# Phosphoglucomutase from Higher Plants: Multiple Forms and Properties

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CONTENTS

# I. SUMMARY

Phosphoglucomutase is widely distributed in nature, and has been purified from several different origins. Their molecular properties and reaction mechanism have been extensively studied. However, information on the enzyme from higher plants is limited. The purpose of the present studies is to purify phosphoglucomutase from potato tubers and to compare the molecular properties and reaction mechanism with those of other sources.

Two fractions (Peaks I and II) having phosphoglucomutase activity were separated by DEAE-cellulose chromatography of the crude enzyme preparation from potato tubers. Upon separate rechromatography under the same conditions, they were respectively eluted at the same positions as initially eluted. Incubation of Peaks I and II with glucose 1,6-bisphosphate which should cause conversion of the dephosphorylated form to the phosphorylated form resulted no change in their eluting positions in the rechromatography. Thus, Peak I and Peak II enzymes correspond neither to the phosphorylated and dephosphorylated forms of a single protein species nor *vice versa*. Their relative amounts in different experiments were fairly constant; the ratios of Peak I activity to Peak II activity in different samples of potato tubers of the variety "Danshaku" were ranged between 0.56 and 0.87, and two samples of the variety "May Queen" of

different ages gave a ratio of about 1.1. All the enzyme solution contained 2-mercaptoethanol, and were chromatographed in the presence of this reducing agent. It is unlikely that the enzyme species found are merely due to chemical and/or enzymic modification of the original enzyme during storage of the tissues or purification of the enzyme. Peak I enzyme was further separated into three fractions on isoelectric focusing. Besides the main enzyme peak of pI 5.76 (Peak Ia), two subsidiary peaks of pI's 5.91 and 6.20 (Peaks Ib and Ic, respectively) were observed. The relative amounts of three fractions on the isoelectric focusing patterns in different experiments were found to be fairly constant; the percentage of the amounts of Ia, Ib and Ic was 56-80, 15-30 and 3-12, respectively.

The resulting main fraction(Peak Ia) was purified 1800-fold over the crude extract with a 9% yield. It was homogeneous as judged by polyacrylamide gel electrophoreses in the absence and presence of sodium dodecyl sulfate. The specific activity, 480 units/mg protein, was 5 times as high as that reported by Pressey, and was comparable to those of the purified mammalian enzymes.

Isoelectric focusing of the Peak II preparation of the first DEAE-cellulose chromatography resulted in a major enzyme peak of pI 5.62 accompanying with a subsidiary peak of pI 5.81 which is supposed to be a contaminating Peak Ia, although the recovery of enzyme activity in this step was

low. These experimental results demonstrated that phospho= glucomutase from potato tubers contains at least two, possibly four different protein species.

On the other hand, both pea and broad bean seeds contained only a single species of phosphoglucomutase which corresponds to Peak II enzyme of potato tubers on the DEAE-cellulose chromatography.

The peak II enzyme of the first DEAE-cellulose chromato= graphy was further purified. The purification involved two Sephadex G-200 chromatography steps and two hydroxylapatite chromatography steps. The specific activity of the final preparation was 27 units/mg protein with a total recovery of 2%.

Chemical and catalytic properties of potato phospho= glucomutase were studied using the purified enzyme species, Peaks Ia, Ib, Ic and II. Their molecular weights are given as all approximately 60,000 daltons by gel filtration on Sephadex G-200. It is close to the values previously reported for the enzymes from other sources. The results of sodium dodecyl sulfate polyacrylamide gel electrophoresis of Peak Ia show that this enzyme is probably composed of a single polypeptide chain with a molecular weight of 62,000. No indication of the presence of subunit structure was obtained as examined under various conditions of denaturation.

The amino acid composition of Peak Ia was compared with those of the enzymes from other sources. It does

not exhibit large differences from the compositions of the enzymes from rabbit muscle and yeast. However, the high values for glycine and serine, and low values for isoleucine, leucine and proline in the potato enzyme are noteworthy.

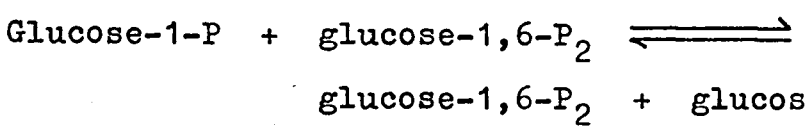
Both Peak Ia and II enzymes absolutely require α-D-glucose 1,6-bisphosphate and $Mg^{2+}$ for activity. The highest activity was obtained when either EDTA or cysteine was added to the above system. The results of kinetic studies indicate that both enzyme species act conforming to the "ping-pong" mechanism. Thus, potato phosphoglucomutase is more like the enzymes from animal origins rather than those from *Micrococcus lysodeikticus*, *Bacillus cereus* and *Saccharomyces cerevisiae*. Both Peak Ia and Peak II enzymes from potato tubers were similarly inhibited by fructose 1,6-bisphosphate and glycerate 2,3-bisphosphate, as found with the beef liver enzyme. A very low concentration of $Be^{2+}$ caused rapid inactivation of both Peak Ia and Peak II enzymes, as observed with the enzyme from rabbit muscle , human muscle, *Micrococcus lysodeikticus*, *Bacillus cereus* and *Saccharomyces cerevisiae*.

Although there are such similarities among the enzyme species of potato phosphoglucomutase, they differ in their kinetic parameters; the *Km* value of Peak Ia enzyme for glucose 1-phosphate is considerably smaller than that of Peak II enzyme. Further, the pH profile of Peak II enzyme

shifted to the alkaline side by about 0.4 pH unit compared with that of Peaks Ia, Ib, and Ic. The conventional fractionation of potato tuber tissues showed all the potato phosphoglucomutase spieces exist in the soluble fraction. The further comparison of both enzymes on chemical levels was difficult because of failure to purify Peak II enzyme to a homogeneous state.

## II. INTRODUCTION

Phosphoglucomutase (EC 2.7.5.1) catalyzes the overall reversible conversion of glucose-1-P to glucose-6-P, in which glucose-1,6-$P_2$ serves as the mediating reactant as shown in Eq. 1. This enzyme is widely distributed in



$$\text{Glucose-1-P} + \text{glucose-1,6-}P_2 \rightleftharpoons \text{glucose-1,6-}P_2 + \text{glucose-6-P} \quad \text{(Eq. 1)}$$

nature, and has been purified from several different origins, i.e., muscle (1,2,3), liver (4), yeast (5,6) and bacteria (7,8). Their molecular properties and reaction mechanism have been studied. Some of the enzymes exist as multiple forms (9).

However, information on the enzyme from higher plants is limited. Although properties of phosphoglucomutase from Phaseolus radiatus (10) and jack bean (11) have been examined using the crude extracts, the isolation in a homogeneous state has never been succeeded from these sources until the present work with potato tubers.

Two attempts to purify the enzyme have so far been made from potato tubers. Boser (12) first purified the enzyme through heat treatment, ammonium sulfate fractionation and calcium phosphate gel treatment. However, the specific activity of his purified preparation was only 1.9 units/mg

protein at 30°C, pH 7.5, far below that of the rabbit muscle enzyme. Pressey (13) later purified the enzyme through ethanol-acetone treatment, ammonium sulfate fractionation and Sephadex G-100 chromatography. Although the specific activity of his preparation is 50 times as high as that of Boser's preparation, it still contained much inert proteins. Joshi et al. (9) have mentioned the separation of potato phosphoglucomutase into two peaks on a DEAE-Sephadex column, although they as well as other workers have not further studied on the multiplicity of the potato enzyme.

Kahl and Stegemann (14) have recently reported that, upon cutting of potato tubers, phosphoglucomutase was rapidly degraded and its degradation was dependent on protein synthesis. The system seems to be suitable to study the role of protein degradation in the regulation of enzyme activity in higher plants. For the physiological studies of phosphoglucomutase in potato tubers, it is of importance to clarify the composition of the multiple forms and isolate the components in a homogeneous state.

On the other hand, with phosphoglucomutases from different sources, some conflicting results have been obtained on their subunit structure and kinetic mechanism. Recent works showed that phosphoglucomutases from rabbit muscle (15) and yeast (6) may be a dimeric enzyme, whereas it has long been believed that all the enzymes including

the above two are composed of each a single polypeptide chain of approximately 60,000 daltons (16). Further, the reaction of phosphoglucomutase from some microorganisms (5,8) follows possibly the "sequential" mechanism, unlike all the enzymes from animal sources which act conforming to the "ping-pong" mechanism (16). Comparison of chemical and enzymic properties of the enzymes among divergent organisms including higher plants should help to understand the structure-function relationship of the enzyme.

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Abbreviations: glucose-1-P, α-D-glucose 1-phosphate; glucose-1,6-$P_2$, α-D-glucose 1,6-bisphosphate; glucose-6-P, D-glucose 6-phosphate; fructose-1-P, D-fructose 1-phosphate; fructose-6-P, D-fructose 6-phosphate; fructose-1,6-$P_2$, D-fructose 1,6-bisphosphate; glycerate-2-P, D-glycerate 2-phosphate; glycerate-3-P, D-glycerate 3-phosphate; glycerate-2,3-$P_2$, D-glycerate 2,3-bisphosphate; SDS, sodium dodecyl sulfate.

## III. EXPERIMENTAL PROCEDURE

Materials  Glucose-1-P, fructose-1-P, fructose-6-P, 1-thio-D-glucose, UDP-glucose, AMP, ADP, ATP, GTP, UTP, NADP, glucose-6-P dehydrogenase, yeast alcohol dehydrogenase and bovine serum albumin were purchased from Sigma Chemical Co. Fructose-1,6-$P_2$, glycerate-2-P, glycerate-3-P and glycerate-2,3-$P_2$ were the products of Boehringer Mannheim. The glucose-1-P was purified on a column (2 x 16 cm) of Dowex 1, x 8 ($Cl^-$), 200-400 mesh, according to the procedure of Hanabusa et al. (8). Other reagents were used without further purification. Glucosyl fluoride and 5-thioglucose were gifted by Dr. M. Ariki. γ-Globulin and ovalbumin were the products of Nutritional Biochemicals Corporation. Rabbit muscle phosphorylase b prepared according to the procedure of Fischer and Krebs (17) was gifted by Mr. S. Shimomura. $BeCl_2$, urea and guanidine-HCl were the highest grade available from Nakarai Chemicals, Ltd. DEAE-cellulose (0.88 meq/g) was obtained from Brown Co. Ampholytes from LKB were used for isoelectric focusing. Sephadex G-50, CM-Sephadex C-50 and Sephadex G-200 were the products of Pharmacia. Hydroxylapatite, Bio-Gel HTP, was purchased from BioRad Co. All other reagents were of special grade obtained from Wako Pure Chemical Industries and Nakarai Chemicals, Ltd.

Assay of enzyme activity In the assay of phosphoglucomutase activity, two methods were employed as follows.

Assay A: The reaction mixture contained 1 µmol of glucose-1-P, 20 nmol of glucose-1,6-$P_2$, 0.29 µmol of NADP, 0.5 unit of glucose-6-P dehydrogenase, 0.1 µmol of EDTA, 5 µmol of $MgCl_2$, 20 µmol of Tris-HCl, and the enzyme in a final volume of 0.5 ml. After preincubation in a cell of 0.2-cm light path at 25°C for 5 min, the reaction was initiated by the addition of the enzyme. The increase in absorbance at 340 nm was recorded with a Hitachi spectrophotometer Model 124 equipped with a temperature controlled cell holder. One unit of enzyme was taken to be that amount of enzyme which catalyzes the conversion of 1 µmol of glucose-1-P to glucose-6-P in 1 min under the assay conditions. During purification Assay A was employed because of its rapidity and independency from the inorganic phosphate present in samples. Specific activity was expressed as units per mg of protein. In some experiments using the purified enzyme, the reaction mixture was scaled up 4-fold, and a cell of 1-cm light path was used. The purified enzyme was diluted with 5 mM Tris-HCl buffer, pH 7.4 containing 0.2 mM EDTA and 20% (v/v) glycerol.

Assay B: The modified Najjar's colorimetric assay (1). This method was employed in experiments of requirements and pH dependency. The reaction mixture contained 1 µmol

of glucose-1-P, 20 nmol of glucose-1,6-$P_2$ and 20 μmol of Tris-HCl, pH 7.5 in a final volume of 0.5 ml. The reaction was started by the addition of the purified enzyme suitably diluted with the buffer. After 5 min, the reaction was stopped by the addition of 0.5 ml of 10 N $H_2SO_4$, and the reaction mixture was heated at 100°C for 7 min. The amount of glucose-1-P was determined from acid-labile phosphate according to the method of Fiske and Subbarow (18). Under optimal conditions, formation of glucose-6-P was proportional to enzyme concentration and was linear with time until about 50% of the glucose-1-P was converted to glucose-6-P.

Phosphorylase activity was measured in the direction of starch synthesis according to the method of Iwata and Fukui (19).

Protein Assay The amount of protein was determined by the method of Lowry *et al.* (20) using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis Polyacrylamide gel electrophoreses in the absence and presence of sodium dodecyl sulfate were performed according to the methods of Davis (21) and Weber and Osborn (22), respectively.

Amino acid composition The amino acid composition was determined after acid hydrolysis at 110°C *in vacuo* for 24 hr and 72 hr, with a Beckman Model 120B amino acid analyzer equipped with the accelerated system. The contents

of threonine, serine and tyrosine were calculated from the results of 24-hr hydrolysis, and those of valine, methionine and isoleucine from the results of 72-hr hydrolysis. For other amino acids except half-cystine and tryptophan, average values of the two hydrolyses were used for calculation. The half-cystine content was determined by the performic acid oxidation method (23). The tryptophan content was estimated from the ultraviolet absorption according to the method of Bencze and Schmid (24). All the values were calculated based on a molecular weight of 62,000, and are shown as the nearest integers.

Cellular fractionation of the enzymes All operations in the fractionation were performed at 4°C.

Method I: The method of Matsushita and Uritani (25) used for the fractionation of the crude extract from sweet potato roots was modified.

A potato tuber (about 200 g) was washed with tap water and cooled in an ice bath for 30 min. The tuber was peeled and cut into two half pieces. Cylinder like pieces were prepared from their parenchymatous tissue with a cork borer. The tissue thus prepared (fresh weight 10 g) was cut into cubic pieces with 2-3 mm length. Then, they were mixed with 10 ml of 50 mM Tris-HCl buffer, pH 7.0 cotaining 0.7 M mannitol, and 1% (w/v) sodium ascorbate, and 1 g of Polyclar AT and given two 30-sec homogenizations in a blender. The homogenate was squeezed through three

layers of gauze cloth.

The filtrate was centrifuged at 900 x g for 5 min to remove mainly starch granules after its pH had been adjusted to 7.0 with 1 N KOH. Then, the supernatant fluid was centrifuged at 14,000 × g for 15 min. The pellet was suspended in 5 ml of 50 mM Tris-HCl buffer, pH 7.0, containing 0.7 M mannitol (washing medium) and centrifuged at 14,000 × g for 15 min to precipitate the mitochondrial fraction. The supernatant material was centrifuged at 100,000 × g for 1 hr (a Beckman preparative ultracentrifuge L3-50, No. 50 rotor) and the resulting supernatant solution (the supernatant fraction) was saved. The pellet was suspended in 5 ml of the washing medium and the suspension was centrifuged at 100,000 × g for 1 hr to precipitate the microsomal fraction. The mitochodrial and microsomal fractions were suspended in the washing medium.

Method II: Lavintman's method (26) for preparation of the potato tuber particulate fraction was modified.

A potato tuber (100 g) cooled in an ice bath were peeled and grated with a grater in the presence of 0.25 ml 2-mercaptoethanol (a final concentration in the juice, 40 mM). The suspension was strained through three layers of gauze cloth and the filtrate was centrifuged at 2000 × g for 10 min, in order to separate starch. The supernatant solution was centrifuged again at 25,000 × g for 10 min after its pH had been adjusted to 7.0. The

pellet was suspended in 2.4 ml 0.1 M Tris-HCl buffer, pH 7.2, containing 0.05% 2-mercaptoethanol and stored at 4°C. In all centrifugation procedures after this, each resulting pellet was suspended in several milliliters of the buffer. The supernatant fluid was then centrifuged at 100,000 × g for 1 hr, and finally, the supernatant solution was centrifuged at 140,000 × g for 2 hr.

Each supernatant solution and suspension thus obtained were assayed for the protein content and enzymic activities.

# IV. RESULTS

## 1. Chromatographic Separation of Potato Phosphoglucomutase

### 1-1. Preparation of crude enzyme

Unless otherwise stated, all operations in the purification were performed at 4°C. All centrifugations were run at 10,000 × g for 10 min.

Potato tubers (Solanum tuberosam var. "Danshaku", 5 kg) purchased at a market were washed in tap water, peeled, sliced in 1 to 2 cm thickness, and soaked for 30 min in 4 liters of a solution containing 0.7% sodium dithionite and 0.7% sodium citrate. The slices were then homogenized in a juice extractor (Hitachi) at the maximum speed. The juice was centrifuged after the addition of 2-mercaptoethanol to a final concentration of 5 mM to prevent enzymic darkening. The supernatant fluid (about 2 liters, pH 6) was filtered through two layers of gauze cloth and its pH was adjusted to 7.5 with 1 N KOH. The pH of the crude extract should be brought to 7.5 as soon as possible, since the optimal pH for stability of the crude enzyme was 7.5, and the activity decreased to less than 50% of the initial in 20 hr at pH 6.0 at 4°C.

The specific activity of the crude extract ranged from 0.25 to 0.54, usually 0.3-0.4, throughout all seasons.

When budded tubers were used, it was lower, 0.16-0.29. The enzyme in the crude extract thus prepared was fairly unstable; as much as 50% of the initial activity was lost at 4°C within a week. However, it was considerably stabilized with glycerol and ethylene glycol. Little activity was lost in the presence of 20% (v/v) glycerol or 30% (v/v) ethylene glycol at pH 7.5 at 4°C for two weeks. Therefore, the buffers used in all the following experiments contained 20% (v/v) glycerol (Fig. 1). In the gel chromatography on Sephadex G-200, the concentration of glycerol was reduced to 10% because of its viscosity.

Fig. 1

To the pale yellow supernatant fluid were added, slowly with continuous stirring, 277 g of solid ammonium sulfate per liter (a 45% saturation), and its pH was readjusted to 7.5 with 1 N KOH. After 30 min the solution was centrifuged. The supernatant fluid was brought to a 75% saturation by the slow addition of 210 g of solid ammonium sulfate per liter, and left for 30 min. The resulting precipitate was collected by centrifugation, and dissolved in about 100 ml of 5 mM potassium phosphate buffer containing 0.2 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$ and 20% (v/v) glycerol, pH 7.4 (Buffer A). The dark brown solution was desalted by passing through

a column (5 × 28 cm) of Sephadex G-50 coarse previously equilibrated with Buffer A. Colored materials were removed from the protein by this chromatography. The protein fractions were combined and centrifuged to remove insoluble materials.

1-2. Chromatography on DEAE-cellulose

The crude enzyme solution (about 160 ml) obtained above was added to the top of a column (5.5 × 29 cm) of DEAE-cellulose previously equilibrated with Buffer A. The column was washed with 800 ml of Buffer A, and eluted with a linear gradient of potassium chloride in the same buffer (0 to 0.45 M KCl in 2 liters) at a flow rate of 2 ml/min. On this chromatography, enzyme activity was eluted in two separate peaks (Fig. 2).

Fig. 2

To confirm the separation of the two peaks (called Peaks I and II in the order of elution), they were separately rechromatographed under the same conditions as in the initial chromatography. Peaks I and II were respectively eluted at the same positions as initially eluted (Fig. 3). Both fractions required glucose-1,6-$P_2$ for activity, showing that they are perhaps the

dephosphorylated forms. The incubation of Peaks I and II with glucose-1,6-$P_2$ which should cause conversion of the dephosphorylated form to the phosphorylated form did not change the elution patterns of rechromatography.

Fig. 3

## 2. Purification of Peak I Enzyme

### 2-1. Further chromatography on DEAE-cellulose and Sephadex G-200

The combined Peak I fractions (390 ml) was dialyzed overnight against one change of Buffer A (each 5 liters). The dialyzed solution was charged on a DEAE-cellulose column (4 x 21 cm) previously equilibrated with Buffer A, and eluted with a linear gradient of potassium chloride in the same buffer (0 to 0.1 M KCl in 600 ml). The flow rate was 0.5 ml/min. The enzyme activity was eluted in a single peak corresponding to an apparent KCl concentration of 0.075 M in the gradient. Fractions of 5 ml were collected and assayed for protein and enzyme activity.

The active fractions were pooled, and concentrated to several milliliters in an ultrafiltration cell (Type MC-2, Bioengineering, Co.) equipped with Diaflo PM 10 membrane (Amicon Far East Ltd.) at a nitrogen pressure

of 2 $kg/cm^2$. Then, it was diluted with 50 ml of 0.1 M potassium phosphate buffer containing 0.2 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$ and 10% (v/v) glycerol, pH 7.4 (Buffer B), and concentrated again in the same manner. The procedure was repeated several times to replace completely the buffer. The concentrated solution (3.5 ml) was applied on the bottom of a Sephadex G-200 column (2.5 × 105 cm) previously equilibrated with Buffer B, and eluted in an up-flow manner with the same buffer through a peristaltic pump. The flow rate was 7.5 ml/hr.

In this chromatography, enzyme activity was eluted in a main protein peak, and two shoulder peaks after and before the main peak had no enzyme activity. Fractions of 5 ml were collected. The active fractions were pooled and concentrated to 3.3 ml through the ultrafiltration.

2-2. Isoelectric focusing

The Peak I enzyme obtained above was subjected to isoelectric focusing in an LKB 8101 column (110 ml) containing 1% ampholytes, pH 5 to 7, in a 0 to 50% glycerol gradient with the anode at the bottom. The enzyme solution was applied to the column at a position about 10 cm upper from the bottom of the column. Electrophoresis was conducted for 93 hr at 1000 v and 0.8 mamp. The temperature was maintained at $0^{o}C$ by circulating ice cold water. After electrophoresis, fractions of 1 ml were collected.

Figure 4 shows the pattern of isoelectric focusing of Peak I enzyme. Besides the main enzyme peak of pI 5.76 (Peak Ia), two subsidiary peaks of pI's 5.91 and 6.20 (Peaks Ib and Ic, respectively) were observed. Fractions 35 to 38 corresponding to Peak Ia were combined and the ampholytes were removed by ultrafiltration.

Fig. 4

The specific activity of the Peak Ia enzyme was increased about 1800-fold over the crude extract, and the total recovery was 9%. Active fractions having different pI's were no longer observed on the repeated isoelectric focusing of this preparation. The specific activity, 480 units/mg protein, was 5 times as high as that reported by Pressey (13), and was comparable to those of the purified mammalian enzymes (1,3,4). The purified enzyme preparation of potato tubers stored at 4°C in Buffer A retained its full activity for one week. Purification procedure of Peak I enzyme is summarized in Table I. The increase of total activity after the ammonium sulfate fractionation may be resulted from removal of such inhibiting materials as heavy metals and polyphenols.

Table I

2-3. Purity of Peak Ia enzyme

Figure 5 shows the results of polyacrylamide gel electrophoreses of the Peak Ia enzyme in the absence and presence of sodium dodecyl sulfate. Both figures show each a single protein band.

Fig. 5

3. Purification of Peak II Enzyme

The peak II enzyme of the first DEAE-cellulose chromatography was further purified. The combined Peak II fractions (230 ml) was brought to a 75% saturation by the addition of solid ammonium sulfate after its pH had been adjusted to 7.5 with 1 N KOH. After 1 hr the resulting precipitate was collected by centrifugation and dissolved in a small volume of Buffer B. The solution was dialyzed against 1 liter of the same buffer and further concentrated by ultrafiltration. Then, it was charged on a Sephadex G-200 column (2.5 × 105 cm) and eluted in the same conditions as in the case of Peak I enzyme. The active fractions were combined and concentrated by ultrafiltration.

The concentrated solution was applied to the second Sephadex G-200 chromatography in the same manner as described above. The active fractions were combined, concentrated

by ultrafiltration, and dialyzed against 1 liter of Buffer A overnight with one change of the buffer.

The dialyzed solution was charged on a hydroxylapatite column (2.8 x 21 cm) previously equilibrated with Buffer A. Then, the column was washed with 120 ml of Buffer A, and eluted with a linear gradient of potassium phosphate in the same buffer (5 to 250 mM potassium phosphate, pH 7.4, in 600 ml) at a flow rate of 5 ml/10 min. In this chromatography, the elution pattern of protein consisted of two adjacent peaks, and the protein peak eluted first accompanied the enzyme activity. The active fractions were combined, concentrated and dialyzed in the same manner as described above.

The concentrated solution was applied again to hydroxylapatite column chromatography in the same manner as in the first hydroxylapatite column chromatography except that a lower linear gradient of potassium phosphate (5 to 125 mM in 600 ml) was used. The active fractions were combined and concentrated by ultrafiltration.

The specific activity of the final preparation was 27 units/mg protein with a total recovery of 2%. Although this preparation still contained much inert proteins, no further purification was achieved by such procedures as heat treatment, fractional precipitation with organic solvents, chromatography on DEAE-Sephadex or ECTEOLA-cellulose, and the third chromatography on Sephadex G-200.

Purification procedure of Peak II enzyme is summarized in Table II.

Table II

Isoelectric focusing of the Peak II preparation of the first DEAE-cellulose chromatography resulted in a major enzyme peak of pI 5.62 accompanying with a subsidiary peak of pI 5.81 which is supposed to be a contaminating Peak Ia, although the recovery of enzyme activity in this step was low.

Fig. 6

4. Chromatography of Other Plant Phosphoglucomutase

Dry pea seeds (Pisum sativum cv. Alaska, 5 g) were soaked for 24 hr at 30°C in distilled water, peeled, and homogenized with the equivalent weight of 0.1 M Tris-HCl buffer, pH 7.5, in a Potter-Elvehjem homogenizer. After centrifugation the crude extract was dialyzed against 2 liters of Buffer A, and added to the top of a column (3 × 21 cm) of DEAE-cellulose previously equilibrated with Buffer A. The column was washed with 250 ml of Buffer A, and eluted with a linear gradient of KCl in the same buffer (0 to 0.45 M KCl in 500 ml). Phosphoglucomutase

activity was eluted in a single peak, whose position agreed well that of Peak II from potato tubers (Fig. 7a).

The crude extracts prepared from dry broad bean seeds (*Vicia Faba L. forma anacarpa*) was chromatographed under the same conditions as above. Although the elution patterns of protein significantly differed among pea and broad bean seeds, phosphoglucomutase activities were eluted at the same position (Fig. 7b).

Fig. 7

## 5. Molecular Properties of Potato Phosphoglucomutase

### 5-1. Molecular weight

The molecular weights of Peak Ia and II enzymes of potato phosphoglucomutase were determined by gel filtration on Sephadex G-200 according to the procedure of Andrews (28). The enzyme samples of Peak Ia and Peak II were separately chromatographed on a column (1.5 × 50 cm) of Sephadex G-200 with 5 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 0.1 M KCl, at a flow rate of 12 ml/hr. The enzyme activities were eluted each as a symmetrical peak at the same elution volume. Peak I before separation by electrofocusing gave the same results as Peak Ia. From the elution volume (Fig. 8a),

the molecular weight of potato phosphoglucomutase was estimated to be 60,000. There is no difference in the molecular weight among Peaks Ia, Ib, Ic, and II.

On polyacrylamide gel electrophoresis in the presence of 0.1% SDS, the Peak Ia treated with 1% SDS for 30 min at 40°C gave a single protein band (Fig. 5). From the mobilities (Fig. 8b), the molecular weight of Peak Ia under these conditions was determined as 62,000, according to the procedure of Weber and Osborn (23). The same value was obtained with the gel pre-run at 8 mA per tube for 2 hr, using the running buffer containing 0.1% 2-mercapto= ethanol (15). In a separate experiment, 0.21 mg of Peak Ia was first treated with 50 mM Tris-HCl, pH 8.5, containing 8 M guanidine-HCl and 72 μM 2-mercaptoethanol at 37°C for 2 hr, and then alkylated with 65 mg of sodium iodoacetate at pH 8-9. The reaction mixture was dialyzed against 0.1 M Tris-HCl, pH 8.0, containing 9 M urea for 5.5 hr and then overnight against sodium phosphate buffer, pH 7.2, containing 0.1% SDS. Polyacrylamide gel electrophoresis of this sample in the presence of 0.1% SDS gave exactly the same results as the one without the pretreatment.

Fig. 8

5-2. Amino acid composition

Peak Ia enzyme was hydrolyzed in 6 N HCl at 110°C *in vacuo* for 24 and 72 hr, and analyzed for amino acid composition on a Beckman 120 B amino acid analyzer equipped with the accelerated system. Table III shows the results of amino acid analyses except for those of half cystine and tryptophan which were separately determined.

Table III

The total amino acid composition of Peak Ia of potato phosphoglucomutase was compared with those of the enzymes from rabbit muscle (7) and yeast (28). The present values of potato phosphoglucomutase (Peak Ia) are fairly similar as a whole to those of the enzymes from rabbit muscle and yeast. However, more serine and glycine contents and less isoleucine, leucine and proline contents in the potato enzyme are noted.

Table IV

## 6. Kinetical Properties of Potato Phosphoglucomutase

### 6-1. Requirements for enzyme activity

Peak Ia and Peak II enzymes showed no activity in the absence of added glucose-1,6-$P_2$. The addition of $MgCl_2$ and EDTA did not induce activation. These results indicate that both enzymes were purified in the dephosphorylated

forms. Effects of $Mg^{2+}$, EDTA and cysteine on the activity of Peak I enzyme were tested in the presence of 40 μM glucose-1,6-$P_2$ (Table V). Peak Ia enzyme exhibited the

Table V

highest activity in the coexistence of $Mg^{2+}$ and EDTA. Cysteine can substitute for EDTA. The enhancement of the enzyme activity by both EDTA and cysteine in the presence of $Mg^{2+}$ showed that they acted as chelating agents removing other inhibiting heavy metal ions. $Mg^{2+}$ was an absolute requirement for the activity of Peak I enzyme, since no or little activity was detected in its absence. The optimal concentration range of $Mg^{2+}$ was from 1 to 10 mM in the presence of 0.2 mM EDTA. Peak II enzyme also exhibited the highest activity in the coexistence of $Mg^{2+}$ and EDTA.

6-2. Optimal pH

The effect of pH on enzyme activity of Peaks Ia, Ib, Ic and II was examined in 10 mM Tris-maleate buffer over the pH range from 6.1 to 9.0. All the subfractions of Peak I showed the highest activity at pH 7.5, while Peak II at a higher pH of 7.9 (Fig. 9).

Fig. 9

6-3. Kinetics

The effect of the concentrations of glucose-1-P and glucose-1,6-$P_2$ on the enzyme activity of Peak Ia was determined in the reaction mixture of Assay A. Figures 10a and 10b show the double reciprocal plots of reaction velocity against glucose-1-P concentration at five different concentrations of glucose-1,6-$P_2$, and against glucose-1,6-$P_2$ at five different concentrations of glucose-1-P. Both plots yielded families of parallel lines. The deviation from straight lines at high concentrations of glucose-1-P in Figure 10a shows that the substrate at high concentrations inhibits enzyme activity. By replotting the two plots, Km values for glucose-1-P and for glucose-1,6-$P_2$ were determined as 60 μM and 1.8 μM, respectively.

Fig. 10

A similar type of plots were also obtained with Peak II enzyme (Fig. 11).

Fig. 11

Km values of Peak Ia and Peak II enzymes are summarized in Table VI.

Table VI

6-4. Effect of metabolites

Effects of metabolites on both enzymes were examined under the conditions of Assay A. The metablites tested had no effect at the concentration used on an auxiliary enzyme, glucose-6-P dehydrogenase. As shown in Table VII both Peak Ia and Peak II enzymes were markedly inhibited by glycerate-2,3-$P_2$ and fructose-1,6-$P_2$.

Table VII

The inhibition of the bisphosphate compounds was further studied with Peak Ia enzyme. Enzyme activity was determined in the absence or presence of the compounds as a function of glucose-1-P or glucose-1,6-$P_2$. The double reciprocal plots of reaction velocity against glucose-1,6-$P_2$ or glucose-1-P concentration (Figs. 12a,b, 14a) show that these compounds competitively inhibited the enzyme activity with respect to glucose-1,6-$P_2$ and uncompetitively with respect to glucose-1-P.

Fig. 12

The inhibition constants, Ki's, for glycerate-2,3-$P_2$

and fructose-1,6-$P_2$ were estimated to be both 40 μM. The compounds inhibited similarly the activity of Peak II enzyme (Figs. 13a,b, 14b). The Ki values of Peak II enzyme are summarized in Table VI.

Figs. 13, 14 Table VI

6-5. Inhibition by beryllium ion

The inhibition of Peak Ia enzyme by $Be^{2+}$ was examined under various conditions. The enzyme was dialyzed against 10 mM Tris-HCl, pH 7.5, containing 20% (v/v) glycerol to remove the EDTA and $MgCl_2$. The dialyzed enzyme (4.6 units) was incubated at 25°C with 10 μM $BeCl_2$ in the absence and presence of various compounds. During incubation 5-μl aliquots of the reaction mixture were taken and tested for the enzyme activity in Assay A system. A very low concentration of $Be^{2+}$ caused rapid inactivation of potato phosphoglucomutase (Fig. 15a), as observed with the enzyme from rabbit muscle (29), human muscle (30), yeast (6), Escherichia coli, Micrococcus lysodeikticus and Bacillus cereus (29). The addition of 1 mM imidazole did not affect the inhibition by $Be^{2+}$. However, it was almost completely retarded by the addition of either 1 mM $MgCl_2$ or 0.94 mM EDTA. The about 20% activation appeared at the zero-time of incubation by these reagents might be caused from the

release of inhibiting metal ions bound to the enzyme. Histidine and cysteine also caused similar activations, but could not retard the $Be^{2+}$ inhibition. The inhibition by $Be^{2+}$ was not reversed by the addition of $Mg^{2+}$ or EDTA (Fig. 15b).

Fig. 15

Similar results were obtained with Peak II enzyme (Fig. 16a,b). However, the activation by $MgCl_2$ and EDTA did not appear, and a higher concentration of $MgCl_2$ required for a similar retardation to Peak Ia.

Fig. 16

7. Subcellular Distribution of Phosphoglucomutase in Potato Tubers

The potato tuber homogenate was fractionated by differential centrifugation into the mitochondrial, microsomal, and soluble fractions according to Method I. The distributions of protein, phosphoglucomutase and phosphorylase activities were determined (Table VIII). In the soluble fraction, 95% of the phosphoglucomutase activity and 110% of the phosphorylase activity were recovered.

Table VIII

Lavintman *et al*. (26) reported the presence of UDPG glucosyltransferase activity in the potato particulate fraction, which consisted mainly of proplastids, and precipitated at 140,000 × *g* for 2 hr. In order to clarify whether phosphoglucomutase activity also existed in the particulate fraction, the 100,000 × *g* supernatant fluid was further centrifuged at 140,000 × *g* for 2 hr according to Method II (Table IX). The particulate fraction thus obtained contained 2.3% of the total protein, 0.7% of the phospho=

Table IX

glucomutase activity and 2.6% of the phosphorylase activity. The specific activity of phosphoglucomutase in this fraction was 0.14 units/mg protein, less than that in the crude extract, whereas that of phosphorylase was 0.65 units/mg protein, comparable to that of the crude extract. It is likely that the particulate fraction contain some phosphorylase but not phosphoglucomutase.

# V. DISCUSSION

## 1. Multiple Forms of Potato Phosphoglucomutase

### 1-1. Multiple forms on DEAE-cellulose column chromatography

Present experimental results demonstrate that phosphoglucomutase from potato tubers consists of two different protein species separable on DEAE-cellulose chromatography. Dawson and Mitchell (30) reported that phosphoglucomutase of the human muscle extract fractionated by ammonium sulfate was separated into four activity peaks by DEAE-cellulose chromatography with a linear gradient of potassium chloride (0-0.1 M), at pH 7.5. Peak I enzyme from potato tubers was no more separated, eluted in a single peak on the second DEAE-cellulose chromatography under the elution conditions similar to those of the human muscle enzyme.

The separation of potato phosphoglucomutase into two species was further confirmed by chromatography on CM-Sephadex. When the ammonium sulfate fraction of potato tubers was subjected to chromatography on a column of CM-Sephadex C-50 (2 x 14 cm) previously equilibrated with 5 mM potassium phosphate buffer containing 0.2 mM EDTA, 1 mM $MgCl_2$ and 10% (v/v) glycerol, pH 6.0, large

amounts of the charged activity was eluted at the breakthrough position. Then, the column was eluted with a linear gradient of potassium phosphate (0.005-0.45 M), pH 6.0, the remaining activity was eluted in a single peak at the mid position of the gradient. The ratio of the eluted activity to the breakthrough was 0.78. The breakthrough and the eluted preparations were separately chromatographed on a DEAE-cellulose column in similar conditions to the first DEAE-cellulose chromatography; they were respectively eluted at the positions corresponding to Peak II and Peak I.

The relative amounts of two enzymes in different experiments were fairly constant; the ratios of Peak I activity to Peak II activity in different samples of potato tubers of the variety "Danshaku" were ranged between 0.56 and 0.87, and two samples of the variety "May Queen" of different ages gave a ratio of about 1.1.

All the enzyme solution contained 2-mercaptoethanol, and were chromatographed in the presence of this reducing agent. It is unlikely that the enzyme species found are merely due to chemical and/or enzymic modification of the original enzyme during storage of the tissues or purification of the enzyme.

Phosphoglucomutase from various sources exists in either the phosphorylated or the dephosphorylated form. Although they are separable on ion-exchange chromatography

(4,31), both Peak I and Peak II enzymes of potato phosphoglucomutase required glucose-1,6-$P_2$ for activity and probably are the dephosphorylated forms. Further, the elution pattern of Peaks I and II was not changed even after incubation of the enzyme with the sugar bisphosphate in which the dephosphorylated form should be converted to the pohsphorylated form. Thus, Peak I and Peak II enzymes correspond neither to the phosphorylated and dephosphorylated forms of a single protein species nor vice versa.

Both pea and broad bean seeds contain only a single species of phosphoglucomutase which corresponds to Peak II enzyme of potato tubers on the DEAE-cellulose chromatography (Fig. 7a,b).

1-2. Multiple forms on isoelectric focusing

The Peak I preparation of potato tubers was further separated into three fractions by isoelectric focusing. When the isoelectric focusing was performed in the presence of 2 mM dithiothreitol, the distribution pattern did not change as compared with in the absence of the thiol reagent. The relative amounts of three fractions on the isoelectric focusing patterns in different experiments were found to be fairly constant; the percentage of the amounts of Ia, Ib and Ic was 56-80, 15-30 and 3-12, respectively.

Harshman and Six (32) reported that the crystalline

preparations of rabbit muscle enzyme were resolved into four phosphorylated forms of the enzyme by isoelectric focusing. They were consisted of the major component of pI 6.80 and three subsidary components whose pI's were more acidic than the major. Their contents were 82, 8, 6, 4%, respectively, of the original enzyme.

The results of the present experiments show that four species of phosphoglucomutase separable on isoelectric focusing exist in potato tubers.

## 2. Molecular Properties of Potato Phosphoglucomutase

### 2-1. Molecular weights

There is no observed difference in molecular weights between four enzyme species of potato phosphoglucomutase (Peaks Ia, Ib, Ic and II); a common molecular weight of approximately 60,000 was obtained. It is close to the values previously reported for the enzymes from other sources (4,16,28). The results of SDS polyacrylamide gel electrophoresis of Peak Ia show that this enzyme is probably composed of a single polypeptide chain with a molecular weight of 62,000.

Although phosphoglucomutases from animals and microorganisms have been believed to be also composed of each a single polypeptide, Duckworth and Sanwal (15) and

Daugherty *et al*. (6) have recently showed that the enzymes from rabbit muscle and Fleischmann's yeast dissociated into subunits with a molecular weight of approximately 30,000. No evidence for the presence of subunit structure, however, was presently obtained with potato phosphoglucomutase (Peak Ia) even under the same conditions as those which they used for dissociation.

On the other hand, Maino and Young (33) reported that two phosphoglucomutases from *Bacillus subtilis* consisted of dimers with an approximate molecular weight of 130,000 and the two enzymes dissociated at pH 6.5 in low concentrations of $Mg^{2+}$. In the present studies, the results of Sephadex G-200 gel chromatography of potato phosphoglucomutases in the presence of 1 m$\underline{\underline{M}}$ $MgCl_2$ show all species have a molecular weight of 60,000.

## 2-2. Amino acid composition

In the present analyses, the high values for glycine and serine were obtained (Table III). There was a possibility that these results were due to some contaminating ampholytes in the enzyme sample, since ampholytes were ninhydrin-positive. To test this posibility, aliquots of ampholytes were subjected to the analysis in the same conditions as Peak Ia enzyme. No peaks arising from the compouds were detected around the glycine and serine peaks on the chromatogram.

The contents of other amino acids in potato phosphoglucomutase (Peak Ia) did not markedly differ from the enzymes of rabbit muscle and yeast (Table IV).

## 3. Kinetical Properties of Potato Phosphoglucomutase

### 3-1. Assay of phosphoglucomutase activity

In Assay method A, the reaction was started by the addition of the enzyme. The time course of reaction has an initial lag phase, even when the purified enzyme was used, the auxilliary enzyme was adequate in amounts and substrates presented over the saturated amount. Since the buffer used through purification steps contained $Mg^{2+}$ and EDTA and the buffer used to dilute the enzyme contained EDTA, the enzyme should be preactivated before it was added to the reaction mixture. Therefore, the initial lag phase is not due to the apparent activation during the reaction, but possibly to the anomerization from the α-isomer of glucose-6-P, the product of phosphoglucomutase, to the β-isomer which is required for glucose-6-P dehydrogenase.

Wurster and Hess (34) have recently reported the occurrence of an enzyme which catalyzes the anomerization of D-glucose-6-P in Escherichia coli, Rhodotorula gracilis, potato tubers, rat muscle, rat liver and rat kidney. If this enzyme was added to the phosphoglucomutase

assay system, the reaction time course should become linear. Although it was not strictly linear, in a practical manner, its linear portion following the initial lag phase was taken for the measurement of activity.

### 3-2. Requirements

For the full activity of phosphoglucomutase, $Mg^{2+}$ and EDTA are absolutely required. However, EDTA can be, at least partly, replaced by cysteine, histidine and imidazole. It is likely that all of these compounds act as chelating reagents. Other divalent metal ions, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Zn^{2+}$ and $Sr^{2+}$, showed inhibiting effects rather than stimulating on the enzyme activity in the range of concentration from 0.5 to 1.0 m*M*.

### 3-3. pH optima

A clear difference was observed among pH optima of two potato phosphoglucomutases, Peaks I and II. The pH profile of Peak II enzyme shifted to the alkaline side by about 0.4 unit, *i.e.*, pH 7.9 compared with that of Peaks Ia, Ib and Ic, pH 7.5. A similar situation has been observed with two phosphoglucomutases from human muscle (3).

## 3-4. Kinetics

Two basically different reaction mechanisms have been proposed for phosphoglucomutase reaction.

[I] E-P + glucose-1-P $\rightleftharpoons$ E + glucose-1,6-$P_2$ (I-1)

E + glucose-1,6-$P_2$ $\rightleftharpoons$ E-P + glucose-6-P (I-2)

[II] E + glucose-1-P + glucose-1,6-$P_2$ $\rightleftharpoons$

E•glucose-1-P•glucose-1,6-$P_2$ $\rightleftharpoons$

E + glucose-6-P + glucose-1,6-$P_2$

Mechanism I first proposed by Najjar and Pullman (35) with the rabbit muscle enzyme involves the two step reactions, I-1 and I-2, where E-P and E represents the phosphorylated and dephosphorylated forms of the enzyme, respectively. In this mechanism, the reaction should follow the "ping-pong" kinetics proposed by Cleland (36). In the absence of products, the rate equation for the mechanism is given as shown in Eq. 1,

$$1)\quad \frac{Vmax}{v} = 1 + \frac{Ka}{[A]} + \frac{Kb}{[B]}$$

where A and B are glucose-1-P and glucose-1,6-$P_2$, Ka and

Kb are Michaelis constants for glucose-1-P and glucose-1,6-$P_2$. Thus, double reciprocal plots give a group of parallel lines when substrate concentration is varied at several fixed levels of coenzyme concentration, and *vice versa*. It was reported that the reaction of phosphoglucomutases from rabbit muscle (37), beef liver (4), shark and flounder muscle (2), Fleischmann's yeast (6), and *Escherichia coli* (7) fitted "ping-pong" mechanism.

However, the reaction mechanism II involving a ternary complex of glucose-1-P (or glucose-6-P), glucose-1,6-$P_2$ and the enzyme was suggested for phosphoglucomutases from *Bacillus cereus* , *Micrococcus lysodeikticus* (8) and *Saccharomyces cerevisiae* (5). This mechanism should follow Cleland's "sequential" kinetics and gives the rate equation in the absence of products as shown in Eq. 2,

$$2)\quad \frac{Vmax}{v} = 1 + \frac{Kb}{[B]} + \frac{Ka}{[A]}\left(1 + \frac{Kib}{[B]}\right)$$

where A and B are glucose-1-P and glucose-1,6-$P_2$, Ka and Kb are Michaelis constants for glucose-1-P and glucose-1,6-$P_2$, and Kib is the inhibition constant(36). In this mechanism all lines of double reciprocal plots meet at a point when substrate concentration is varied at several fixed levels of coenzyme concentration, and *vice versa*.

Thus, the reaction mechanisms of phosphoglucomutases

from the different origins are not identical. No kinetic data has been reported with higher plant phosphoglucomutase. The present kinetic results with Peak Ia and Peak II enzymes from potato tubers conform to the criterion of the "ping-pong" mechanism. Therefore, potato phosphoglucomutase is more like the enzymes from animal origins(2,4,37) rather than those from Micrococcus lysodeikticus (8), Bacillus cereus (8) and Saccharomyces cerevisiae (5).

Although all the enzyme species of potato phosphoglucomutase act in the same reaction mechanism, they differ in their kinetic parameters; Peak Ia enzyme for glucose-1-P is considerably smaller than that of Peak II enzyme.

### 3-5. Metabolite inhibition

Both Peak Ia and Peak II enzymes from potato tubers were similarly inhibited by fructose-1,6-$P_2$ and glycerate-2,3-$P_2$, as found with the beef liver enzyme (4).

Duckworth et al. (38) reported that phosphoglucomutases from rabbit muscle and Escherichia coli were inhibited by several nucleotides, especially by ADP, ATP, GMP, GDP, GTP, dTDP, coenzyme A and several coenzyme A derivatives. Further, Maino and Young (33) reported that several nucleotides inhibited both phosphoglucomutases I and II from Bacillus subtilis separated by DEAE-cellulose, and of the compounds tested, GTP and UTP inhibited virtually 100% of the control activity at a concentration of 1.0 mM. Therefore, the

effects of nucleotides on the activity of potato phospho= glucomutase were examined. Both ADP and ATP inhibited about 10% of the control activity of both Peak Ia and Peak II enzymes at a concentration of 1.0 mM(Table VII), although they inhibited 43% of the control activity of the rabbit enzyme at the same concentration (38). UTP and GTP also inhibited the Peak Ia enzyme; only 10 and 17% of the control activity at a concentration of 1 mM, respectively (the data are not shown ).

All the synthetic glucose analogs, 5-thio-D-glucose, glucosyl fluoride, and 1-thio-D-glucose gave no significant effect on the activity of Peak Ia enzyme in the range of concentration from 0.5 to 10 mM, although they acted as inhibitors of phosphorylase (39,40). Glucose, maltose, and sucrose gave also no effect on the activity of Peak Ia enzyme at a concentration of 1 mM.

### 3-6. Inhibition by beryllium ion

Both Peak Ia and Peak II enzymes from potato tubers were similarly inhibited by $BeCl_2$ at very low concentrations, and the inhibitions were retarted by chelating agents, as observed with two phosphoglucomutases from human muscle (3).

## 4. Subcellular Distribution of Potato Phosphoglucomutase

The results from the two independent fractionation experiments show that all phosphoglucomutase species Peaks I and II exist in the soluble fraction.

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Fig. 1. Effect of polyhydric alcohols on the stability of the crude enzyme. The crude extract was incubated at pH 7.5 at 4°C in the absence (●) or in the presence of polyhydric alcohols (10% (v/v) glycerol, ▲; 20% (v/v) glycerol, ■; 10% (v/v) ethylene glycol, △; 20% (v/v) ethylene glycol, □). During incubation aliquots of the mixtrure were taken and assayed for the enzyme activity in the Assay A system.

Fig. 2. Chromatography of potato phosphoglucomutase on a DEAE-cellulose column. Fractions of 20 ml were collected and analyzed for protein and enzyme activity. Details of experimental procedures are given in the text.

Fig. 3. Rechromatography of Peak I and Peak II enzymes. A sample of the desalted ammonium sulfate fraction containing 196 mg protein and 1860 units of enzyme activity was charged on a DEAE-cellulose column (3 x 21 cm) previously equilibrated with Buffer A. The column was washed with 300 ml of Buffer A and eluted with a linear gradient of KCl in the same buffer (0 to 0.45 M in 600 ml) at a flow rate of 1 ml/min. Fractions of 10 ml were collected. Peak I (fraction number 20-33) and Peak II (fraction number 34-56) fractions were separately dialyzed against one change of Buffer A. Then, each sample was rechromato= graphed under the same conditions as above. a) The first

chromatography of the desalted ammonium sulfate fraction; b) rechromatography of Peak I; c) rechromatography of Peak II.

Fig. 4. Separation of Peak I enzyme by isoelectric focusing. Details of experimental procedures are given in the text.

Fig. 5. Polyacrylamide gel electrophoreses of the purified potato phosphoglucomutase (Peak Ia). a) A sample containing 21 μg of protein was analyzed without SDS treatment. Protein was stained with Amido black. b) A sample containing 21 μg of protein was treated with 1% SDS for 30 min at 40°C and then analyzed in the presence of 0.1% SDS. Protein was stained with Coomassie brilliant blue.

Fig. 6. Isoelectric focusing of Peak II enzyme. The Peak II enzyme (protein, 156 mg; activity, 480 units) dialyzed against Buffer A was subjected to isoelectric focusing in the same conditions as Peak I enzyme.

Fig. 7. Chromatography of pea and broad bean phospho= glucomutases on a DEAE-cellulose column. a) Pea. b) Broad bean. Details of experimental procedures are given in the text.

Fig. 8. Estimation of the molecular weight of potato phosphoglucomutase. a) Gel filtration on Sephadex G-200. b) Polyacrylamide gel electrophoresis in the presence of SDS. Calibrations were made using γ-globulin (1, mol. wt. $16 \times 10^4$), rabbit muscle phosphorylase b (2, mol. wt. $9.7 \times 10^4$), bovine serum albumin (3, mol. wt. $6.8 \times 10^4$), ovalbumin (4, mol. wt. $4.3 \times 10^4$), yeast alcohol dehydrogenase (5, mol. wt. $3.7 \times 10^4$), Peaks Ia and II(O).

Fig. 9. Effect of pH on the activities of Peak Ia, Ib, Ic, and Peak II enzymes. a) Peak Ia enzyme (●—●), Peak II enzyme (O—O). b) Peak Ib enzyme (●····●), Peak Ic enzyme (O—O). Assay B was used except that Tris-HCl was replaced by Tris-maleate.

Fig. 10. Double reciprocal plots of reaction velocity against substrate concentrations in Peak Ia enzyme reaction. a) Glucose-1,6-$P_2$ concentration varied. 1) 0.50; 2) 0.75; 3) 1.25; 4) 2.50; 5) 10.0. b) Glucose-1-P concentration varied. 1) 10.0; 2) 12.5; 3) 15.0; 4) 20.0; 5) 25.0. All in μM. Enzyme activity was measured by Assay A.

Fig. 11. Double reciprocal plots of reaction velocity against substrate concentrations in Peak II enzyme reaction. a) Glucose-1,6-$P_2$ concentration varied. 1) 0.50; 2) 0.75; 3) 1.25; 4) 2.50; 5) 10.0. b) Glucose-1-P concentration

varied. 1) 10.0; 2) 12.5; 3) 15.0; 4) 20.0; 5) 25.0. All in μM. Enzyme activity was measured by Assay A.

Fig. 12. Double reciprocal plots of reaction velocity against glucose-1,6-$P_2$ concentration in the presence of the bisphosphate in Peak Ia enzyme reaction. a) Fructose-1,6-$P_2$ b) Glycerate-2,3-$P_2$ (concentrations are indicated in the figures.) The concentration of glucose-1-P was 25 μM in both cases. Enzyme activity was measured by Assay A.

Fig. 13. Double reciprocal plots of reaction velocity against glucose-1,6-$P_2$ concentration in the presence of the bisphosphate in Peak II enzyme reaction. a) Fructose-1,6-$P_2$ b) Glycerate-2,3-$P_2$ (concentrations are indicated in the figures.) The concentration of glucose-1-P was 25 μM in both cases. Enzyme activity was measured by Assay A.

Fig. 14. Double reciprocal plots of reaction velocity against glucose-1-P concentration in the presence of the bisphosphate in Peak Ia and Peak II enzyme reactions. a) Peak Ia enzyme (●, none; ■, 100 μM fructose-1,6-$P_2$; ▲, 200 μM glycerate-2,3-$P_2$). b) Peak II enzyme (●, none; ■, 100 μM fructose-1,6-$P_2$; ▲, 200 μM glycerate-2,3-$P_2$). The concentration of glucose-1,6-$P_2$ was 2.5 μM. Enzyme activity was measured by Assay A.

Fig. 15. Inhibition of the activity of Peak Ia enzyme by beryllium ion. a) The dialyzed Peak Ia enzyme (4.6 units) was incubated in 0.99 ml of 10 mM Tris-HCl, pH 7.5, containing 20% (v/v) glycerol at 25°C. After preincubation for 5 min, 10 µl of 1 mM $BeCl_2$ was added to the incubation mixture in the absence (●) or in the presence of various compounds (1 mM $MgCl_2$, ○; 0.94 mM EDTA, △; 1 mM imidazole, ×). During incubation 5-µl aliquots of the mixture were taken and assayed for the enzyme activity in the Assay A system. ---- control (without $BeCl_2$). b) The enzyme (4.6 units) was incubated with 10 µM $BeCl_2$ in 0.99 ml of 10 mM Tris-HCl, pH 7.5, containing 20% (v/v) glycerol at 25°C, and 0.94 mM EDTA (△) or 1 mM $MgCl_2$ (○) was added at the time indicated by the arrow. The enzyme activity was assayed in the Assay A system. ---- control (without $BeCl_2$).

Fig. 16. Inhibition of the activity of Peak II enzyme by beryllium ion. a) The dialyzed Peak II enzyme (4.2 units) was incubated in 0.99 ml of 10 mM Tris-HCl, pH 7.5, containing 20% (v/v) glycerol at 25°C. After preincubation for 5 min, 10 µl of 1 mM $BeCl_2$ was added to the incubation mixture in the absence (●) or in the presence of various compounds (1 mM $MgCl_2$, ○; 10 mM $MgCl_2$, ■; 0.94 mM EDTA, △; 1 mM imidazole, ×). During incubation 5-µl aliquots of the mixture were taken and assayed for the enzyme activity in the Assay A system. ---- control (without $BeCl_2$).

b) The enzyme (4.2 units) was incubated with 10 μM $BeCl_2$ in 0.99 ml of 10 mM Tris-HCl, pH 7.5, containing 20% (v/v) glycerol at 25°C, and 0.94 mM EDTA (▲) or 10 mM $MgCl_2$ (■) (indicated by A) or 1 mM $MgCl_2$ (○) (indicated by B) was added at the time indicated by the arrows. The enzyme activity was assayed in the Assay A system. ---- control (without $BeCl_2$).

Table I Purification of Peak I enzyme

| Step | Fraction | Total protein (mg) | Total activity (units) | Specific activity (units/mg protein) |
|---|---|---|---|---|
| Crude extract | Peaks I & II | 28,000 | 7,600 | 0.27 |
| Ammonium sulfate | Peaks I & II | 4,100 | 12,000 | 2.9 |
| 1st DEAE-cellulose | Peak I | 480 | 3,800 | 7.9 |
| | Peak II | 2,200 | 5,500 | 2.5 |
| 2nd DEAE-cellulose | Peak I | 56 | 3,100 | 55 |
| Sephadex G-200 | Peak I | 29 | 2,400 | 83 |
| Isoelectric focusing | Peak I | 2.3 | 1,100 | 480 |

From 5 kg potato tubers

Table II Purification of Peak II enzyme

| Step | Fraction | Total protein (mg) | Total activity (units) | Specific activity (units/mg protein) |
|---|---|---|---|---|
| Crude extract | Peaks I & II | 24,000 | 11,000 | 0,46 |
| Ammonium sulfate | Peaks I & II | 5,100 | 18,000 | 3,5 |
| DEAE-cellulose | Peak I | 620 | 7,000 | 11 |
| | Peak II | 2,400 | 8,300 | 3,5 |
| 1st Sephadex G-200 | Peak II | 400 | 2,100 | 5,3 |
| 2nd Sephadex G-200 | Peak II | 170 | 1,300 | 7,6 |
| 1st Hydroxylapatite | Peak II | 40 | 640 | 16 |
| 2nd Hydroxylapatite | Peak II | 14 | 370 | 26 |

From 5 kg of potato tubers

Table III Amino acid analysis of Peak Ia enzyme

| | 24 hr Hydrolysis | 72 hr Hydrolysis | Average | Residures per 62,000 g | Nearest Integer |
|---|---|---|---|---|---|
| | (μmol) | | (μmol) | | |
| Lysine | 0.0998 | 0.0938 | 0.0938 | 47.5 | 48 |
| Histidine | 0.0325 | 0.0315 | 0.0320 | 15.7 | 16 |
| Arginine | 0.0415 | 0.0376 | 0.0396 | 19.5 | 20 |
| Aspartic acid | 0.116 | 0.106 | 0.111 | 54.5 | 55 |
| Threonine | 0.0586 | 0.0507 | *0.0586 | 28.8 | 29 |
| Serine | 0.137 | 0.109 | *0.137 | 67.2 | 67 |
| Glutamic acid | 0.110 | 0.116 | 0.113 | 55.5 | 56 |
| Proline | 0.0457 | 0.0504 | 0.0481 | 23.6 | 24 |
| Glycine | 0.142 | 0.141 | 0.142 | 69.5 | 70 |
| Alanine | 0.0958 | 0.0925 | 0.0942 | 46.2 | 46 |
| Valine | 0.0672 | 0.0691 | #0.0691 | 33.9 | 34 |
| Methionine | 0.00900 | 0.0113 | #0.0113 | 5.55 | 6 |
| Isoleucine | 0.0512 | 0.0522 | #0.0522 | 25.6 | 26 |
| Leucine | 0.0726 | 0.0714 | 0.0720 | 35.3 | 35 |
| Tyrosine | 0.0370 | 0.0318 | *0.0370 | 18.2 | 18 |
| Phenylalaine | 0.0422 | 0.0411 | 0.0417 | 20.4 | 20 |
| Tryptophan | | | †0.0178 | 8.74 | 9 |
| Half-cystine | | | +0.00650 | 3.00 | |

* The results of 24 hr hydrolysis
\# The results of 72 hr hydrolysis
† Tryptophan determined spectrophotometrically (24).
\+ Half-cystine determined by performic acid oxidation (23).

Table IV Comparative amino acid composition of phosphoglucomutase

| | Potato Tuber[a] | Rabbit muscle[b] | Yeast[c] |
|---|---|---|---|
| Lys | 48 | 46 | 42 |
| His | 16 | 12 | 9 |
| Arg | 20 | 27 | 15 |
| Asp + Asn | 55 | 59 | 68 |
| Thr | 29 | 29 | 33 |
| Ser | 67 | 24 | 35 |
| Glu + Gln | 56 | 43 | 52 |
| Pro | 24 | 29 | 26 |
| Gly | 70 | 49 | 50 |
| Ala | 46 | 49 | 40 |
| Val | 34 | 39 | 33 |
| Met | 6 | 10 | 3 |
| Ile | 26 | 48 | 39 |
| Leu | 35 | 43 | 36 |
| Tyr | 18 | 15 | 23 |
| Phe | 20 | 31 | 26 |
| Trp | 9 | | 6 |
| 1/2 Cys | 3 | 10 | 6 |

a This investigation
b Original values (41) recalculated for a molecular weight of 62,000 (7).
c Hirose *et al.* (28).

Table V Effect of $Mg^{2+}$ and chelating reagents on the activity of Peak Ia enzyme

| Addition | Concentration (mM) | Relative activity ( % ) |
|---|---|---|
| $MgCl_2$ + EDTA | 10 + 0.2 | 100 |
| $MgCl_2$ + Cysteine | 10 + 10 | 94 |
| $MgCl_2$ | 10 | 71 |
| " | 1 | 48 |
| EDTA | 0.01 | 5 |
| " | 0.2 | 0 |
| Cysteine | 1 | 5 |
| " | 10 | 2 |
| None | | 0 |

Assay B was used except that the concentrations of $MgCl_2$ and chelating reagents were changed as shown in the table.

Table VI Kinetic parameters of Peak Ia and Peak II enzymes

| Substrate or inhibitor | $K_m$ or $K_i$ (μM) | |
|---|---|---|
| | Peak Ia | Peak II |
| Glucose-1-P | 60 | 120 |
| Glucose-1,6-$P_2$ | 1.8 | 2.2 |
| Fructose-1,6-$P_2$ | 40 | 32 |
| Glycerate-2,3-$P_2$ | 40 | 20 |

Assay A was used with varied concentrations of substrates and inhibitors.

Table VII Effects of metabolites on the activities of Peak Ia and Peak II enzymes

| Addition ( 1 mM ) | Relative activity (%) | |
|---|---|---|
| | Peak Ia | Peak II |
| None | 100 | 100 |
| Fructose-1-P | 95 | 94 |
| Fructose-6-P | 100 | 99 |
| Fructose-1,6-$P_2$ | 40 | 49 |
| Glycerate-2-P | 87 | 87 |
| Glycerate-3-P | 86 | 90 |
| Glycerate-2,3-$P_2$ | 44 | 49 |
| UDPG | 95 | 95 |
| AMP | 96 | 94 |
| ADP | 89 | 92 |
| ATP | 90 | 91 |

Assay A was used with the additions shown in the table.

Table VIII Distribution of phosphoglucomutase in potato tuber tissue (1)

| Fraction | Protein | | Phosphoglucomutase | | Phosphorylase | |
|---|---|---|---|---|---|---|
| | Total content (mg) | Recovery ( % ) | Total activity (units) | Recovery ( % ) | Total activity (units) | Recovery ( % ) |
| Crude | 18.6 | 100 | 27.5 | 100 | 24.8 | 100 |
| 14,000 x g ppt | 1.7 | 9.1 | 0.1 | 0.4 | 0.3 | 1.2 |
| 100,000 x g ppt | 0.8 | 4.3 | 0.0 | 0.0 | 0.1 | 0.4 |
| 100,000 x g sup | 18.9 | 102 | 26.0 | 94.5 | 27.3 | 110 |

From 10 g fresh tissue

Table IX Distribution of phosphoglucomutase in potato tuber tissue ( II )

| Fraction | Protein | | Phosphoglucomutase | | Phosphorylase | |
|---|---|---|---|---|---|---|
| | Total content (mg) | Recovery ( % ) | Total activity (units) | Recovery ( % ) | Total activity (units) | Recovery ( % ) |
| Crude | 449 | 100 | 194 | 100 | 252 | 100 |
| 25,000 x g ppt | 36.1 | 8.0 | 6.9 | 3.6 | 8.8 | 3.5 |
| 100,000 x g ppt | 21 | 4.7 | 1.9 | 1.0 | 4.4 | 1.7 |
| 140,000 x g ppt | 10.3 | 2.3 | 1.4 | 0.7 | 6.7 | 2.7 |
| 140,000 x g sup | 354 | 78.7 | 140 | 72.3 | 187 | 74.0 |

From 100 g fresh tissue

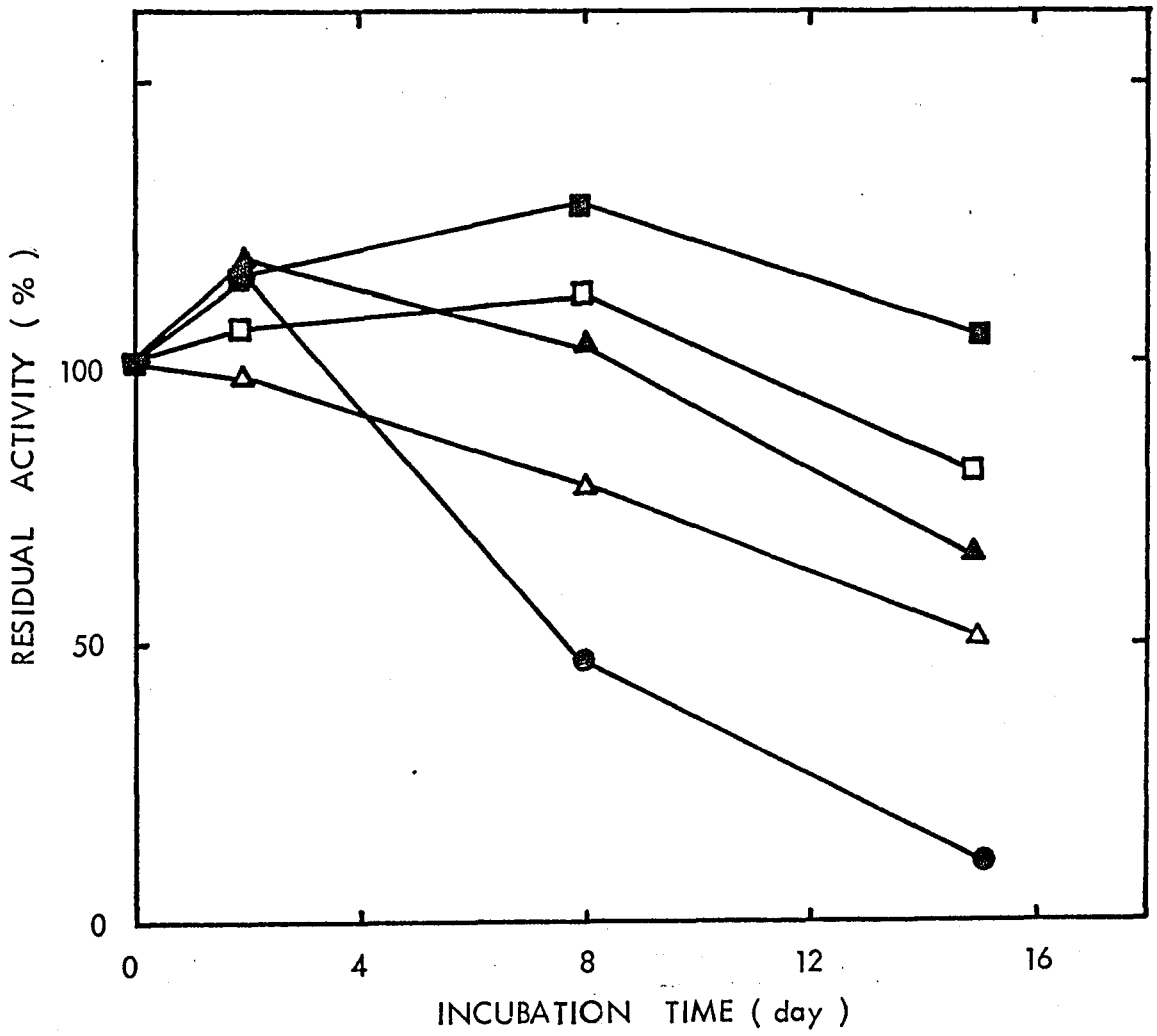


Fig. 1

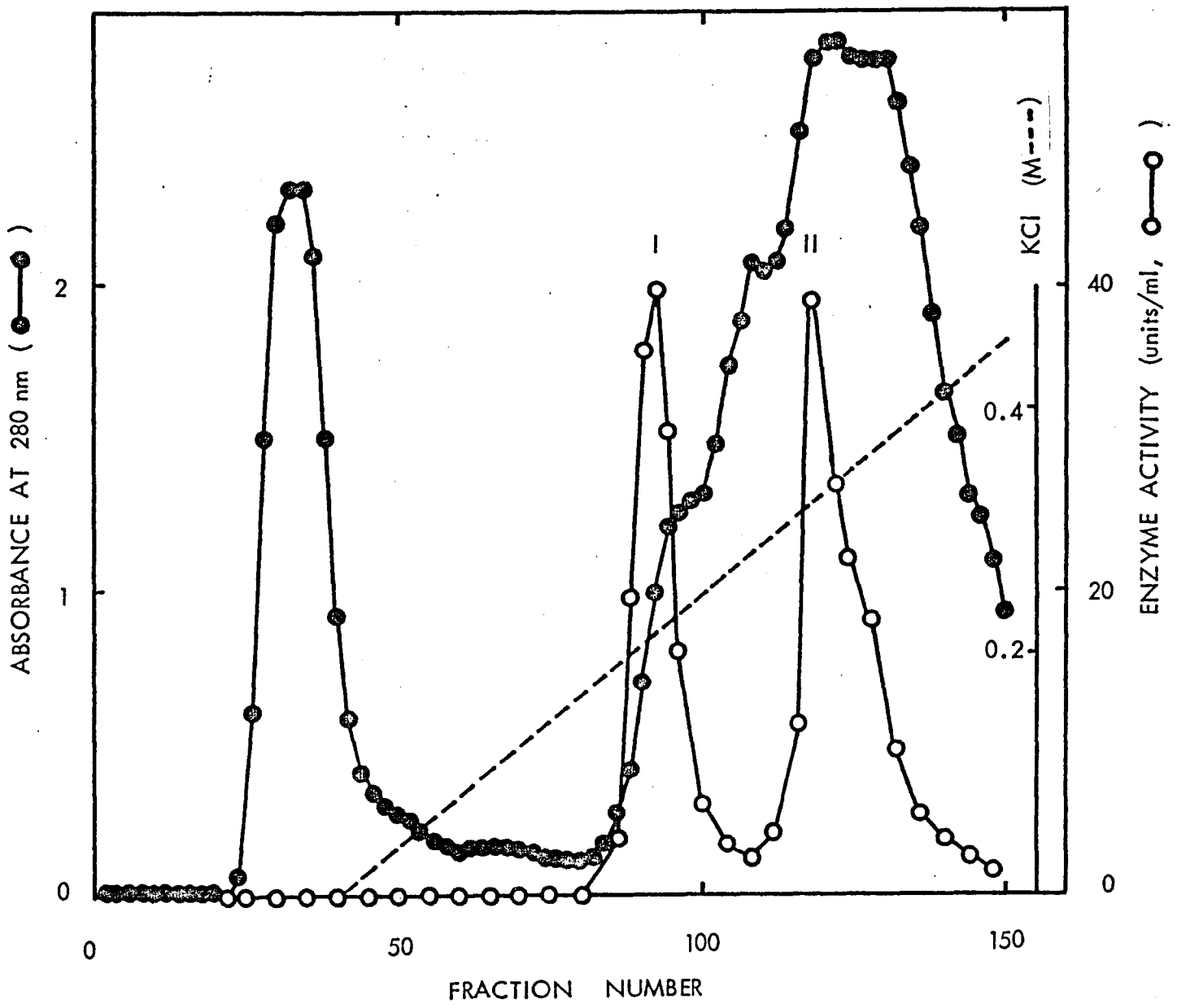
ABSORBANCE AT 280 nm (●—●)
ENZYME ACTIVITY (units/ml, ○—○)
KCl (M---)
FRACTION NUMBER
I
II
0
1
2
0
20
40
0.2
0.4
0
50
100
150

Fig. 2

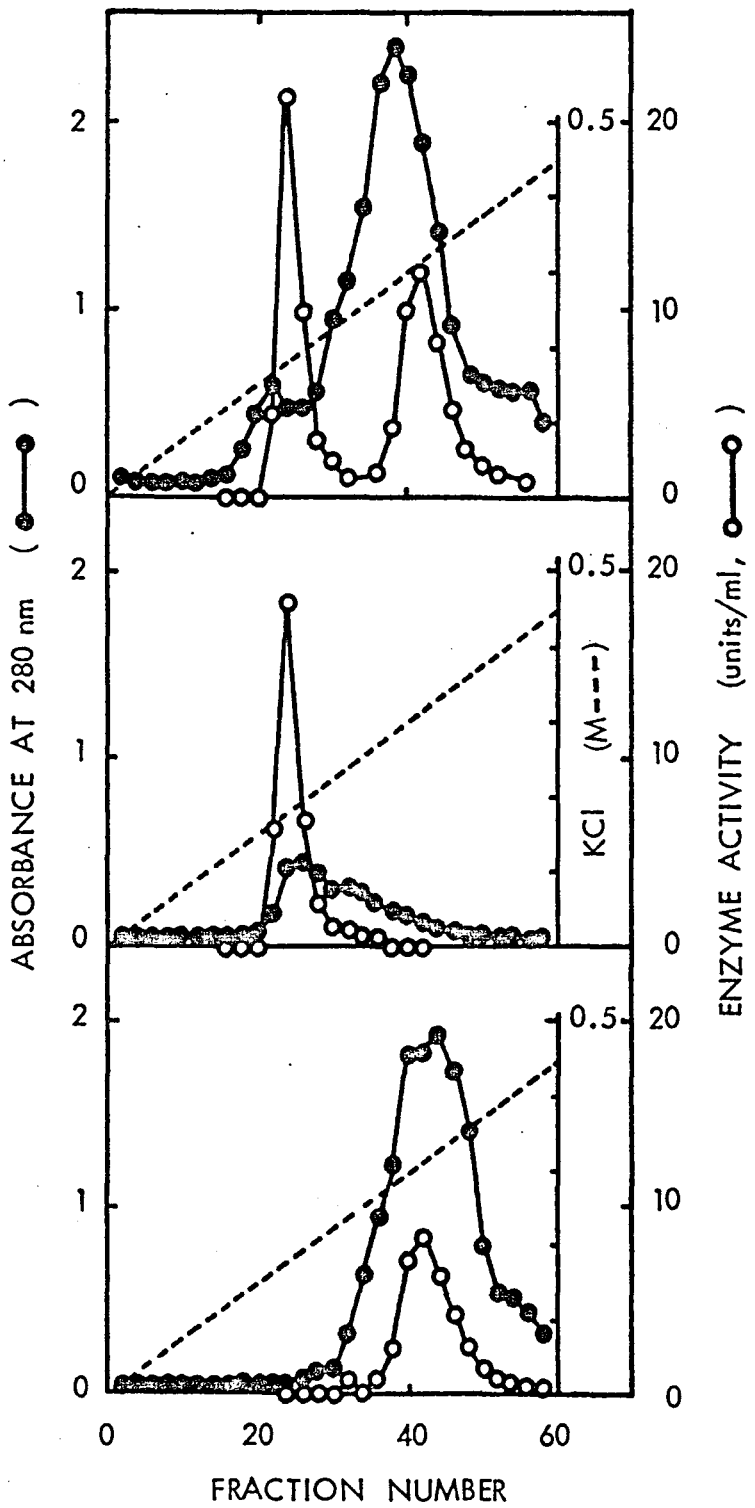
ABSORBANCE AT 280 nm ( ● — ● )
ENZYME ACTIVITY (units/ml, ○ — ○ )
KCl (M- - - -)
FRACTION NUMBER
0
20
40
60
0
1
2
0
10
20
0.5

Fig. 3

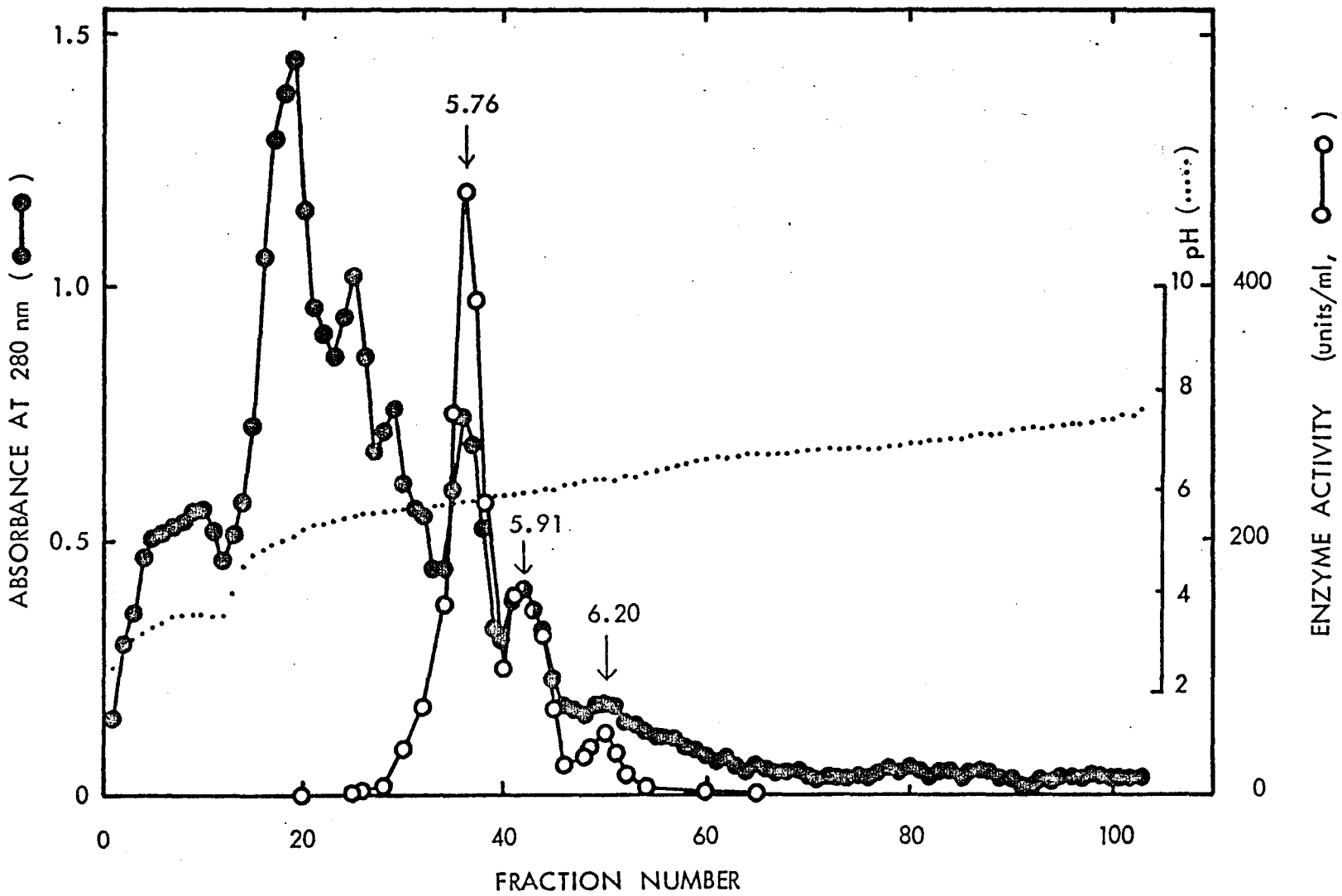
1.5
1.0
0.5
0
ABSORBANCE AT 280 nm ( )
5.76
5.91
6.20
pH (.....)
10
8
6
4
2
400
200
0
ENZYME ACTIVITY (units/ml, )
0
20
40
60
80
100
FRACTION NUMBER

Fig. 4

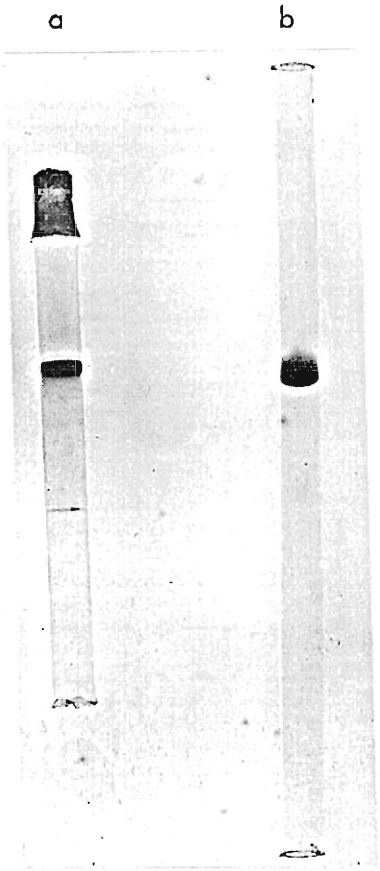


Fig.5

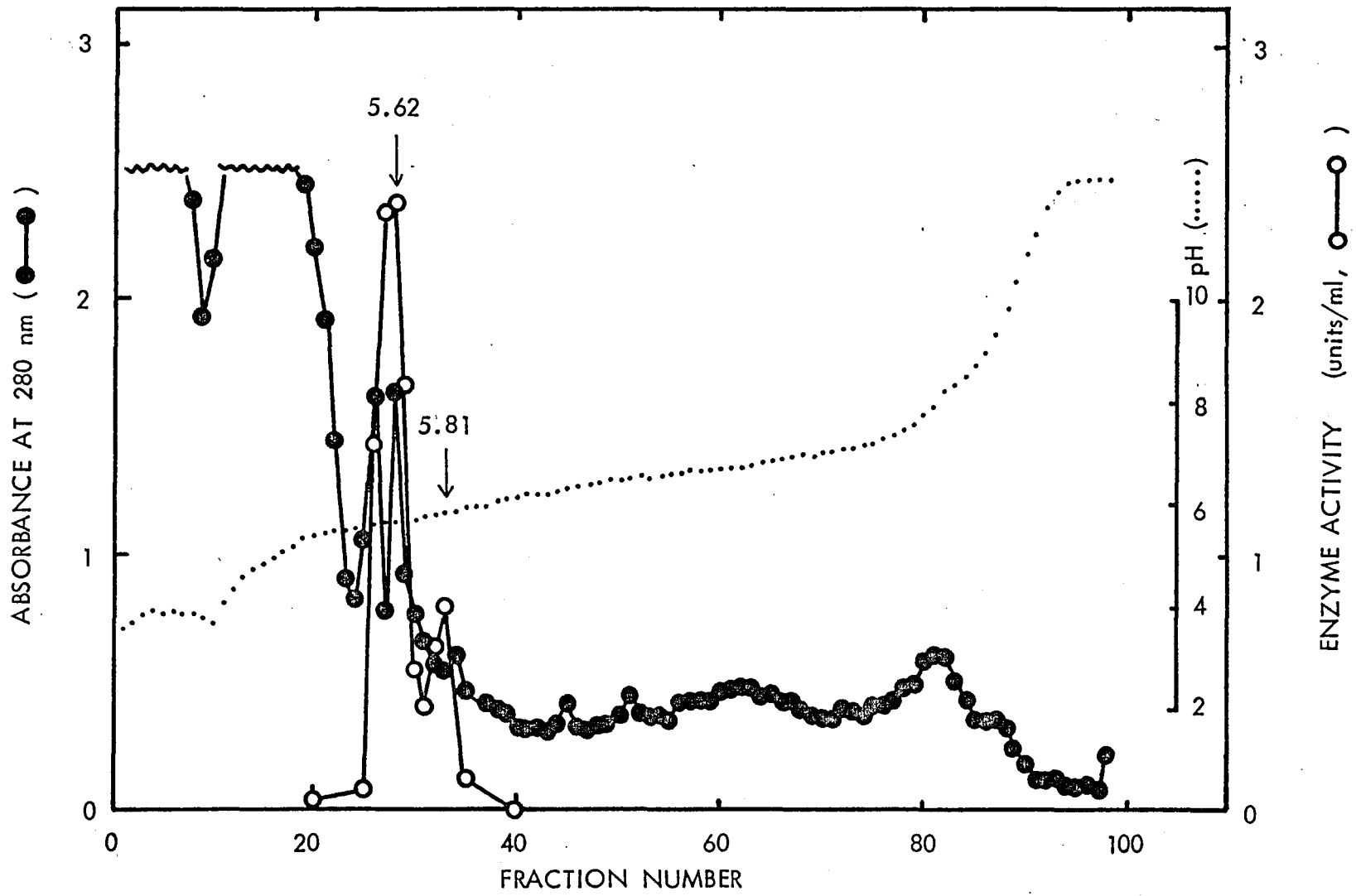
ABSORBANCE AT 280 nm (●—●)
ENZYME ACTIVITY (units/ml, ○—○)
pH (.....)
FRACTION NUMBER
5.62
5.81
0
1
2
3
0
20
40
60
80
100
2
4
6
8
10

Fig. 6

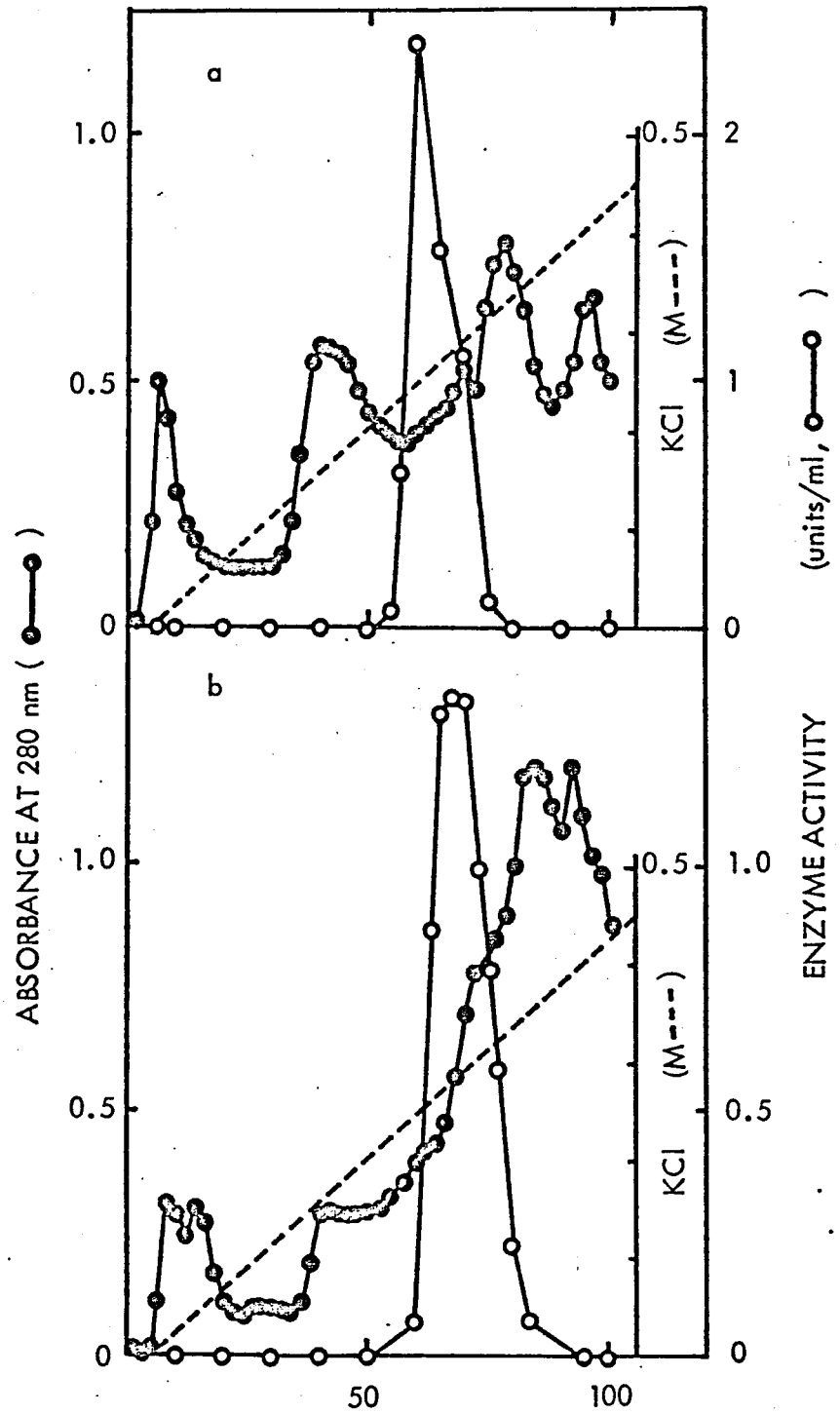
a
b
1.0
0.5
0
50
100
0.5
1
2
KCl (M---)
ABSORBANCE AT 280 nm ( )
ENZYME ACTIVITY (units/ml, )

Fig. 7

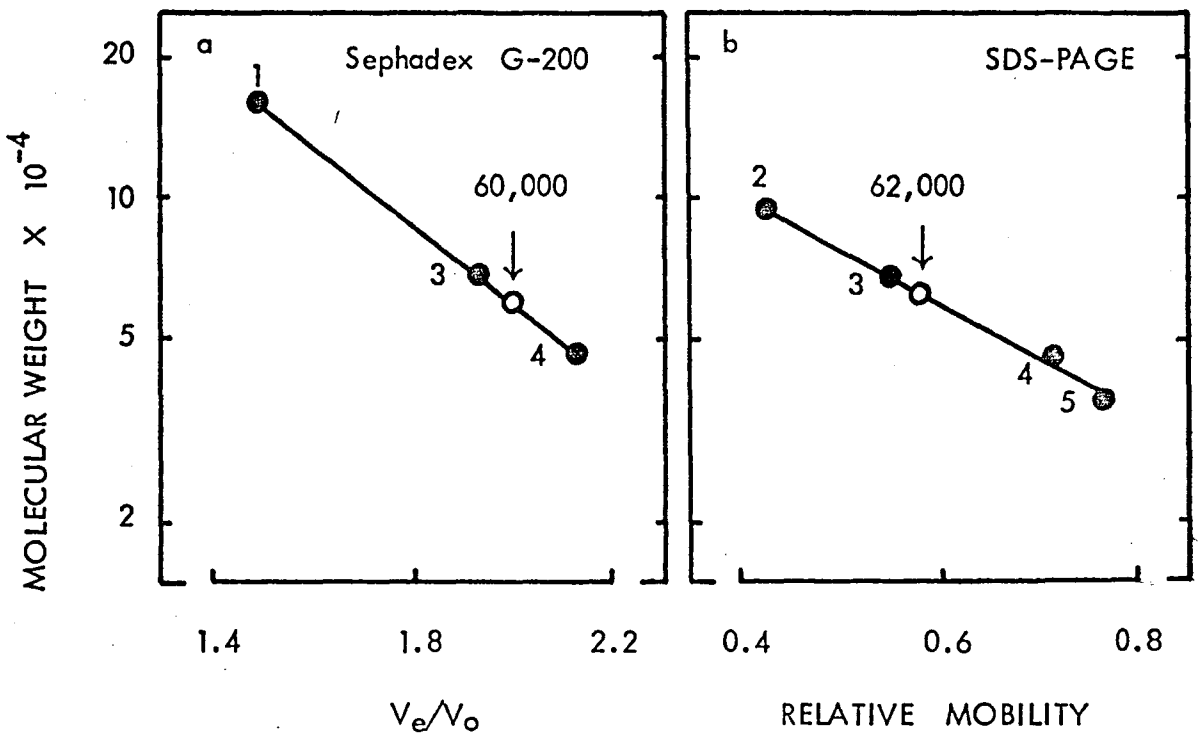
a
Sephadex G-200
b
SDS-PAGE
MOLECULAR WEIGHT X 10^-4
20
10
5
2
1
2
3
4
5
60,000
62,000
1.4
1.8
2.2
0.4
0.6
0.8
Ve/Vo
RELATIVE MOBILITY

Fig.8

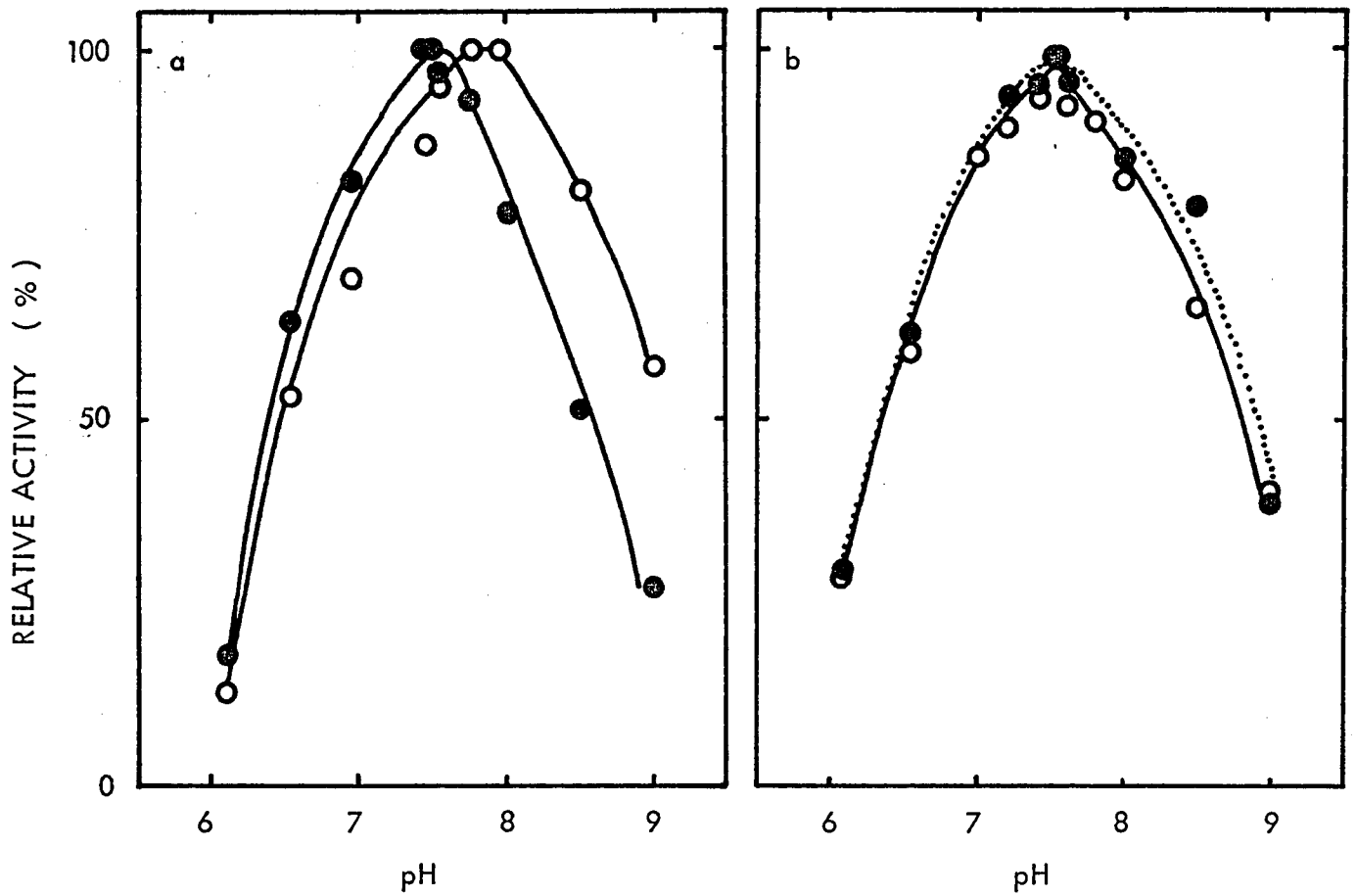


Fig. 9

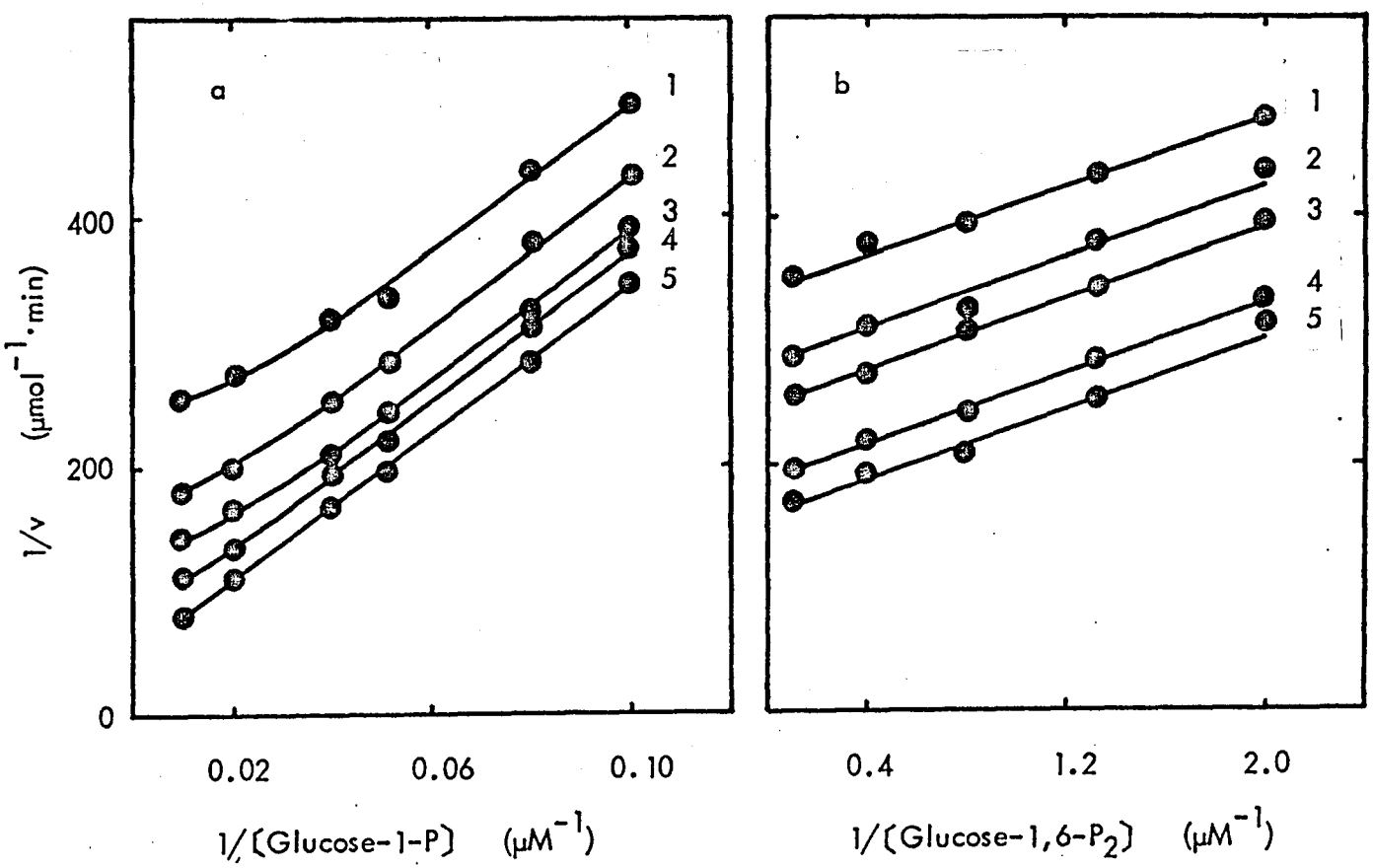
a
b
1
2
3
4
5
400
200
0
1/v (μmol⁻¹·min)
0.02
0.06
0.10
1/[Glucose-1-P] (μM⁻¹)
0.4
1.2
2.0
1/[Glucose-1,6-P2] (μM⁻¹)

Fig. 10

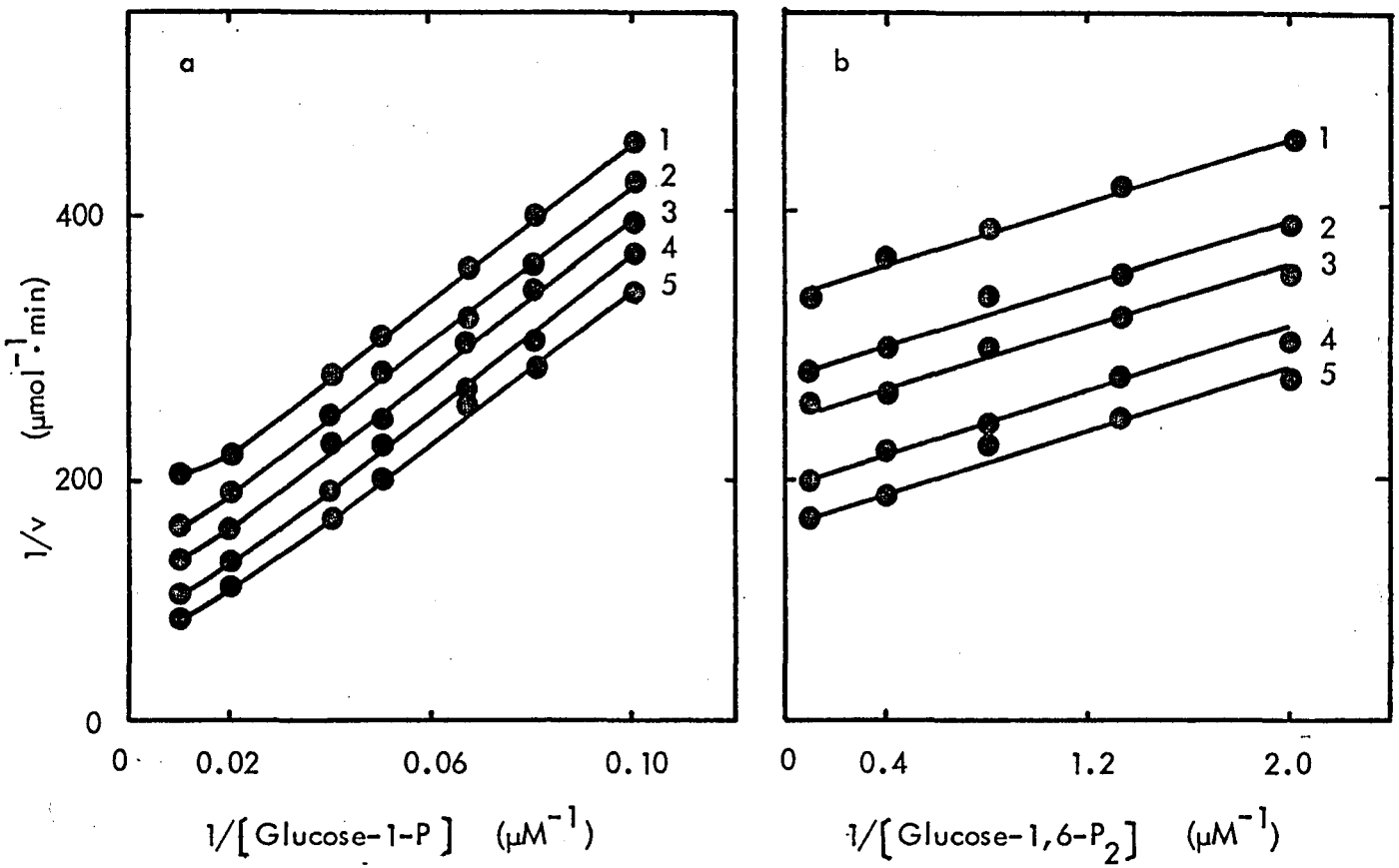
a
b
1
2
3
4
5
400
200
0
1/v (μmol⁻¹·min)
0
0.02
0.06
0.10
1/[Glucose-1-P] (μM⁻¹)
0.4
1.2
2.0
1/[Glucose-1,6-P2] (μM⁻¹)

Fig. 11

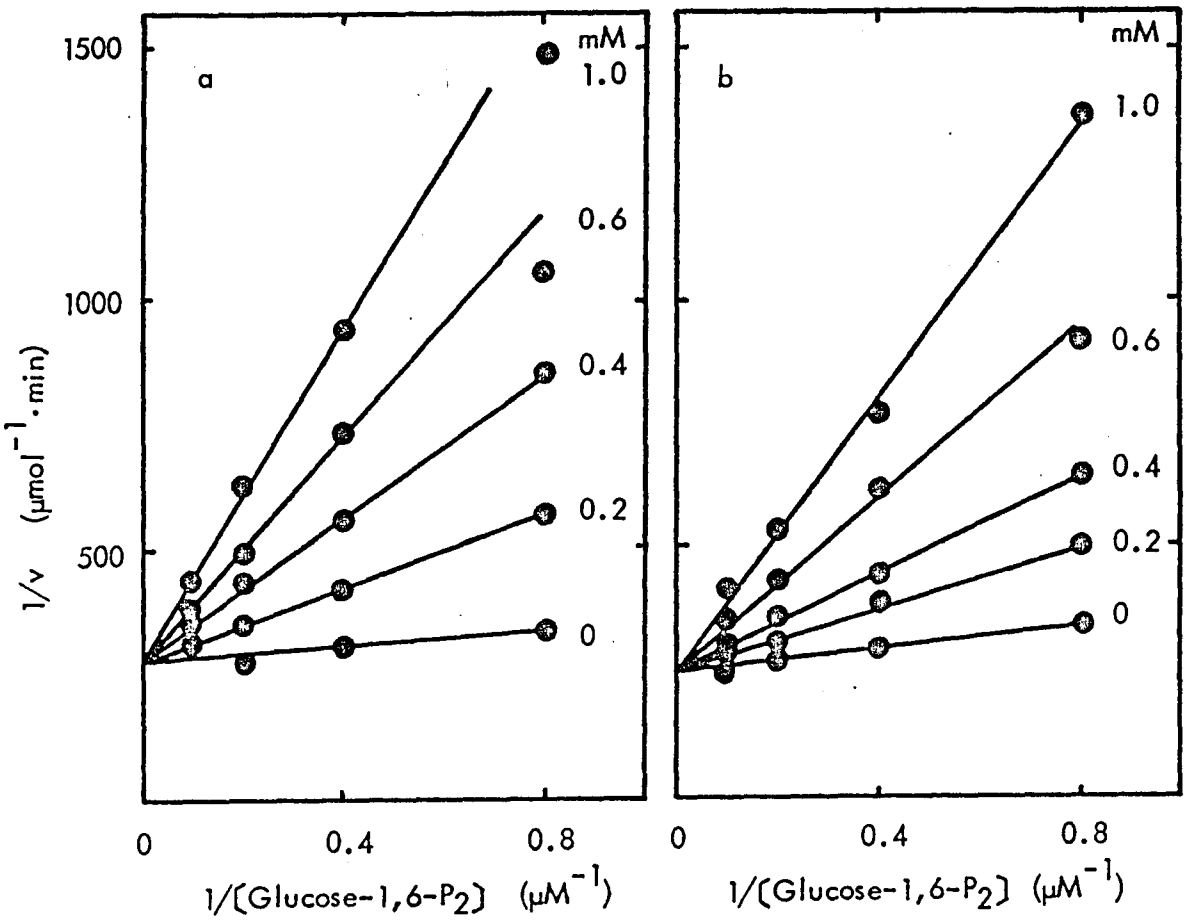
a
b
mM
1.0
0.6
0.4
0.2
0
1500
1000
500
1/v (μmol⁻¹·min)
0
0.4
0.8
1/[Glucose-1,6-P2] (μM⁻¹)

Fig. 12

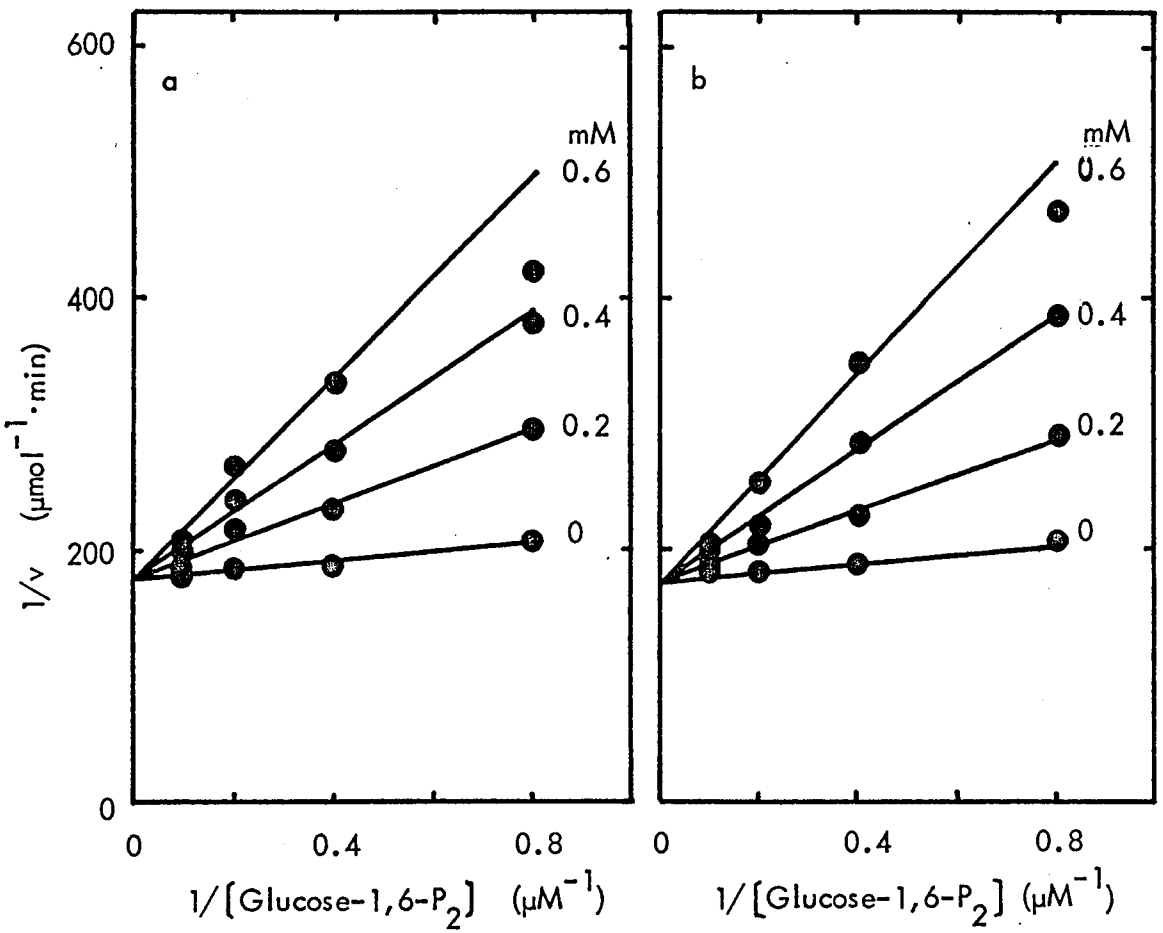
600
400
200
0
a
b
mM
0.6
0.4
0.2
0
1/v (μmol⁻¹·min)
0
0.4
0.8
1/[Glucose-1,6-P2] (μM⁻¹)

Fig. 13

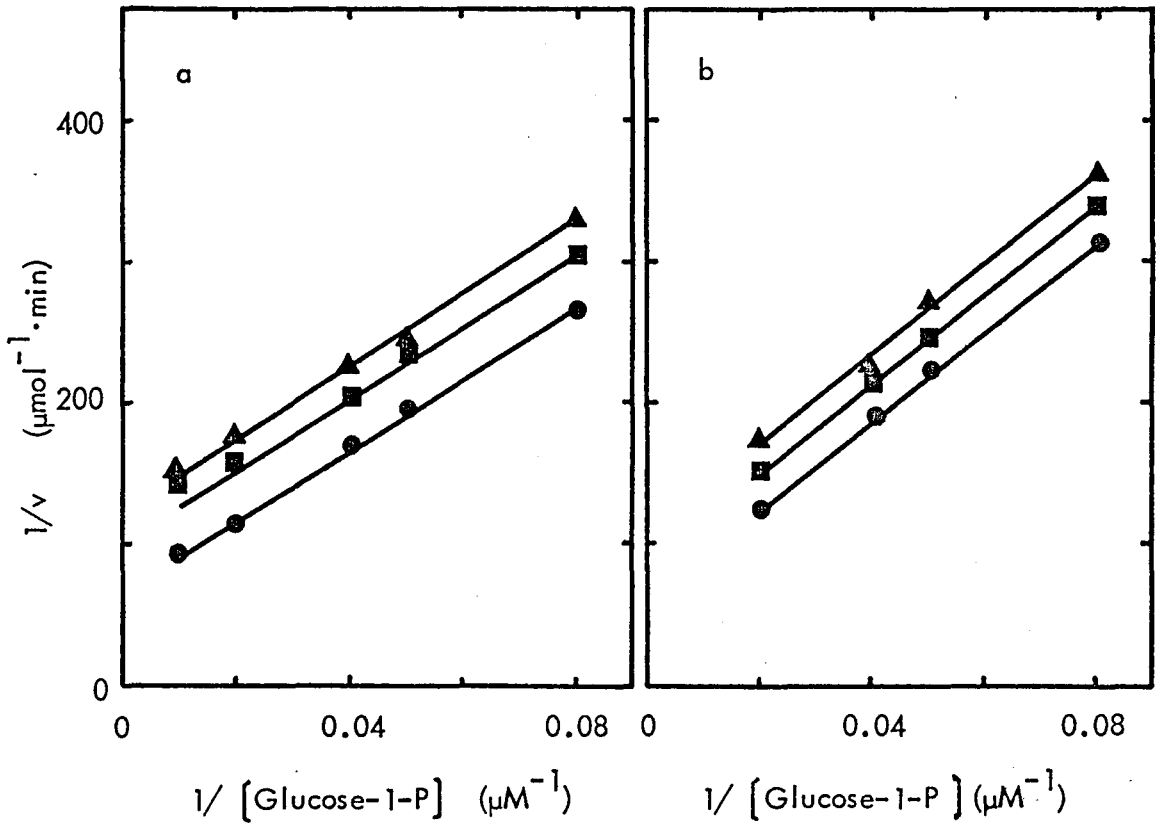


Fig. 14

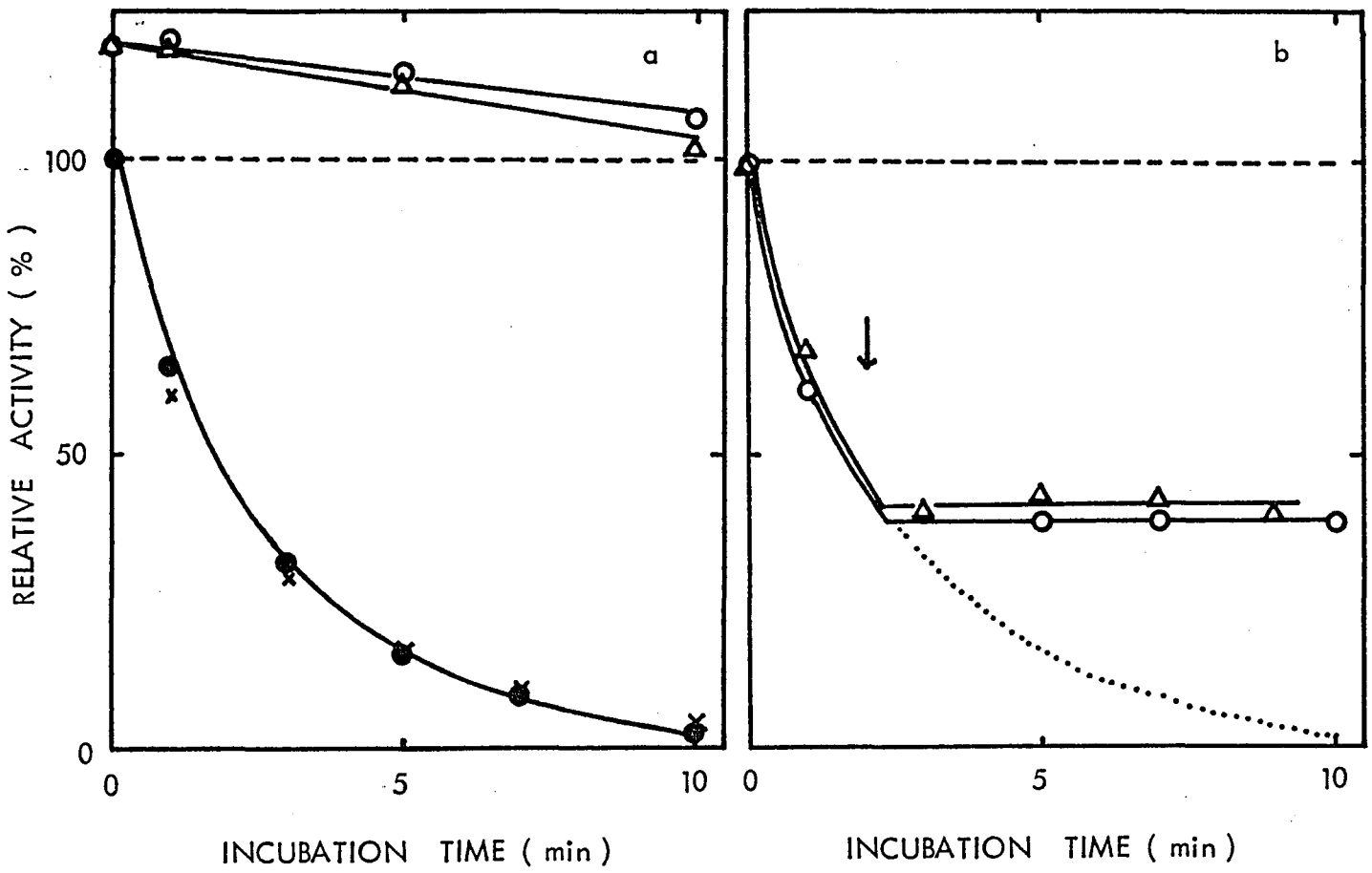
a
b
100
50
0
RELATIVE ACTIVITY ( % )
0
5
10
INCUBATION TIME ( min )
0
5
10
INCUBATION TIME ( min )

Fig. 15

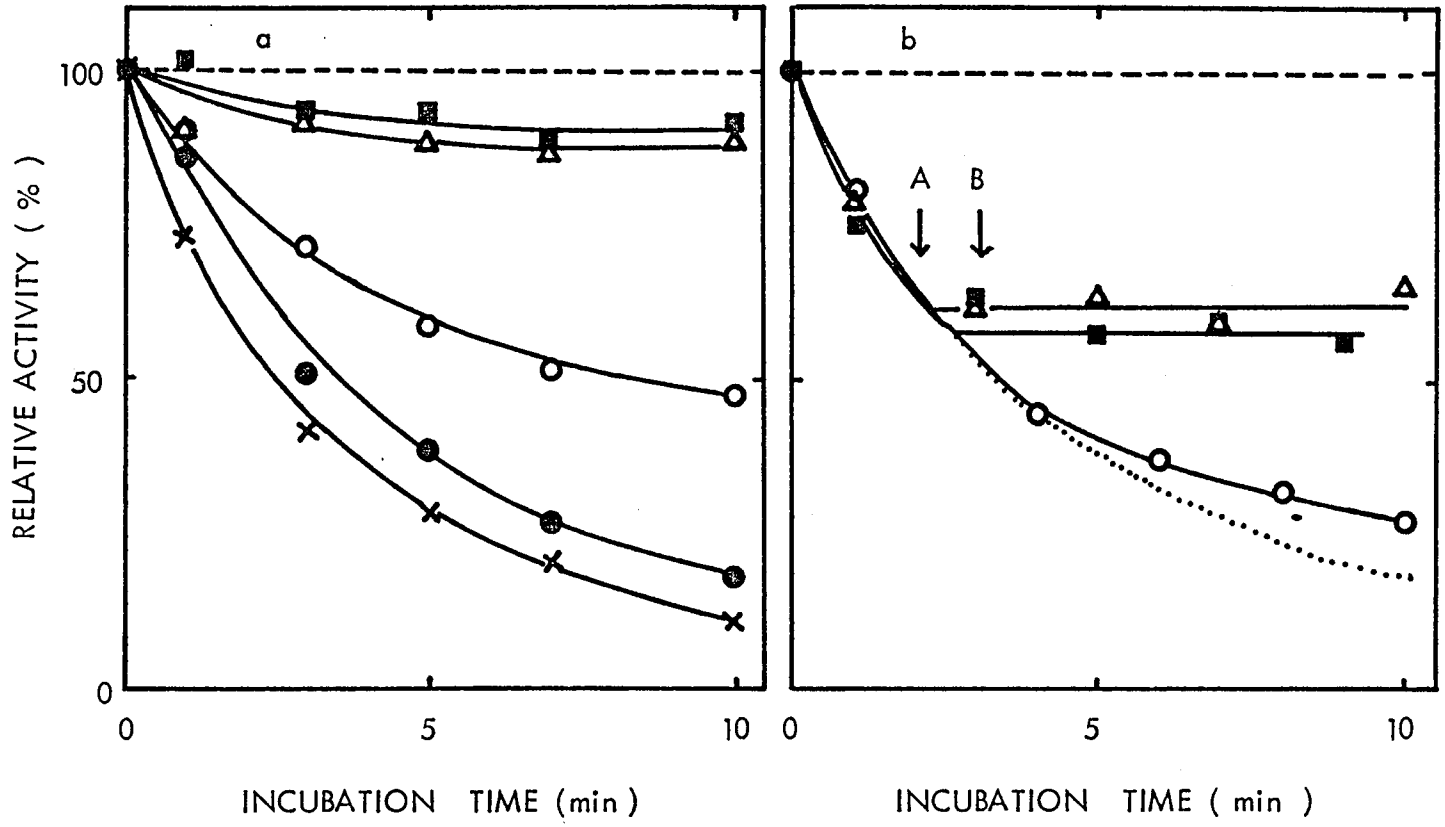
a
b
100
50
0
RELATIVE ACTIVITY ( % )
A
B
0
5
10
0
5
10
INCUBATION TIME (min )
INCUBATION TIME ( min )

Fig. 16

## List of Publications

The present thesis will be published in the following papers.

1) Purification and Multiple Forms of Phosphoglucomutase from Potato Tubers, S. Takamiya and T. Fukui, Plant Cell Physiol. in press (1978).
2) Chemical and Catalytic Properties of Potato Phosphoglucomutase, S. Takamiya and T. Fukui, J. Biochem. in preparation.