentrations with or without equimolar concentrations of Mg^{+2} ion.

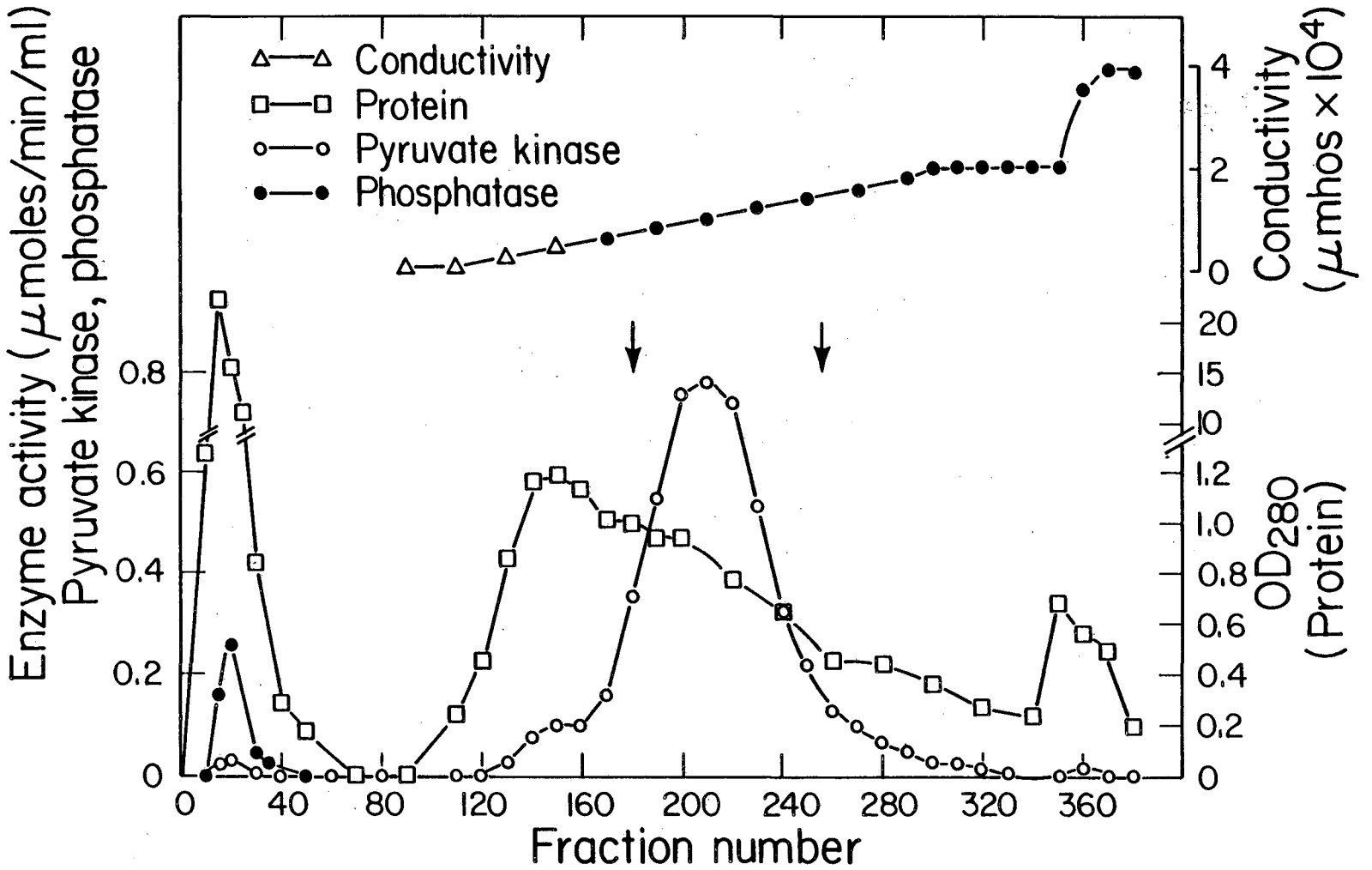


Figure 1. Affi-Gel Blue Chromatography

XBL 805-4178

The dialyzed G-25 fraction (94ml) was applied to a column of Affi-Gel Blue (2.5 x 18cm). Pyruvate kinase activity was eluted as described in the text. The initial wash fraction contained 8ml each. The gradient and final wash fractions contained 3ml each. The flow rate was 0.75ml/min.

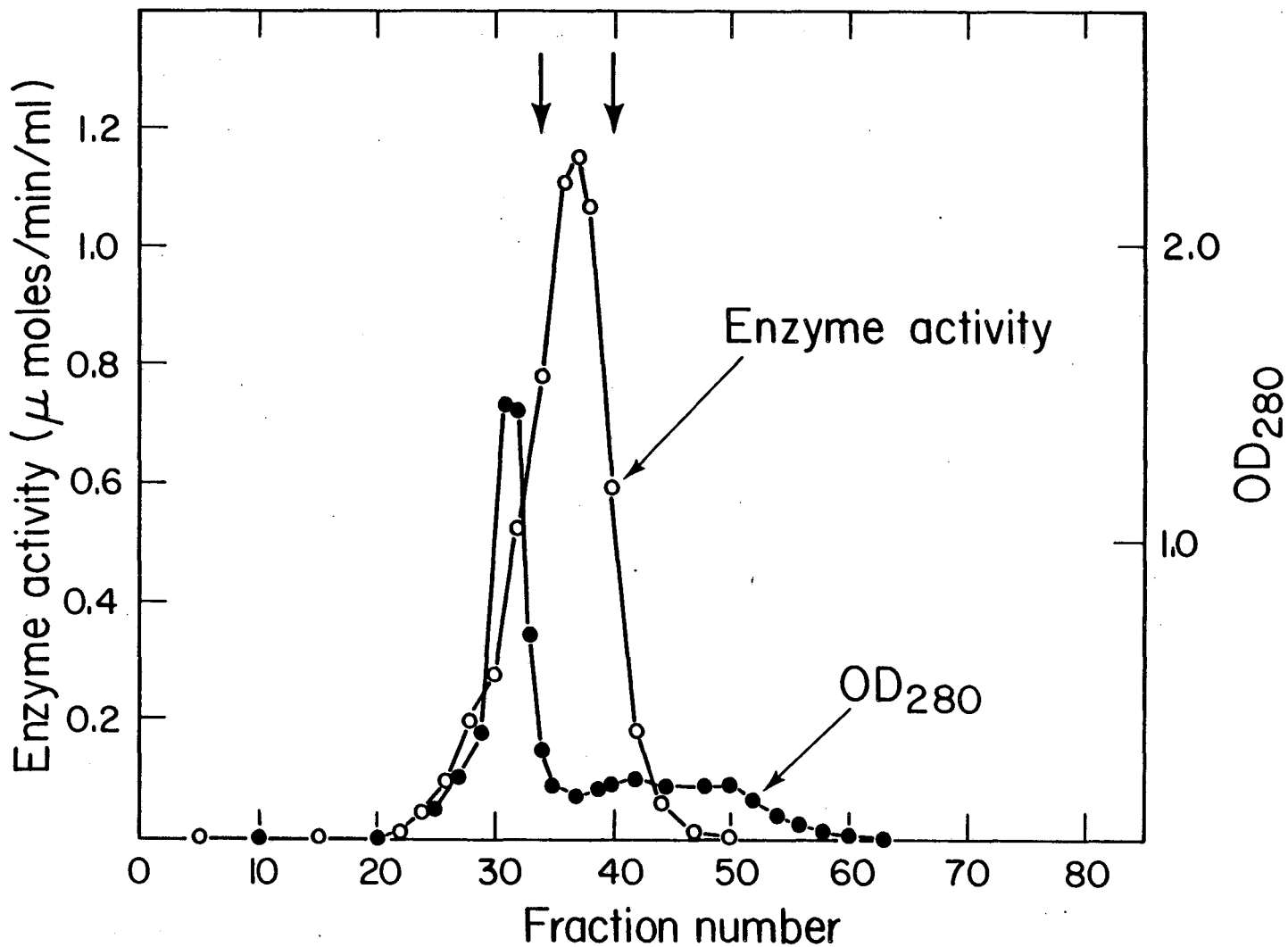
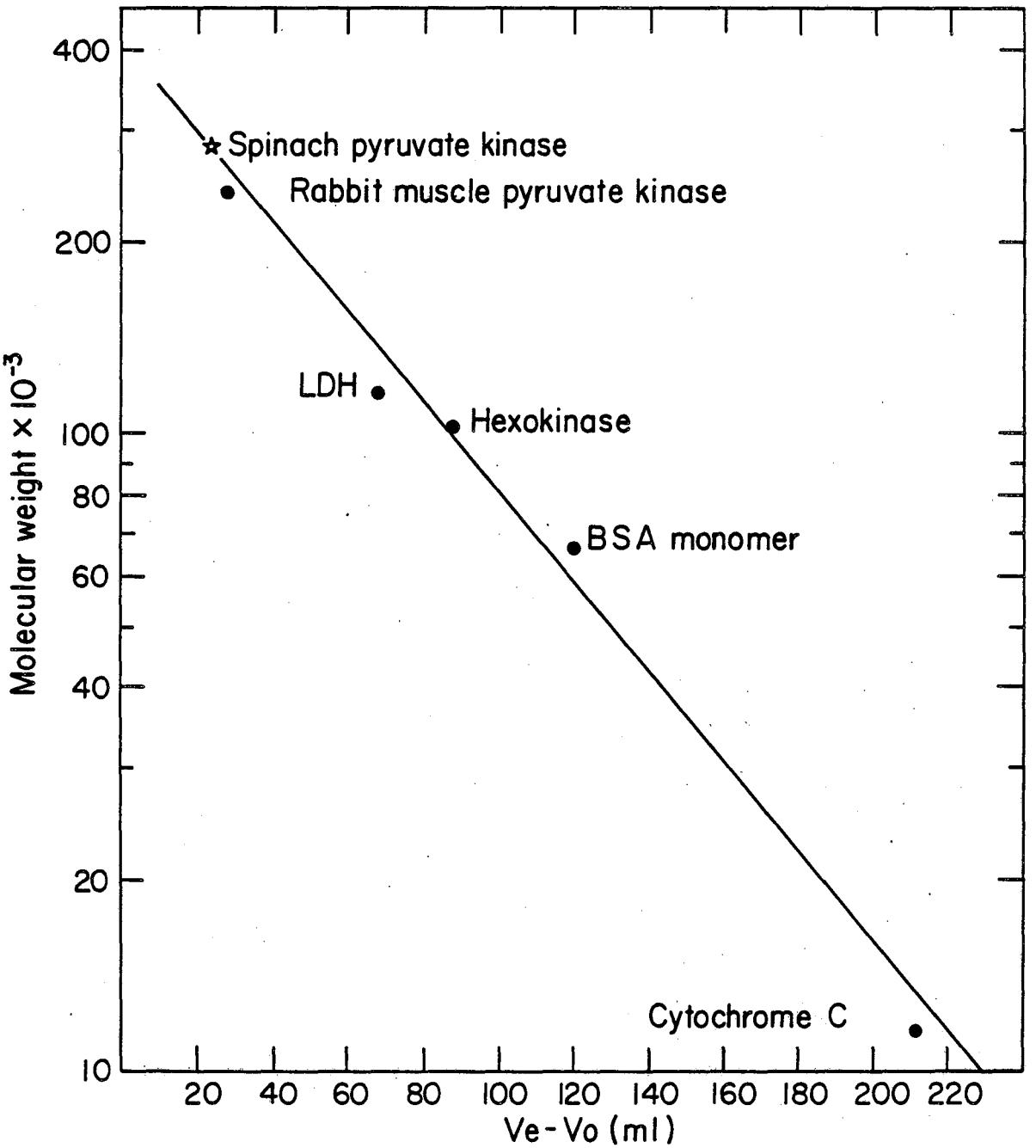


Figure 2. Sephacryl S-200 Chromatography

XBL 805-4179

The concentrated and dialyzed Affi-Gel Blue fraction (10ml) was applied to a column of Sephacryl S-200 (2.5 x 90cm). Pyruvate kinase activity was eluted as described in the text. Fractions of 4ml were collected at a flow rate of 1ml/min.



XBL 808-4289

Figure 3. Molecular Weight Estimation

A column (2.5 x 80cm) of Sephacryl S-200 equilibrated with Buffer A was calibrated with molecular weight standards as described in Material and Methods. The same column was used for the rechromatography of a 1 ml aliquot of the S-200 fraction obtained from the pyruvate kinase purification procedure. Fractions of 3.1 ml each were collected at a flow rate of 0.75 ml/min.

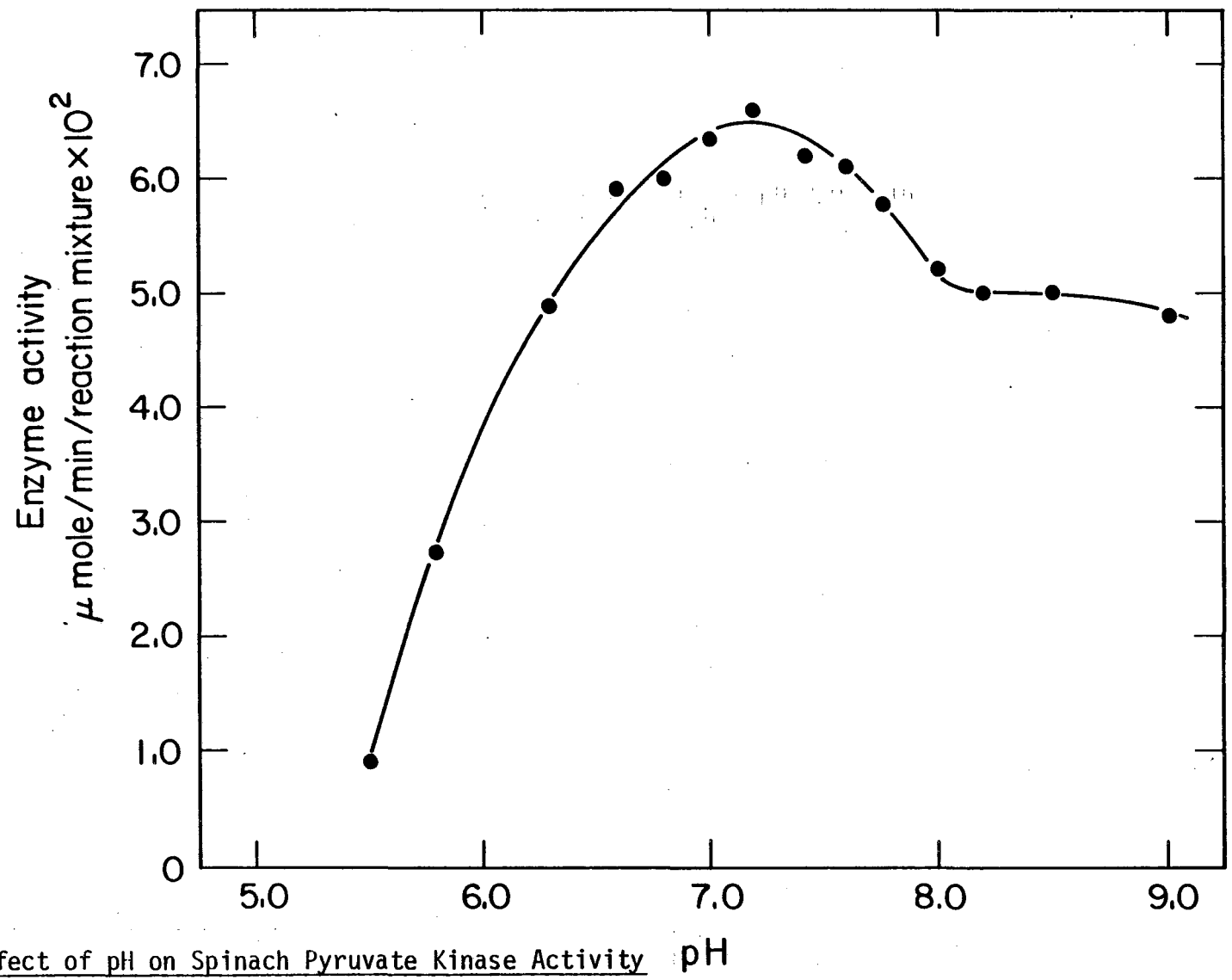
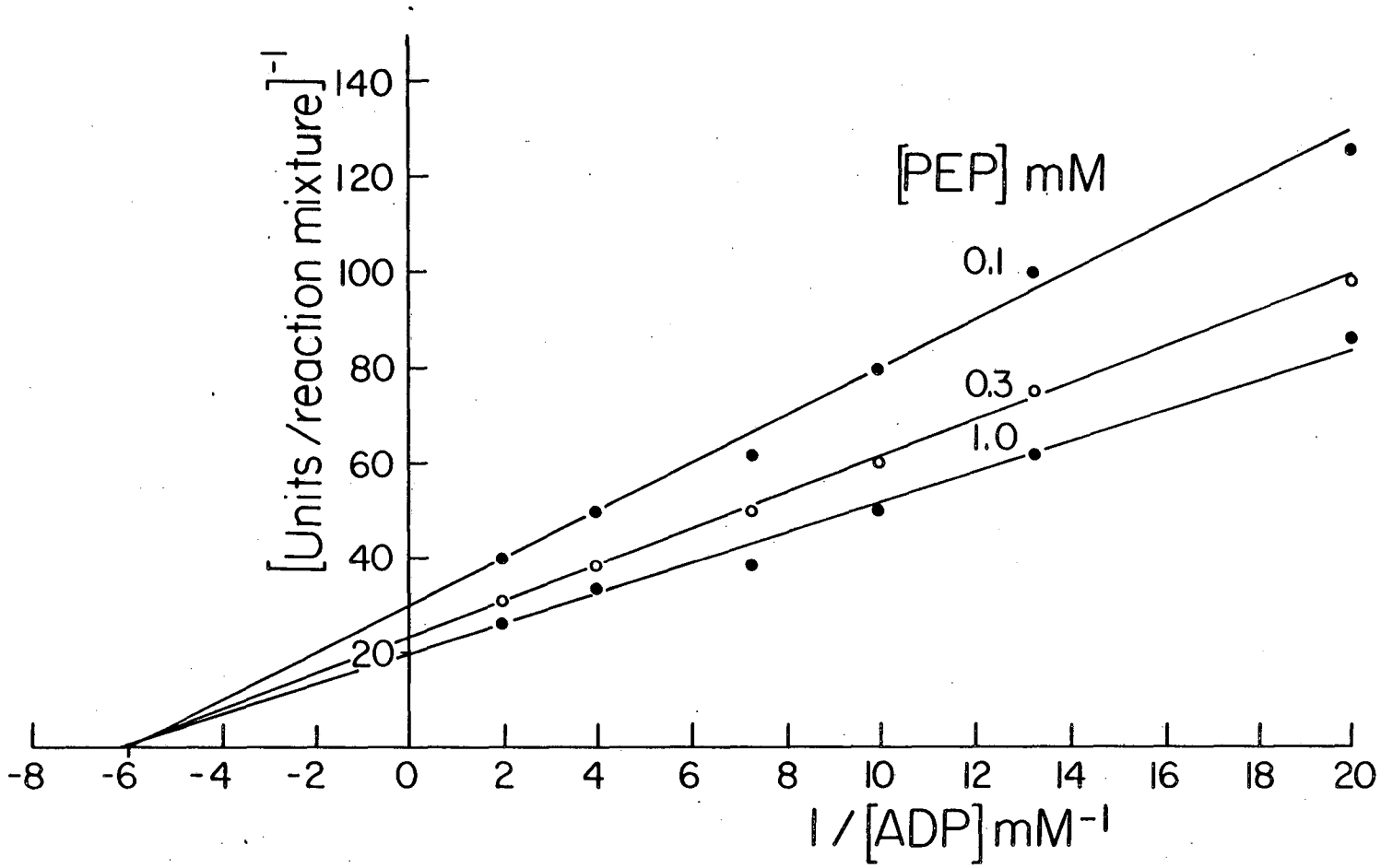


Figure 4. Effect of pH on Spinach Pyruvate Kinase Activity

The standard reaction mixture as described in Material and Methods was used except that MES was employed to buffer the reaction solution between pH's 5.5 - 7.0 and Tris was used between pH's 8.0 and 9.0. The enzyme activity was measured using both MES and imidazole buffers at pH 7.0 and Tris and imidazole at pH 8.0. The enzyme activity was found not to be buffer dependent at those pH's.

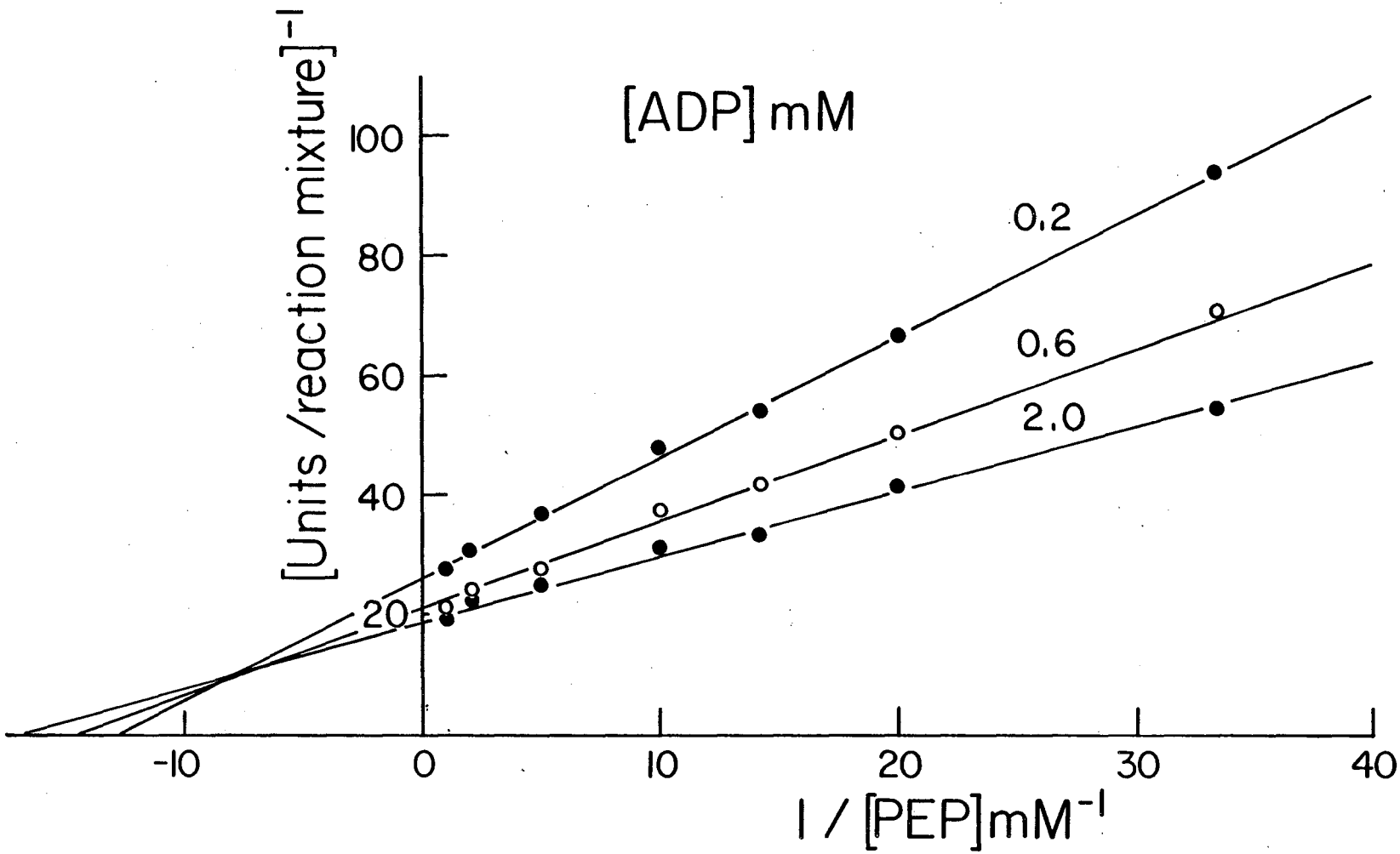
XBL 805-4180



XBL 808-4291

Figure 5A. Kinetics of Spinach Leaf Pyruvate Kinase with Respect to ADP and PEP

Assays were conducted as described in Material and Methods. ADP was varied at various fixed PEP concentrations.



XBL 808-4290

Figure 5B. Kinetics of Spinach Leaf Pyruvate Kinase with Respect to ADP and PEP

Assays were conducted as described in Material and Methods. PEP was varied at various fixed ADP.

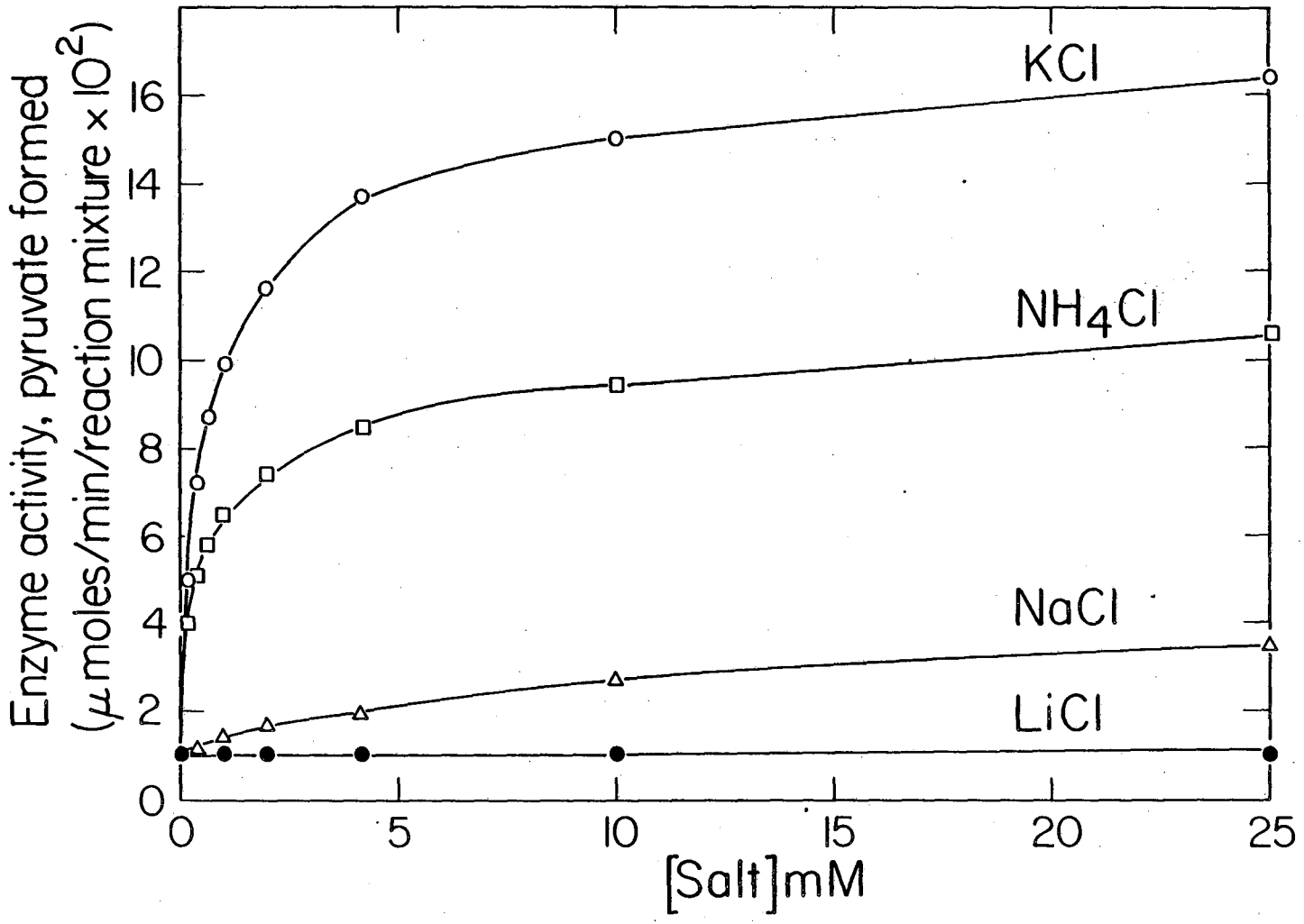


Figure 6. Dependence of Spinach Leaf Pyruvate Kinase on Concentration of Various Monovalent Cations

XBL 805-4176

Enzyme assays were performed as described in Material and Methods. The chloride salts were used in this experiment.

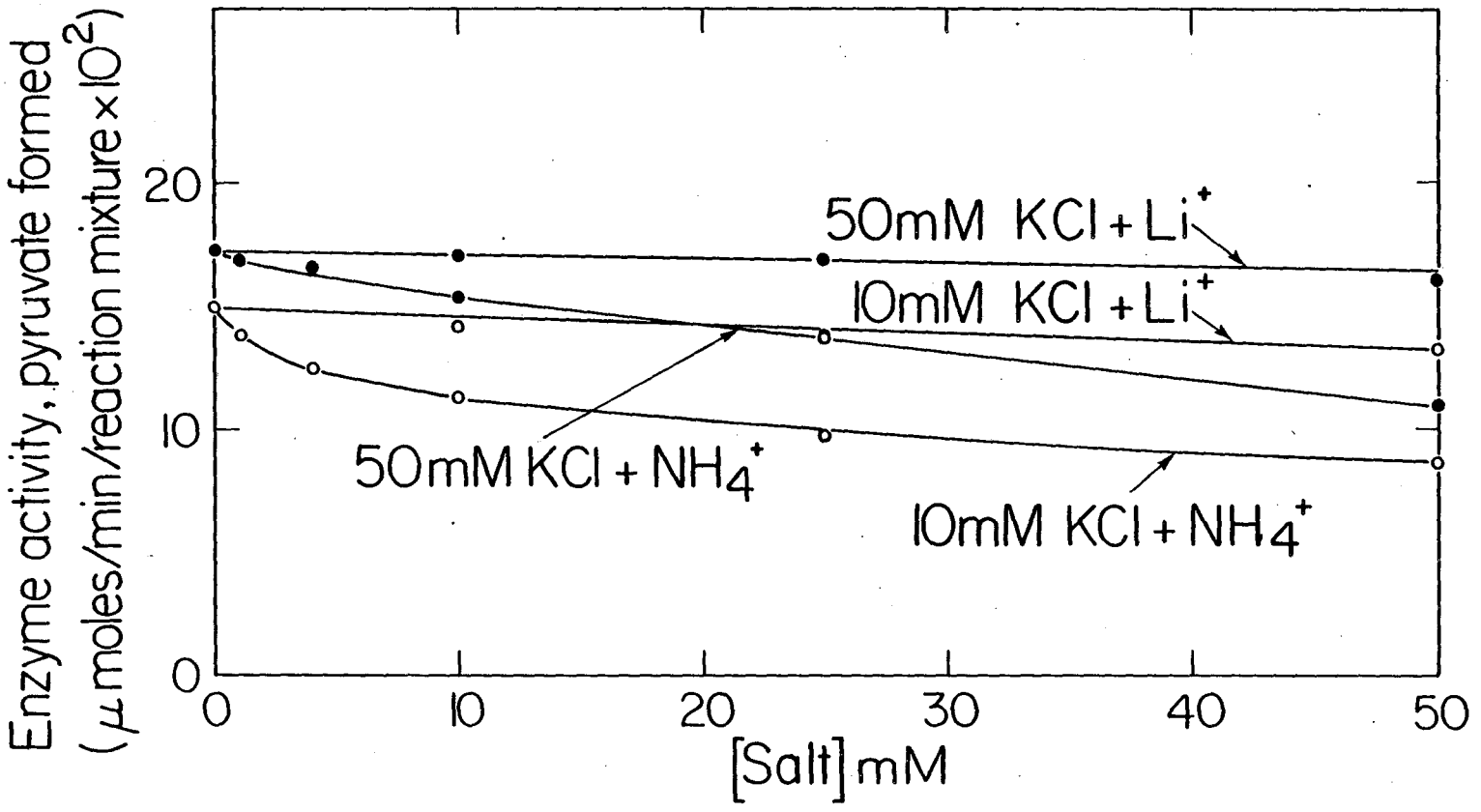
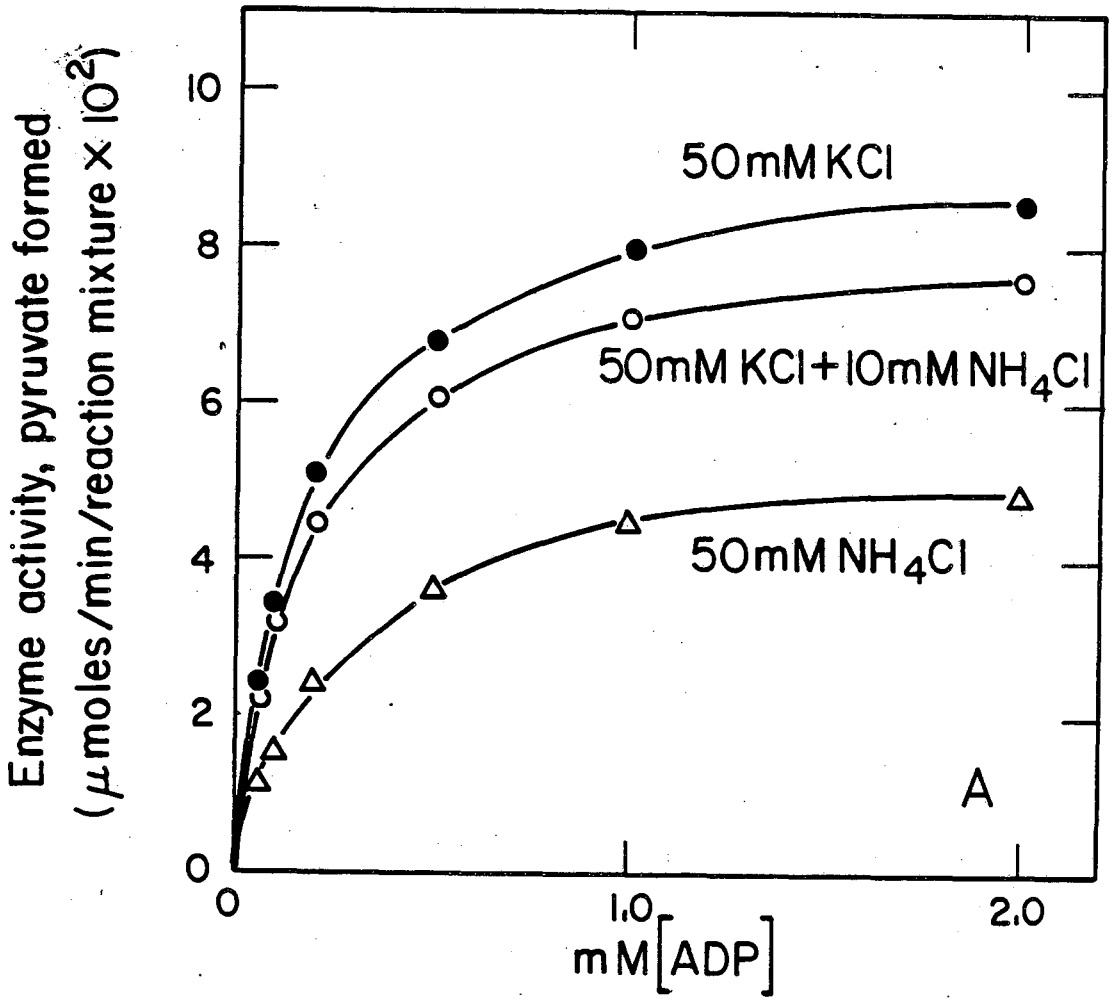


Figure 7. Inhibition of Spinach Pyruvate Kinase by Ammonium ion in the Presence of KCl XBL 805-4177

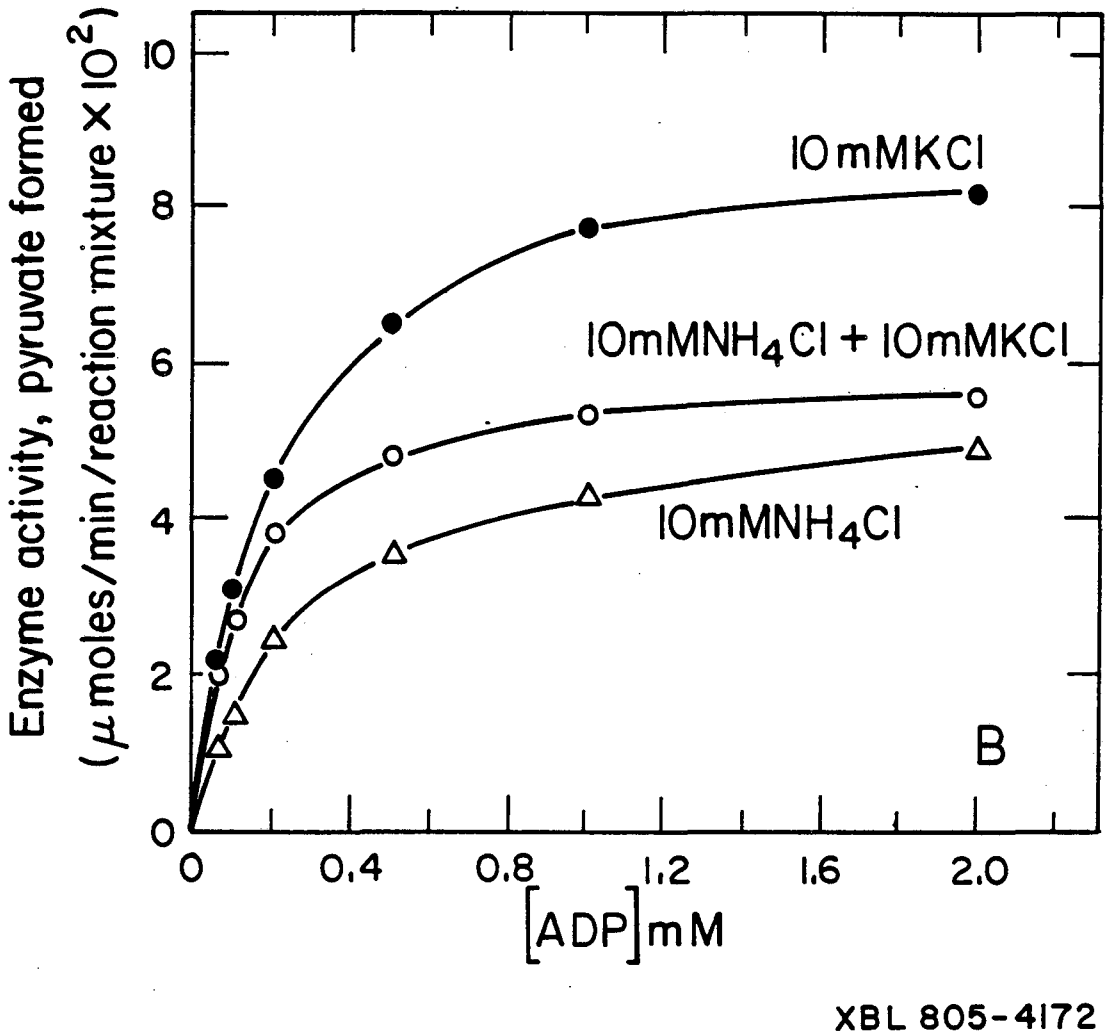
NH_4Cl and LiCl concentrations were varied in the presence of fixed levels of KCl. Assays were performed as described in Materials and Methods.



XBL 805-4173

Figure 8A. Kinetics of Spinach Pyruvate Kinase with Respect to ADP under Varying Monovalent Cation Conditions

Substrate kinetics are measured in standard assay mixtures containing 50mM NH_4Cl or 50mM KCl with and without added NH_4Cl.



XBL 805-4172

Figure 8B. Kinetics of Spinach Pyruvate Kinase with Respect to ADP under Varying Monovalent Cation Conditions

Substrate kinetics are measured in standard assay mixtures containing 10mM NH_4Cl or 10mM KCl with and without added NH_4Cl.

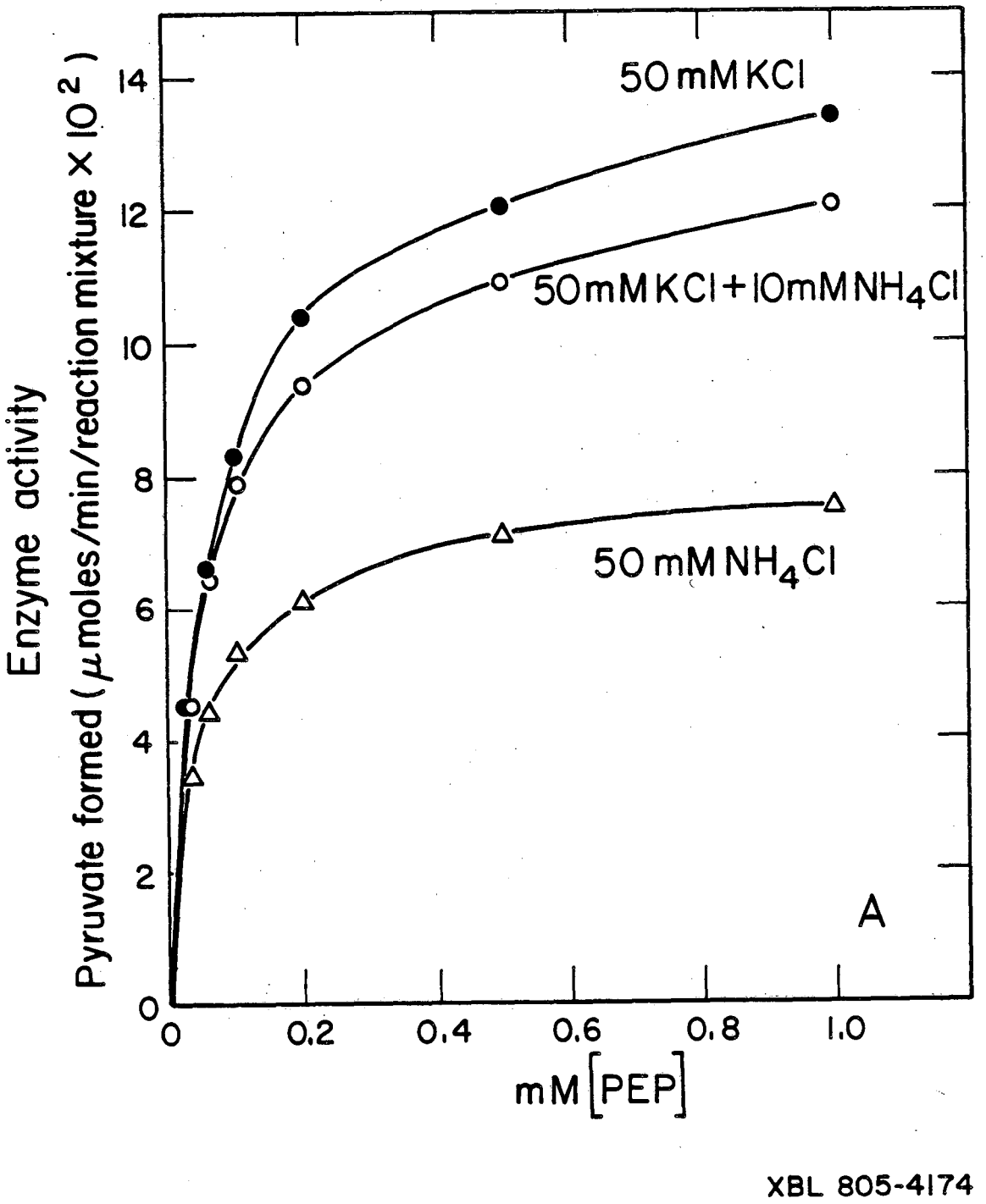
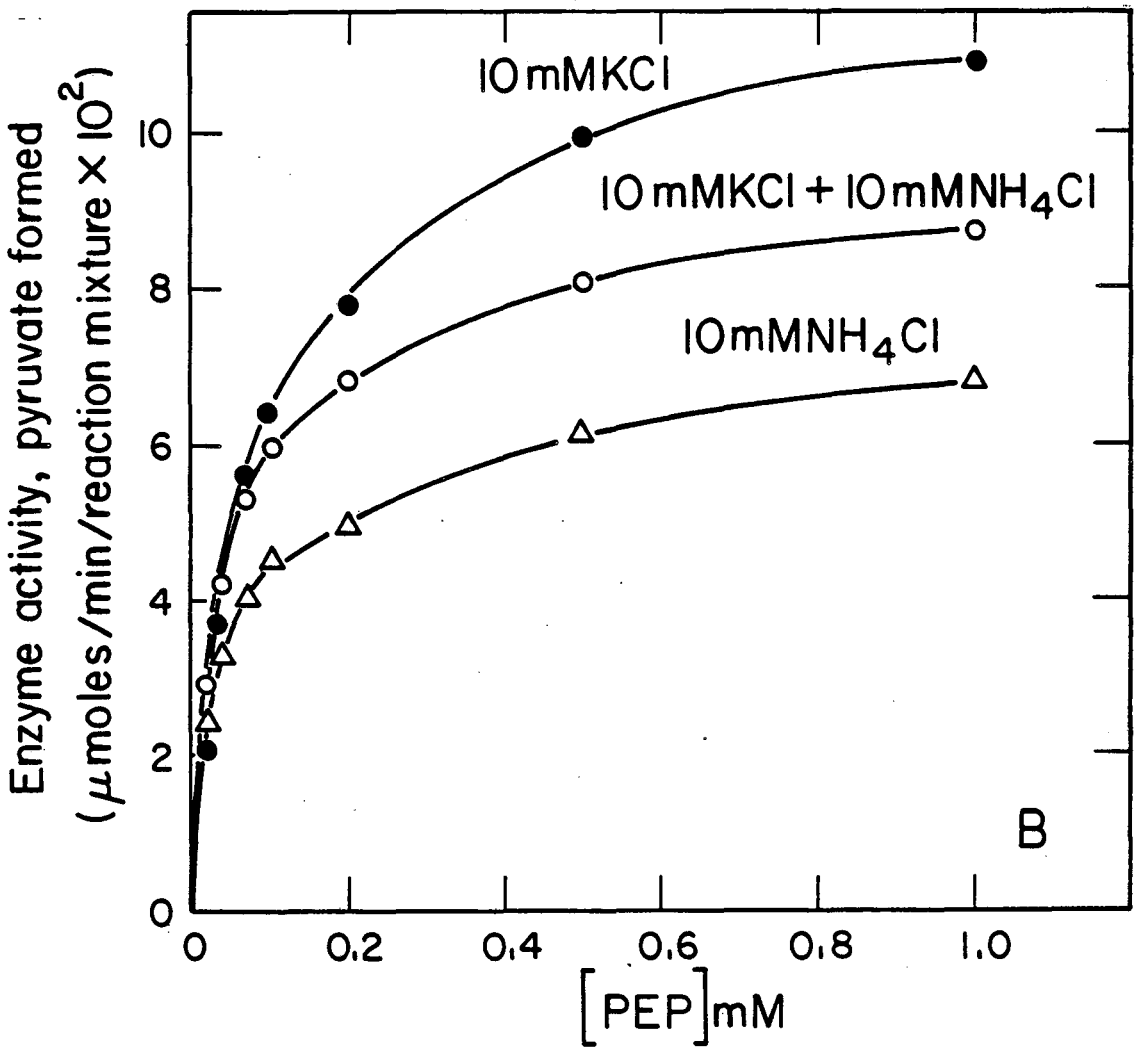


Figure 9A. Kinetics of Spinach Pyruvate Kinase with Respect to PEP under varying Monovalent Cation Conditions

Substrate kinetics are measured in standard assay mixtures containing 50mM NH_4Cl or 50mM KCl with and without added NH_4Cl.



XBL 805-4171

Figure 9B. Kinetics of Spinach Pyruvate Kinase with Respect to PEP under varying Monovalent Cation Conditions

Substrate kinetics are measured in the standard assay mixture containing 10mM NH_4Cl or 10mM KCl with or without added NH_4Cl.

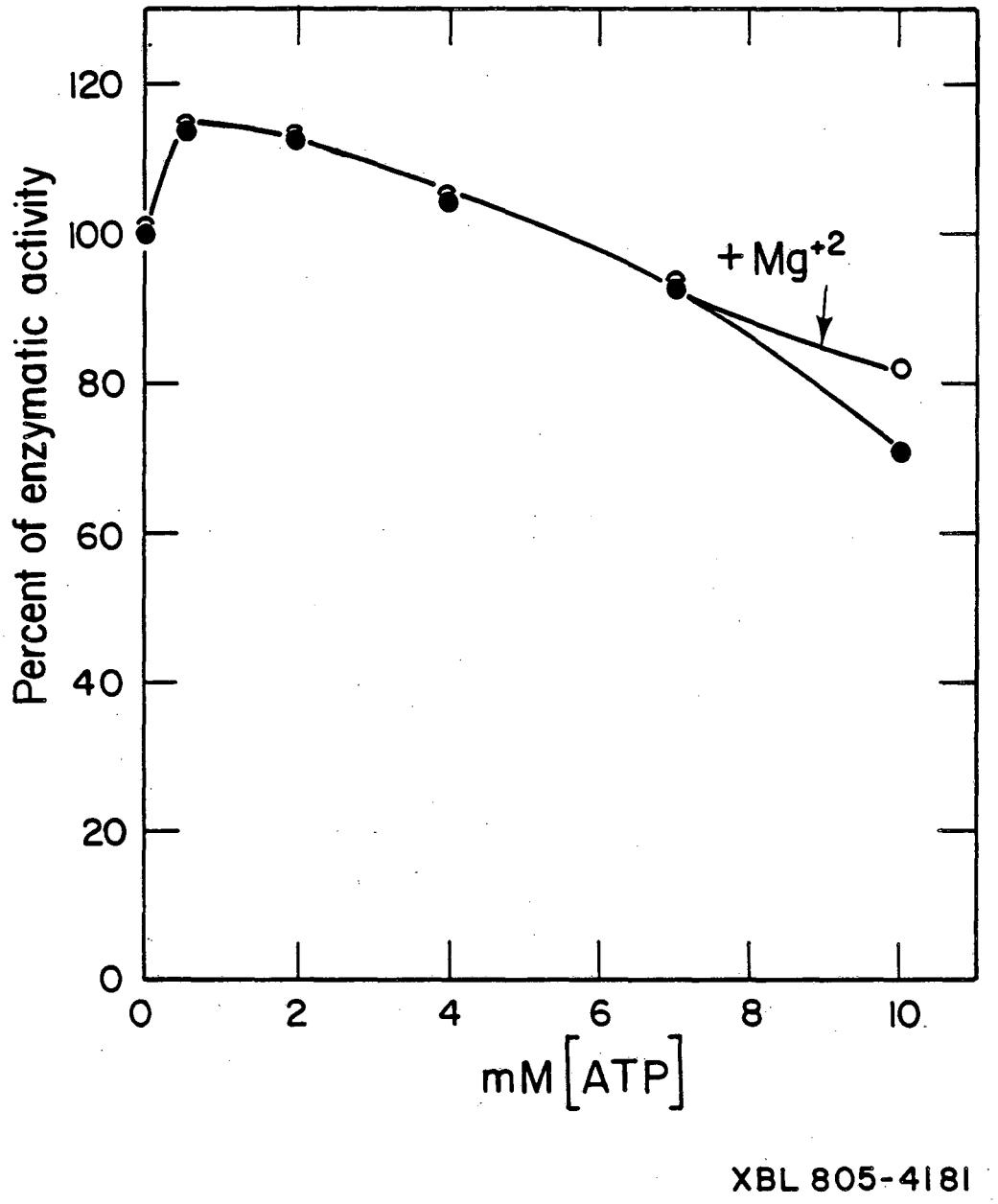
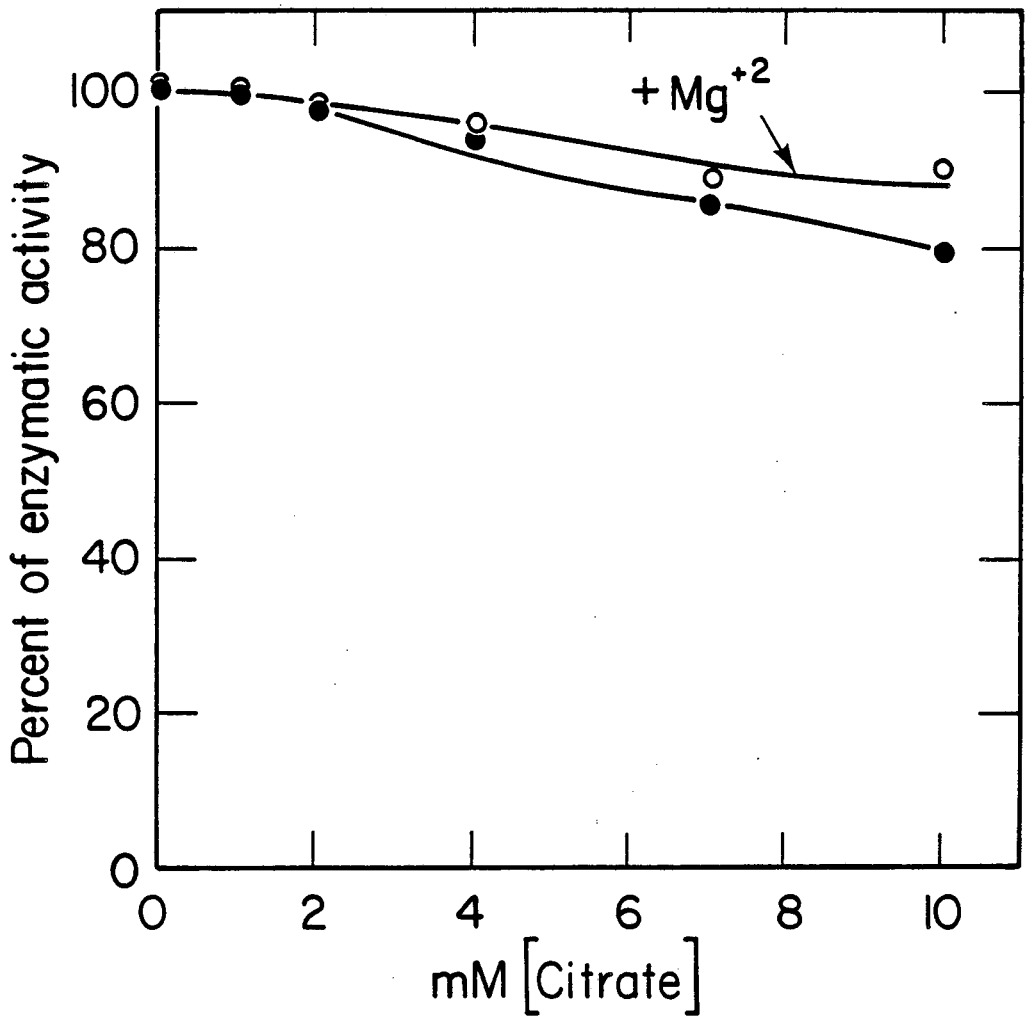


Figure 10. Effect of ATP on Spinach Pyruvate Kinase

Assays were performed as described in Material and Methods except that Tris ATP was added at varying concentrations with or without equimolar concentrations of Mg^{+2} ion.



XBL 805-4175

Figure 11. Effect of Citrate on Spinach Pyruvte Kinase

Assays were performed as described in Material and Methods except that citrate was added to the reaction mixture at varying concentrations with or without equimolar concentrations of Mg^{2+} ion.

This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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