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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

2

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

4

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

$$\text{Glucose-1-P} + \text{glucose-1,6-}P_2 \rightleftharpoons$$
$$\text{glucose-1,6-}P_2 + \text{glucose-6-P} \quad \text{(Eq. 1)}$$
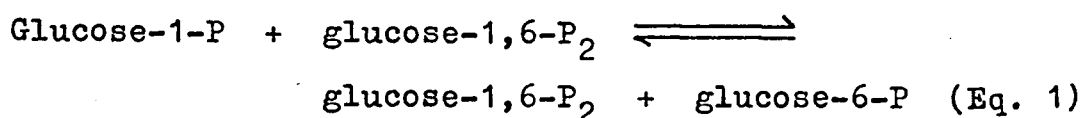
nature, and has been purified from several different origins, i.e., muscle (1,2,3), liver (4), yeast (5,6) and bacteria (7,8). Their molecular properties and reaction mechanism have been studied. Some of the enzymes exist as multiple forms (9).

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

7

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.

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.

<u>Assay of enzyme activity</u>   In the assay of phosphogluco=
mutase 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 m$\underline{M}$ Tris-HCl buffer, pH 7.4 containing 0.2 m$\underline{M}$ 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 $\underline{\underline{N}}$ 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 $4^\circ C$.

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 x 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 x g for 15 min to precipitate the mitochondrial
fraction. The supernatant material was centrifuged at
100,000 x 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 x 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 x g for 10 min, in order to separate starch. The
supernatant solution was centrifuged again at 25,000 x g
for 10 min after its pH had been adjusted to 7.0. The

pellet was suspended in 2.4 ml 0.1 $\underline{M}$ Tris-HCl buffer, pH 7.2, containing 0.05% 2-mercaptoethanol and stored at $4^\circ C$. 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 x $\underline{g}$ for 1 hr, and finally, the supernatant solution was centrifuged at 140,000 x $\underline{g}$ 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 $4^{\circ}C$. All centrifugations
were run at $10,000 \times g$ for 10 min.

Potato tubers (<u>Solanum</u> <u>tuberosam</u> 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 m$\underline{M}$ 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 $\underline{N}$ 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 $4^{\circ}C$.

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 $\underline{N}$ 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 m$\underline{M}$ potassium phosphate

buffer containing 0.2 m$\underline{M}$ EDTA, 1 m$\underline{M}$ 2-mercaptoethanol,

1 m$\underline{M}$ MgCl$_2$ and 20% (v/v) glycerol, pH 7.4 (Buffer A).

The dark brown solution was desalted by passing through

16

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 $\underline{M}$ 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 $\underline{M}$ 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 $\underline{M}$ 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 x 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°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 $4^\circ C$ 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 $\underline{N}$ 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 m$\underline{M}$ potassium phosphate, pH 7.4, in 600 ml) at a flow rate of 5 ml/10 min. In this chroma= tography, 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 m$\underline{M}$ 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 $30^\circ C$ in distilled water, peeled, and homogenized with the equivalent weight of 0.1 $\underline{M}$ 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 $\underline{M}$ 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 $40^{\circ}C$ 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 m$\underline{M}$ Tris-HCl, pH 8.5, containing
8 $\underline{M}$ guanidine-HCl and 72 µM 2-mercaptoethanol at $37^{\circ}C$ for
2 hr, and then alkylated with 65 mg of sodium iodoacetate
at pH 8-9. The reaction mixture was dialyzed against
0.1 $\underline{M}$ Tris-HCl, pH 8.0, containing 9 $\underline{M}$ 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 $\underline{N}$ HCl at $110^{\circ}C$ 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

26

forms. Effects of Mg^{2+}, EDTA and cysteine on the activity
of Peak I enzyme were tested in the presence of 40 $\mu\underline{M}$

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 $m\underline{M}$
in the presence of 0.2 $m\underline{M}$ 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 $m\underline{M}$ 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

27

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, $\underline{Km}$ values for glucose-1-P and for glucose-1,6-P_2 were determined as 60 $\mu\underline{M}$ and 1.8 $\mu\underline{M}$, respectively.

Fig. 10

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

Fig. 11

$\underline{Km}$ 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, $\underline{Ki}$'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 $\underline{M}$), 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 m$\underline{M}$ potassium phosphate buffer containing 0.2 m$\underline{M}$ EDTA, 1 m$\underline{M}$ 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 $\underline{M}$),
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 m$\underline{\text{M}}$ 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 mM $MgCl_2$ 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.

37

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$\underline{M}$.
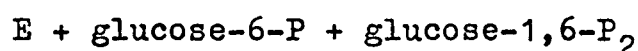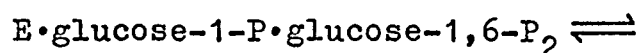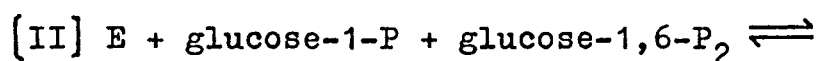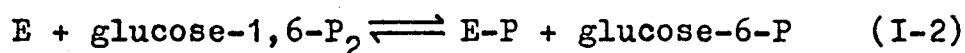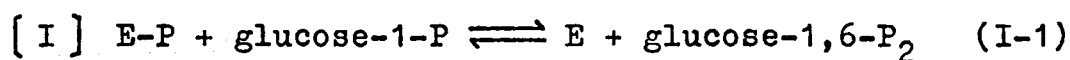
3-3. pH optima

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

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

$\underline{Kb}$ 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 $\underline{vice}$ $\underline{versa}$.
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 $\underline{Escherichia}$ $\underline{coli}$
(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
$\underline{Bacillus}$ $\underline{cereus}$, $\underline{Micrococcus}$ $\underline{lysodeikticus}$ (8) and
$\underline{Saccharomyces}$ $\underline{cerevisiae}$ (5). This mechanism should follow
Cleland's "sequential" kinetics and gives the rate equation
in the absence of products as shown in Eq. 2,

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

where A and B are glucose-1-P and glucose-1,6-P_2, $\underline{Ka}$ and
$\underline{Kb}$ are Michaelis constants for glucose-1-P and glucose-1,6-P_2,
and $\underline{Kib}$ 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 $\underline{vice}$ $\underline{versa}$.

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 phosphogluco=
mutase 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 m$\underline{M}$. 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 m$\underline{M}$(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 m$\underline{M}$,
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 m$\underline{M}$, 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 m$\underline{M}$.

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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ACKNOWLEDGMENTS

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 $\underline{M}$ 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. 16×10^4), rabbit muscle phosphorylase b (2, mol. wt. 9.7×10^4), bovine serum albumin (3, mol. wt. 6.8×10^4), ovalbumin (4, mol. wt. 4.3×10^4), yeast alcohol dehydrogenase (5, mol. wt. 3.7×10^4), 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 $\mu\underline{M}$. 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 $\mu\underline{M}$ 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 $\mu\underline{M}$ 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 $\mu\underline{M}$ fructose-1,6-P_2; ▲, 200 $\mu\underline{M}$ glycerate-2,3-P_2). b) Peak II enzyme (●, none; ■, 100 $\mu\underline{M}$ fructose-1,6-P_2; ▲, 200 $\mu\underline{M}$ glycerate-2,3-P_2). The concentration of glucose-1,6-P_2 was 2.5 $\mu\underline{M}$. 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 m$\underline{M}$ Tris-HCl, pH 7.5, containing 20% (v/v) glycerol at 25°C. After preincubation for 5 min, 10 μl of 1 m$\underline{M}$ BeCl$_2$ was added to the incubation mixture in the absence (●) or in the presence of various compounds (1 m$\underline{M}$ MgCl$_2$, O ; 0.94 m$\underline{M}$ EDTA, △ ; 1 m$\underline{M}$ 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 $\mu$$\underline{M}$ BeCl$_2$ in 0.99 ml of 10 m$\underline{M}$ Tris-HCl, pH 7.5, containing 20% (v/v) glycerol at 25°C, and 0.94 m$\underline{M}$ EDTA (△) or 1 m$\underline{M}$ MgCl$_2$ (O) 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 m$\underline{M}$ Tris-HCl, pH 7.5, containing 20% (v/v) glycerol at 25°C. After preincubation for 5 min, 10 μl of 1 m$\underline{M}$ BeCl$_2$ was added to the incubation mixture in the absence (●) or in the presence of various compounds (1 m$\underline{M}$ MgCl$_2$, O ; 10 m$\underline{M}$ MgCl$_2$, ■ ; 0.94 m$\underline{M}$ EDTA, △ ; 1 m$\underline{M}$ 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 $\mu\underline{M}$ $BeCl_2$ in 0.99 ml of 10 $m\underline{M}$ Tris-HCl, pH 7.5, containing 20% (v/v) glycerol at 25°C, and 0.94 $m\underline{M}$ EDTA (Δ) or 10 $m\underline{M}$ $MgCl_2$ ($\blacksquare$) (indicated by A) or 1 $m\underline{M}$ $MgCl_2$ ($\mathbf{O}$) (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 (μmol)	72 hr Hydrolysis (μmol)	Average (μmol)	Residures per 62,000 g	Nearest Integer
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 Total content (mg)	Recovery (%)	Phosphoglucomutase Total activity (units)	Recovery (%)	Phosphorylase Total activity (units)	Recovery (%)
Crude	18.6	100	27.5	100	24.8	100
14,000 × $\underline{g}$ ppt	1.7	9.1	0.1	0.4	0.3	1.2
100,000 × $\underline{g}$ ppt	0.8	4.3	0.0	0.0	0.1	0.4
100,000 × $\underline{g}$ 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 Total content (mg)	Recovery (%)	Phosphoglucomutase Total activity (units)	Recovery (%)	Phosphorylase Total activity (units)	Recovery (%)
Crude	449	100	194	100	252	100
25,000 × g ppt	36.1	8.0	6.9	3.6	8.8	3.5
100,000 × g ppt	21	4.7	1.9	1.0	4.4	1.7
140,000 × g ppt	10.3	2.3	1.4	0.7	6.7	2.7
140,000 × g sup	354	78.7	140	72.3	187	74.0

From 100 g fresh tissue

Fig. 1

ENZYME ACTIVITY (units/ml, O——O)

KCl (W---)

FRACTION NUMBER

ABSORBANCE AT 280 nm (●——●)

Fig. 2

Fig. 3

ENZYME ACTIVITY (units/ml, ○——○)

pH (·····)

ABSORBANCE AT 280 nm (●——●)

FRACTION NUMBER

5.76

5.91

6.20

Fig. 4

a   b

Fig. 5

ENZYME ACTIVITY (units/ml, O————O)

pH (·······)

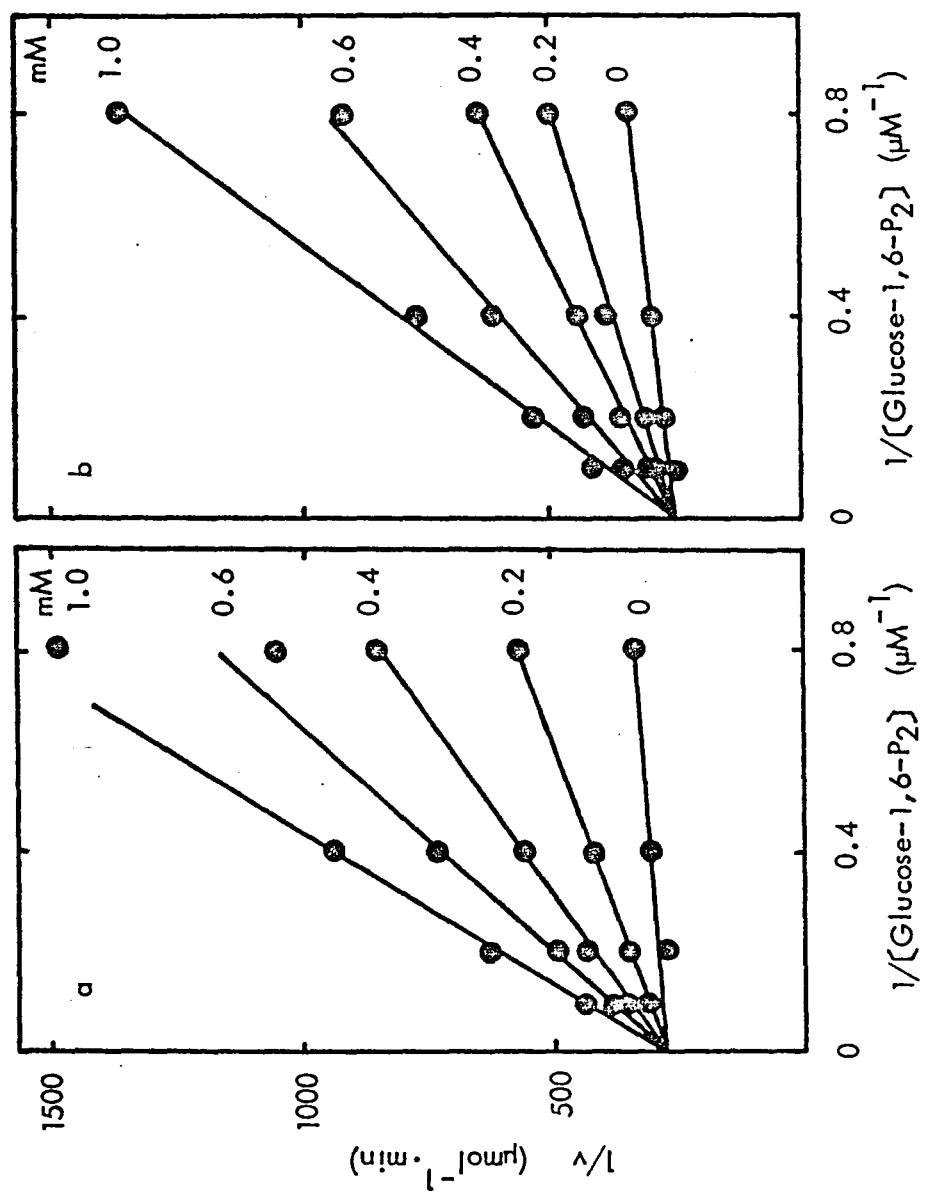ABSORBANCE AT 280 nm (●——●)

5.62

5.81

FRACTION NUMBER

Fig. 6

Fig. 7

Fig. 8

RELATIVE ACTIVITY (%)

pH

pH
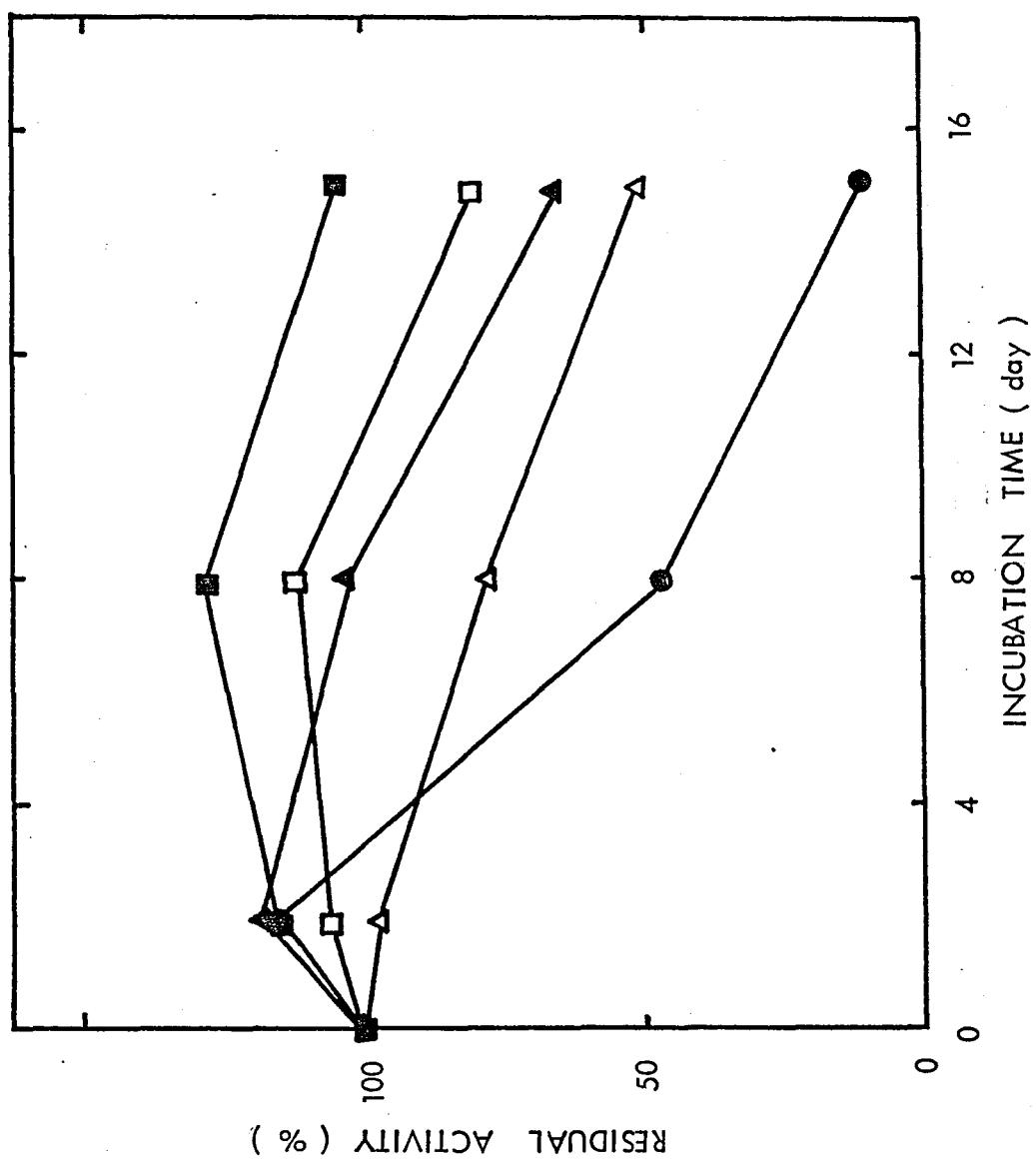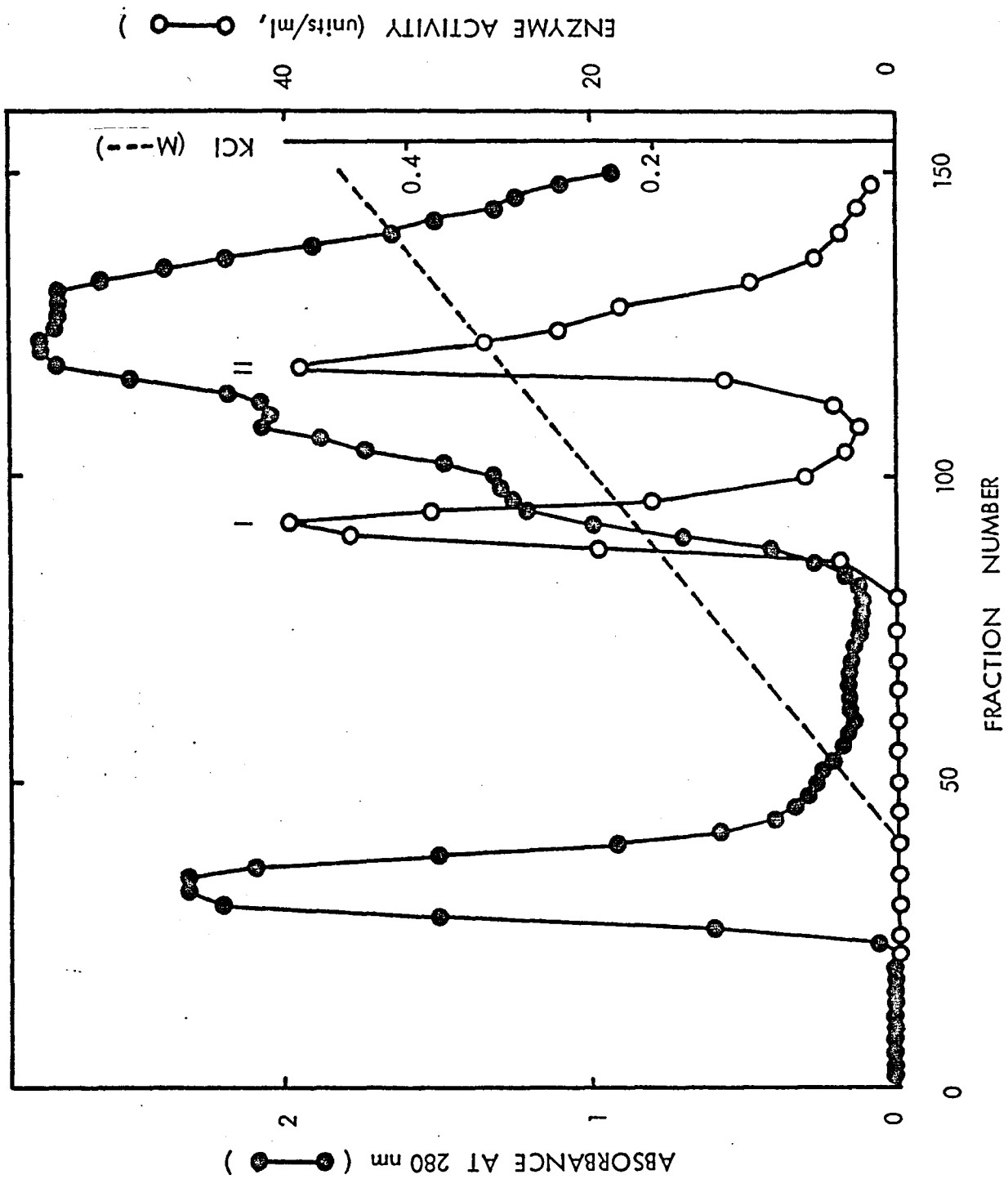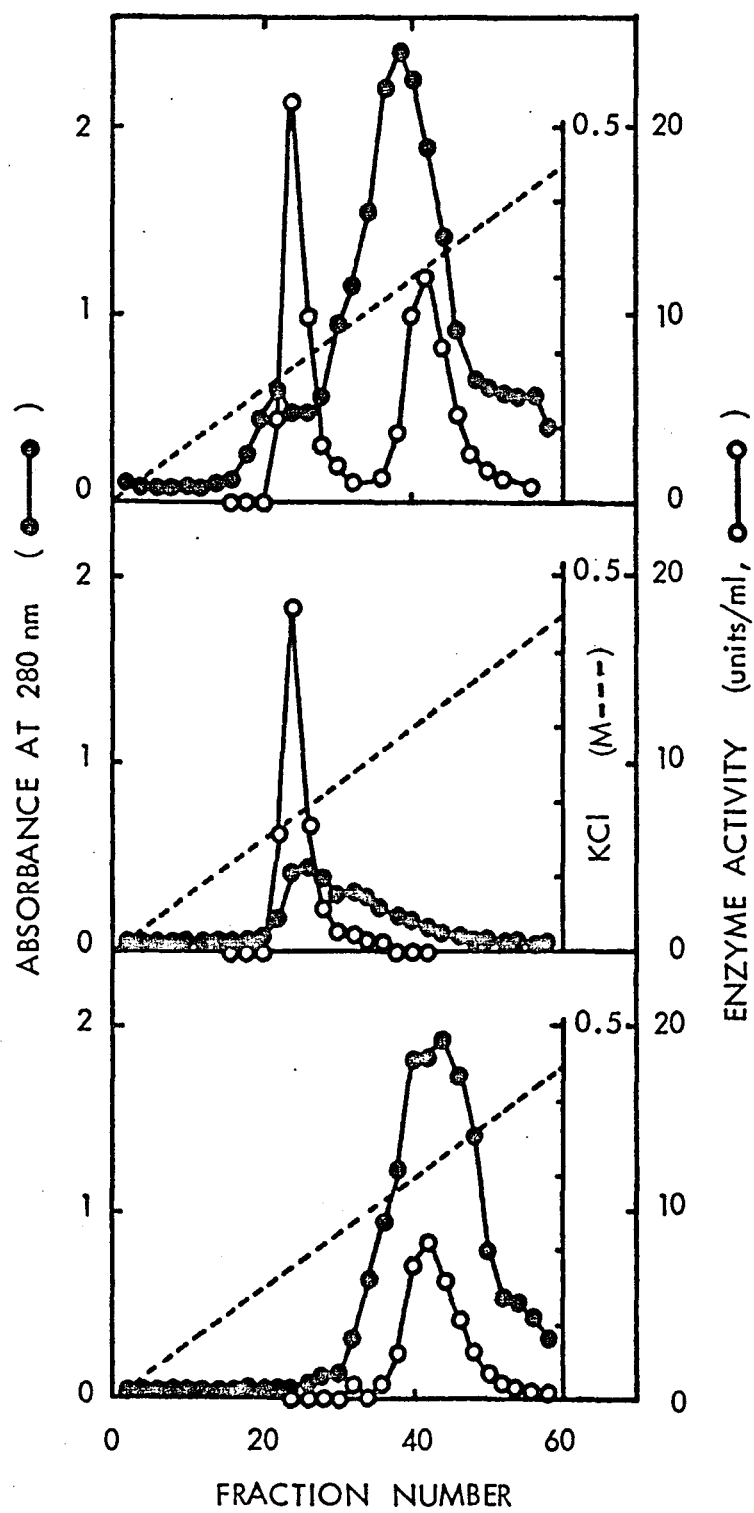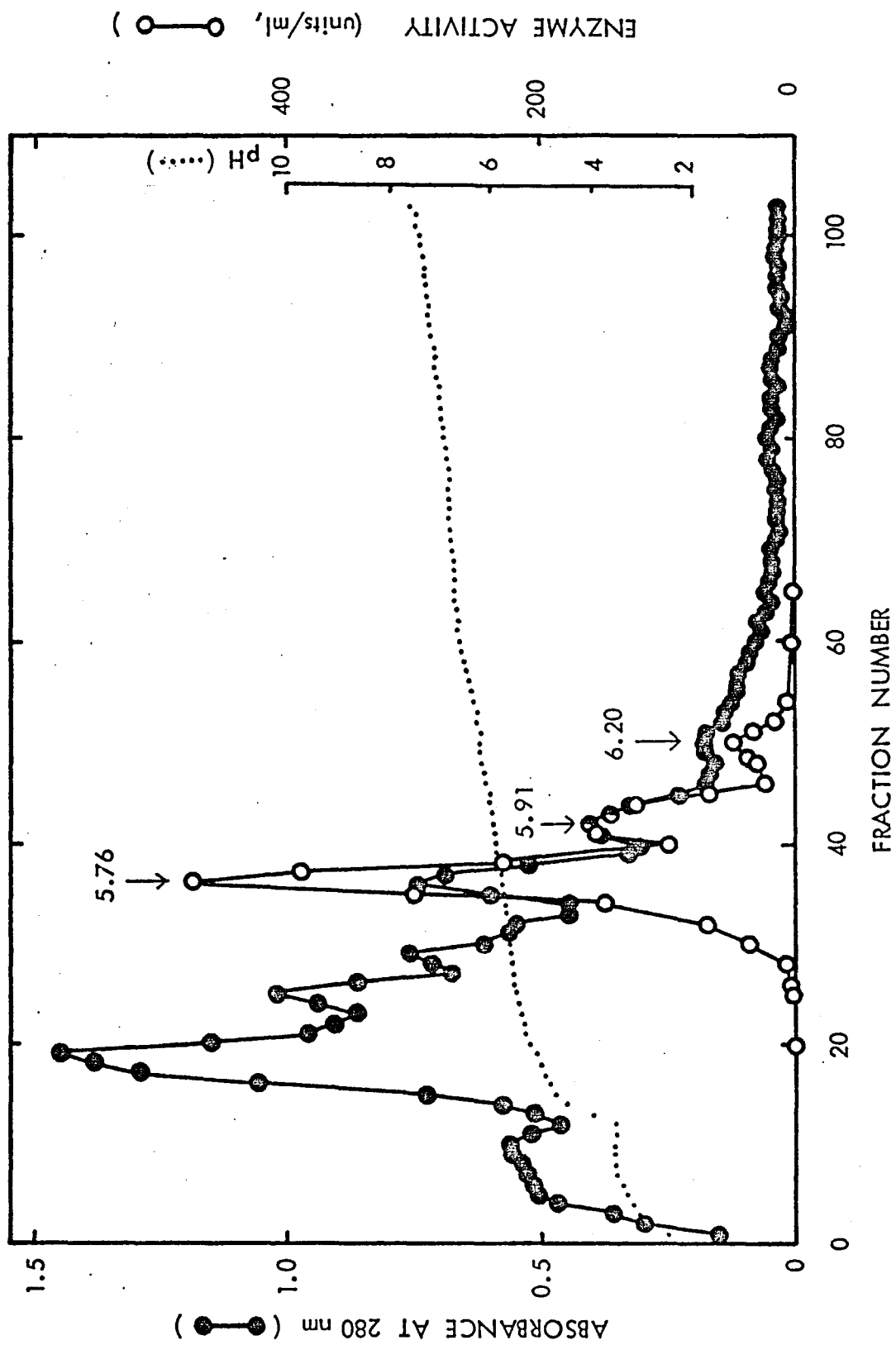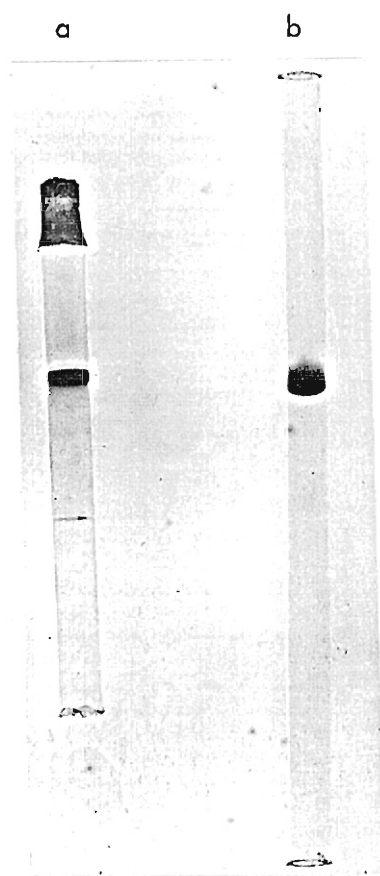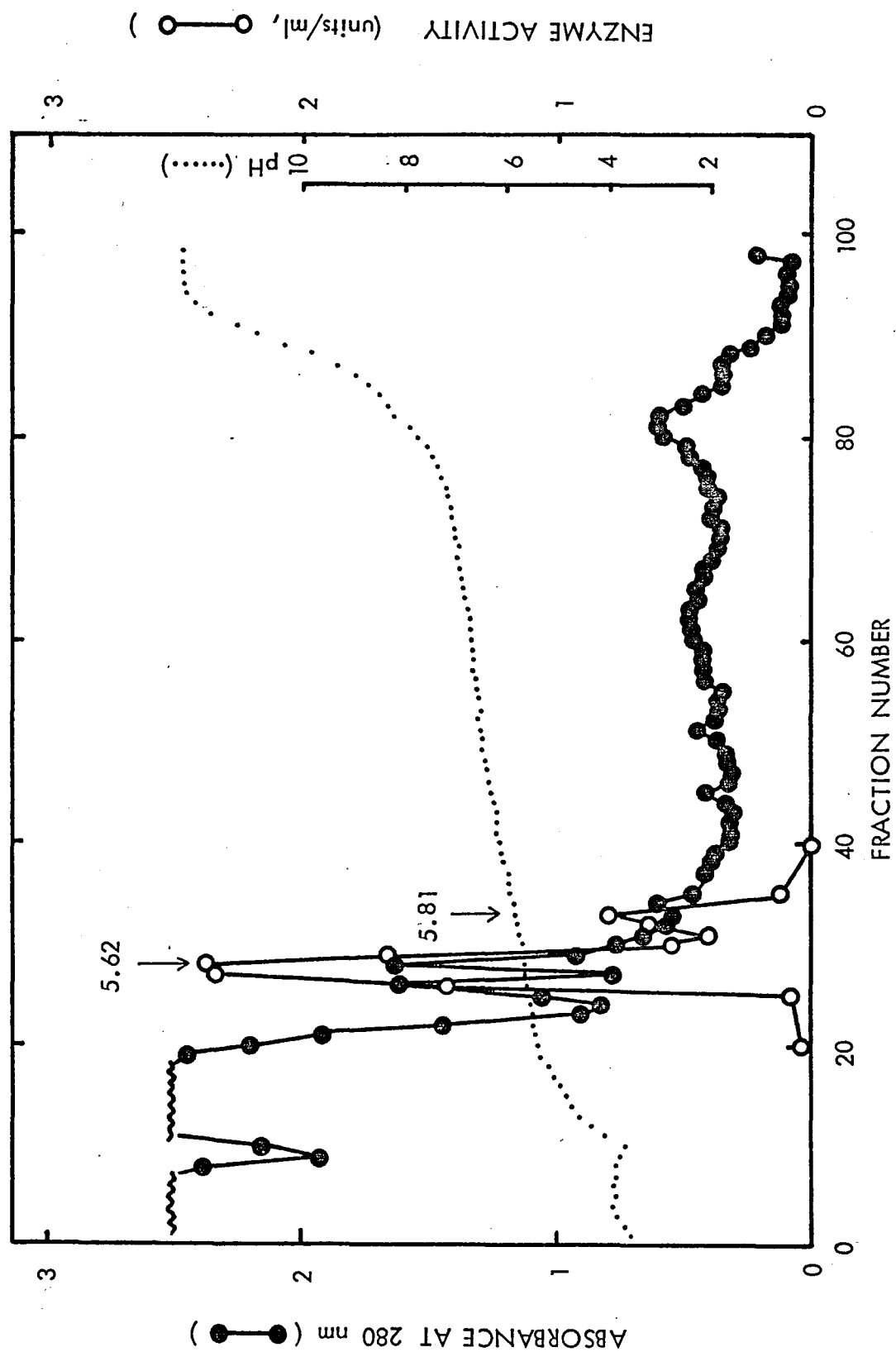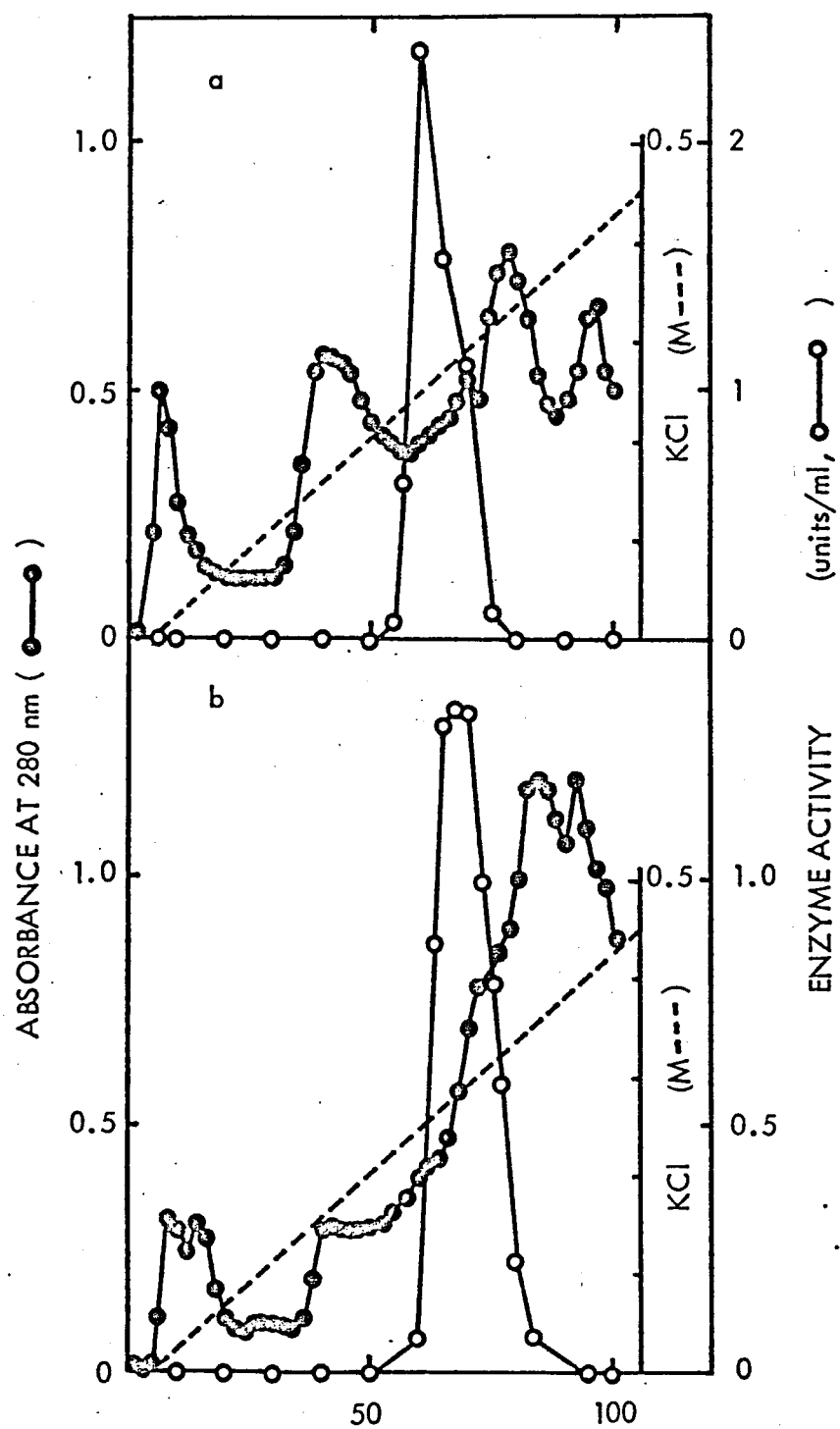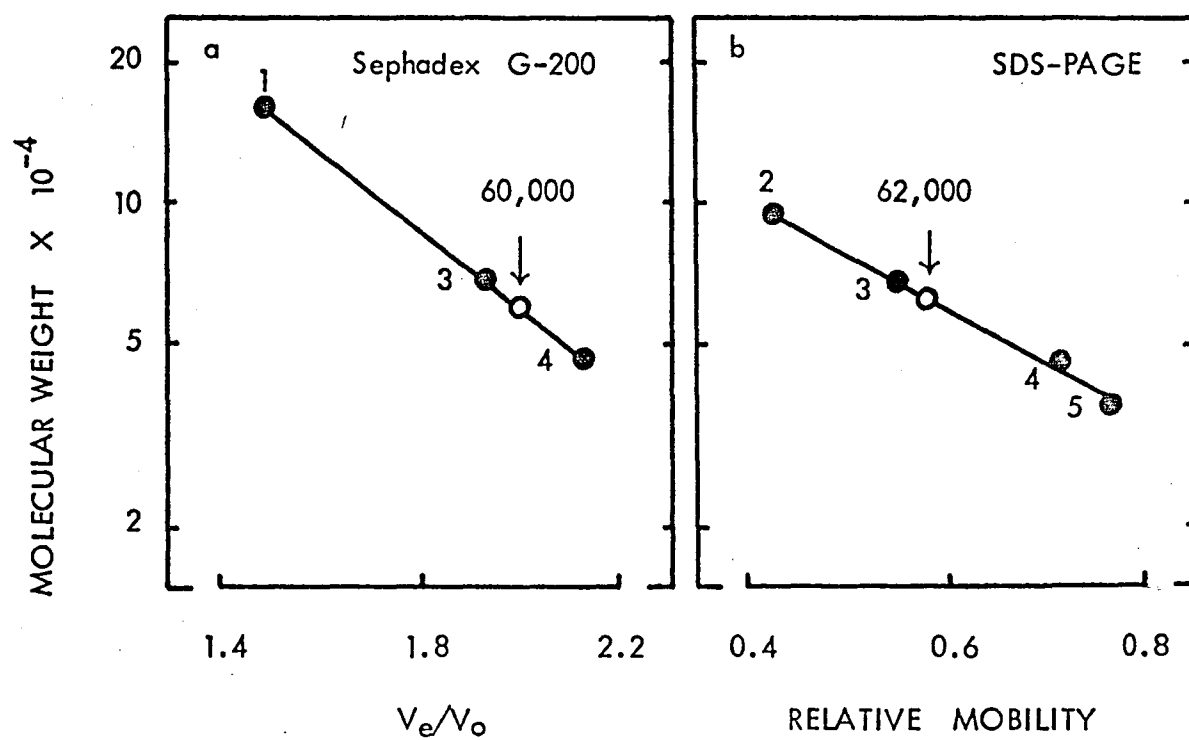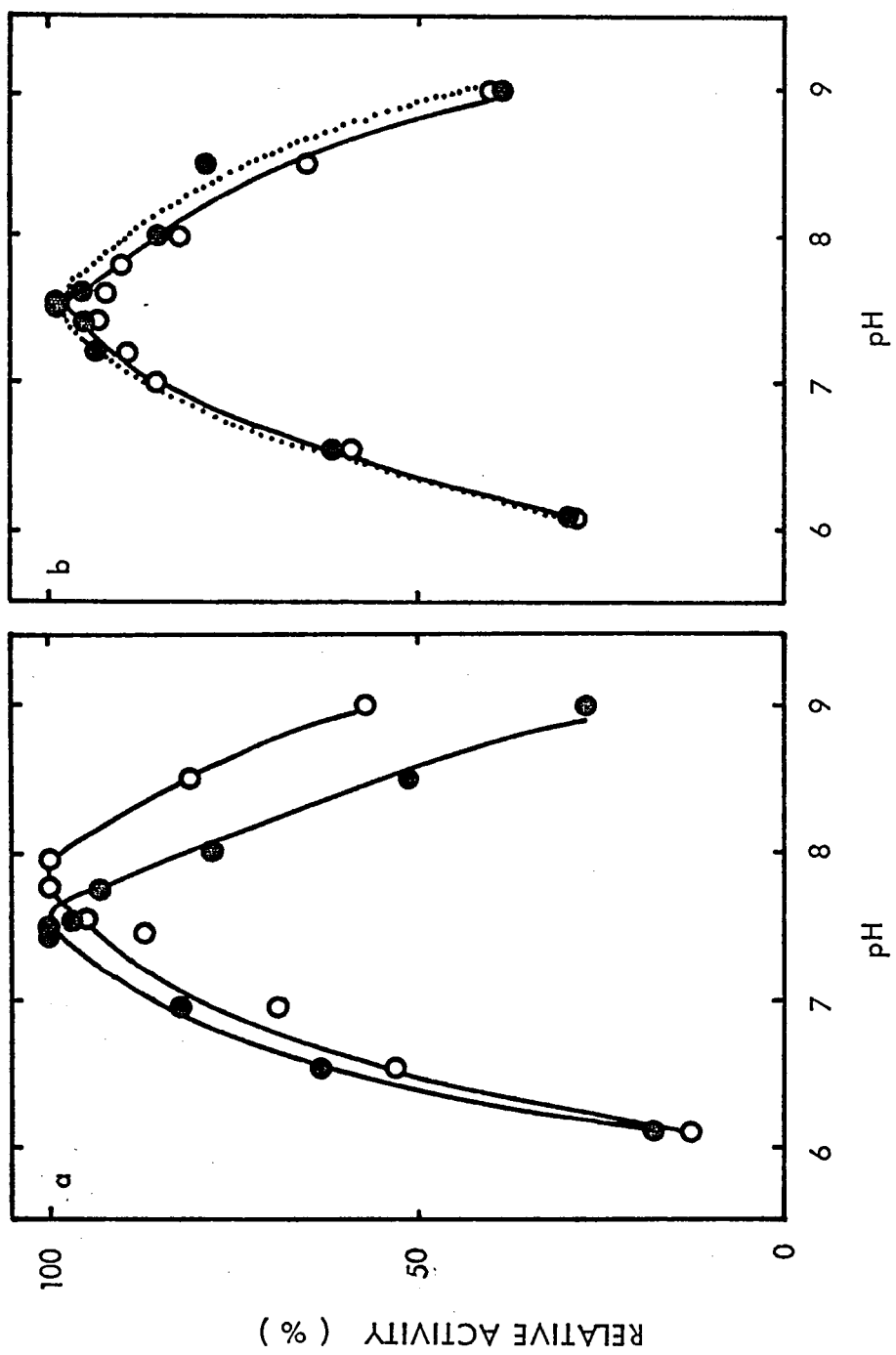
Fig. 9
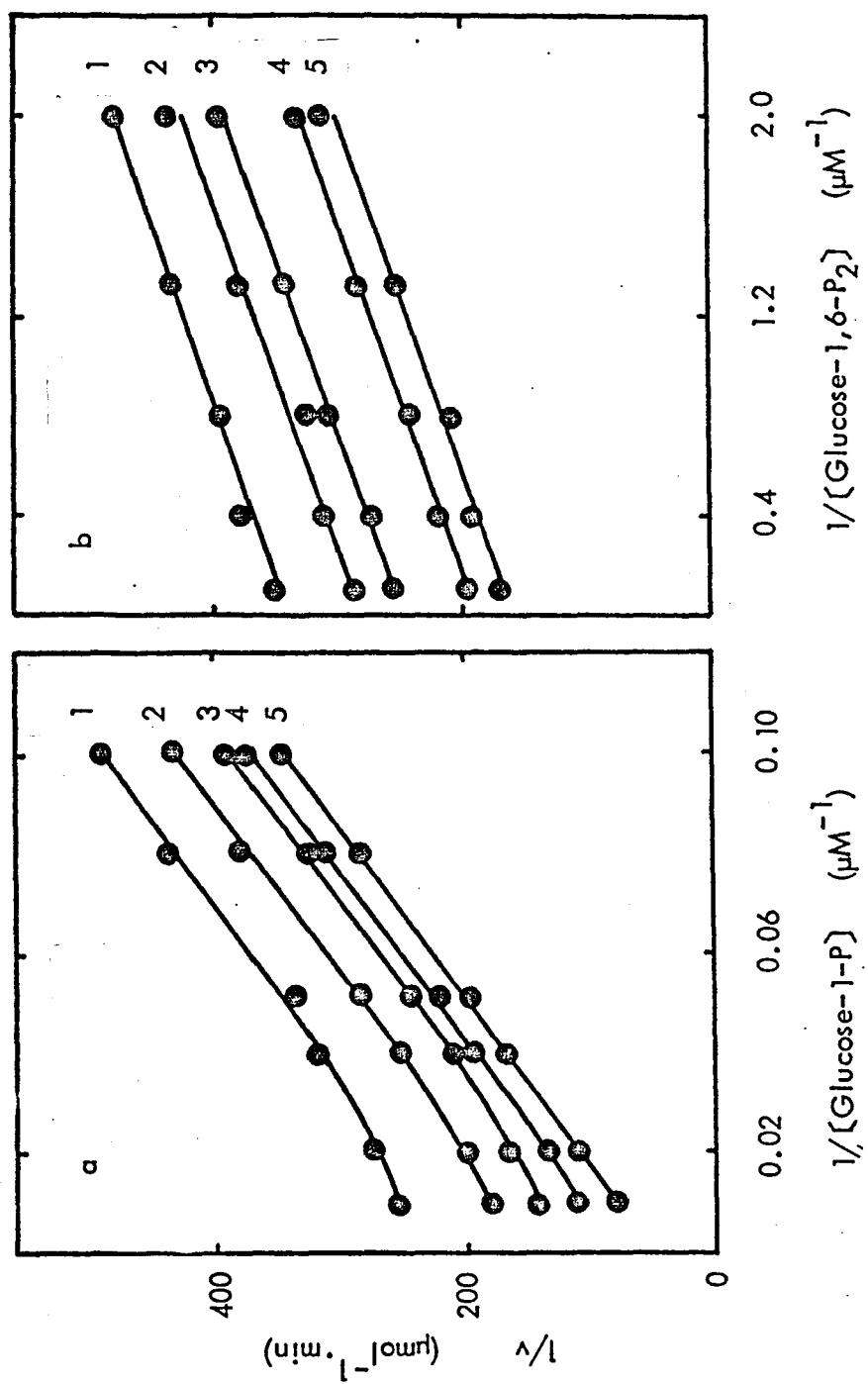
Fig. 10

Fig. 11

Fig. 12

Fig.13

Fig. 14

RELATIVE ACTIVITY (%)

INCUBATION TIME (min)

INCUBATION TIME (min)

Fig. 15

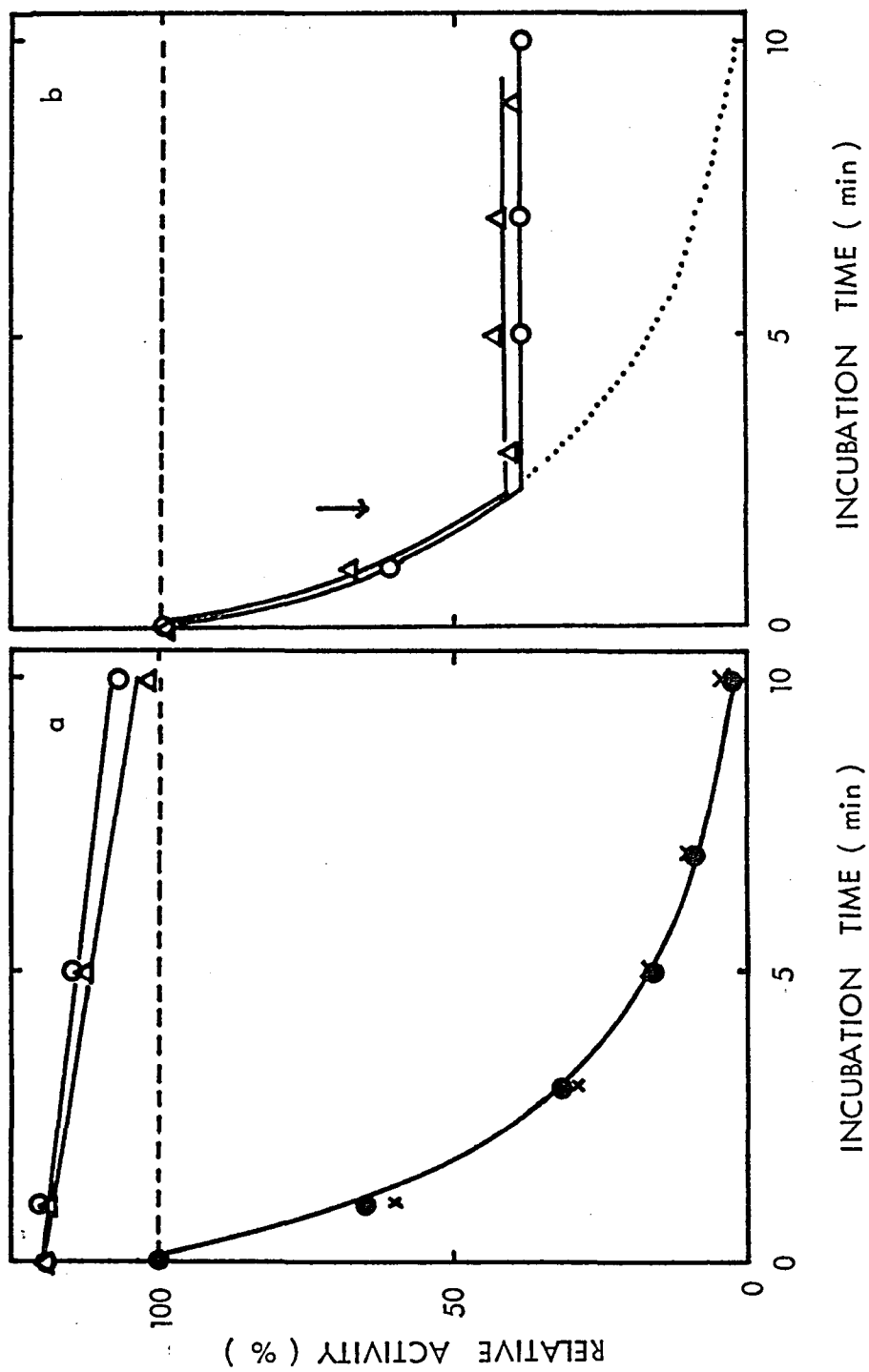RELATIVE ACTIVITY (%)
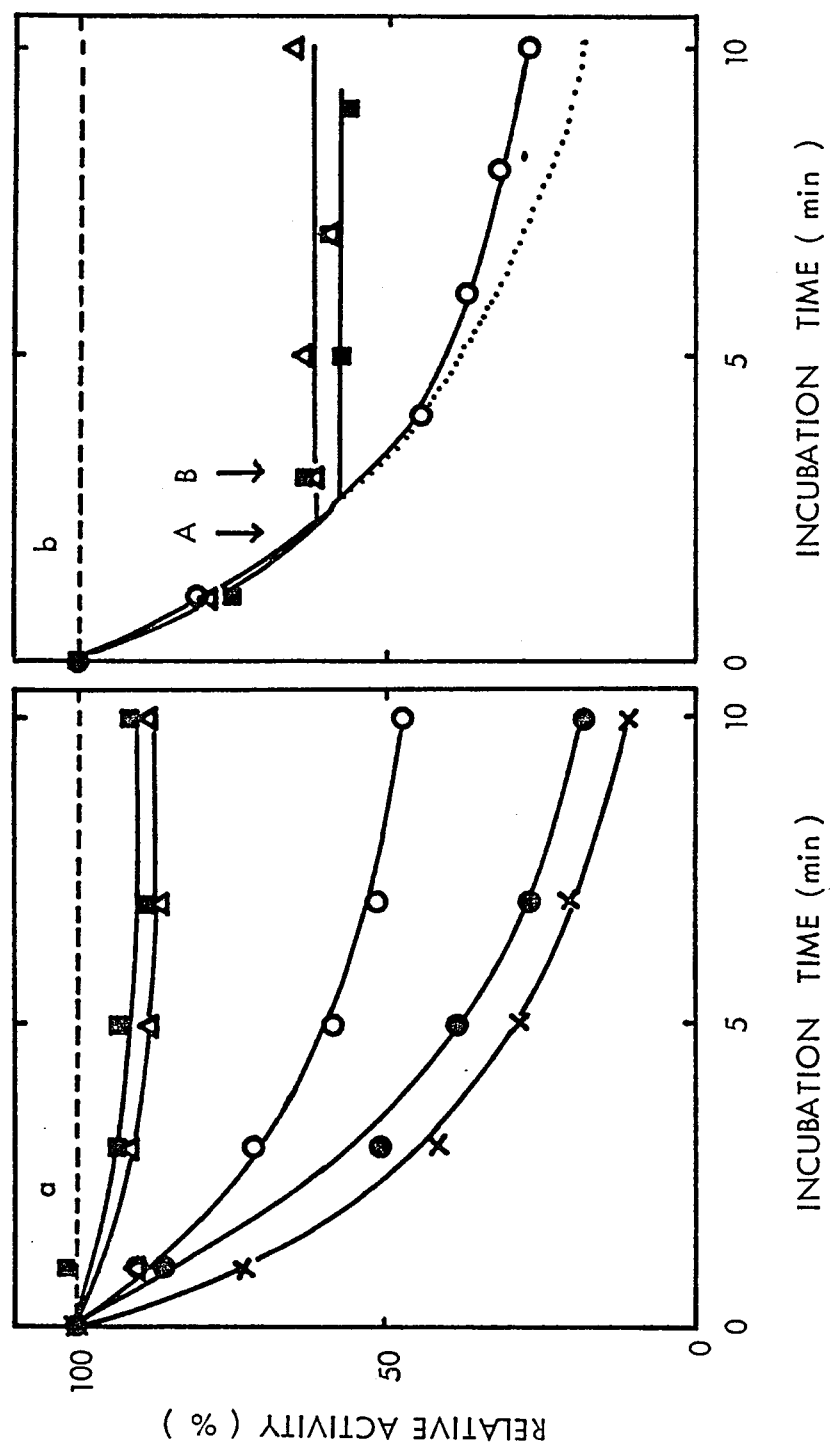
INCUBATION TIME (min)

INCUBATION TIME (min)
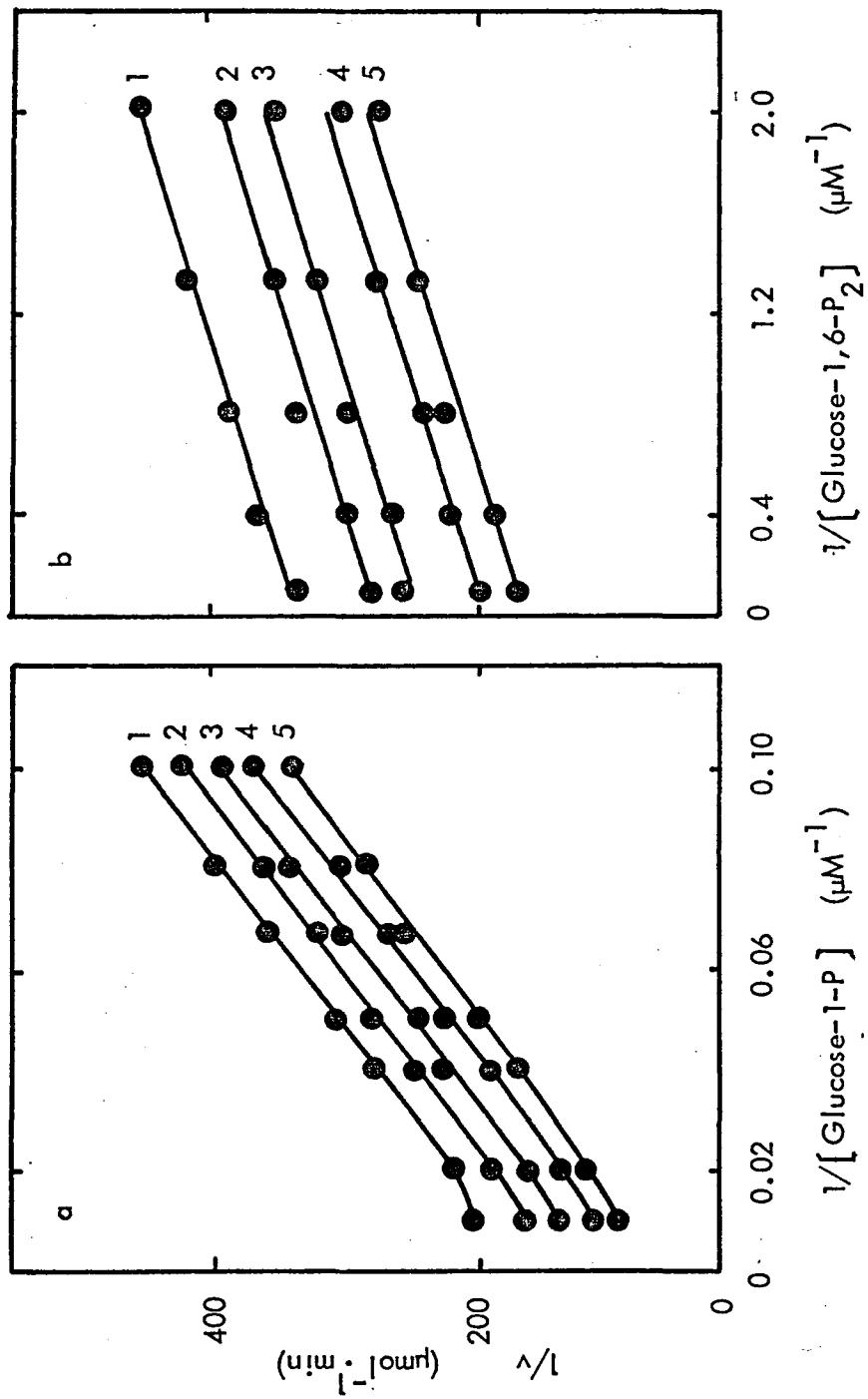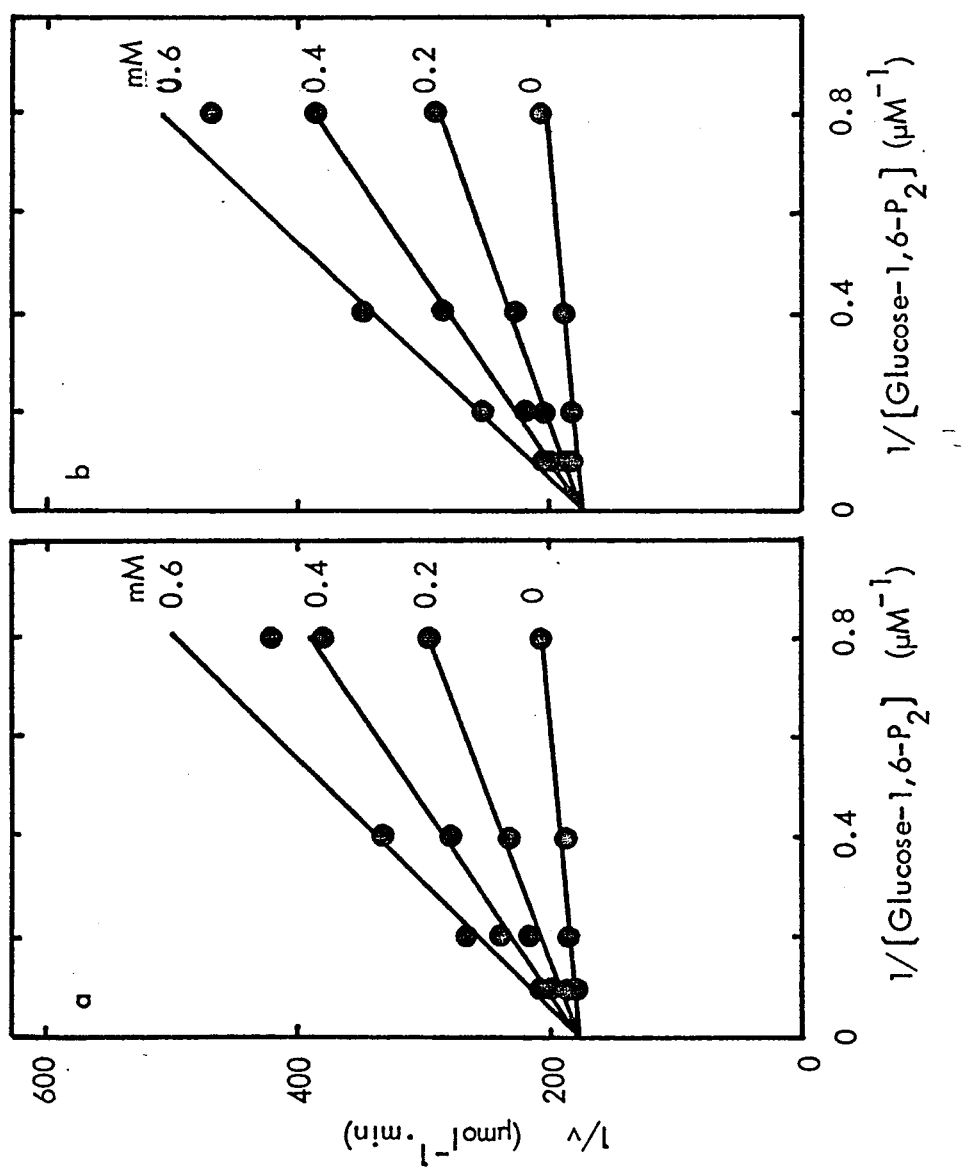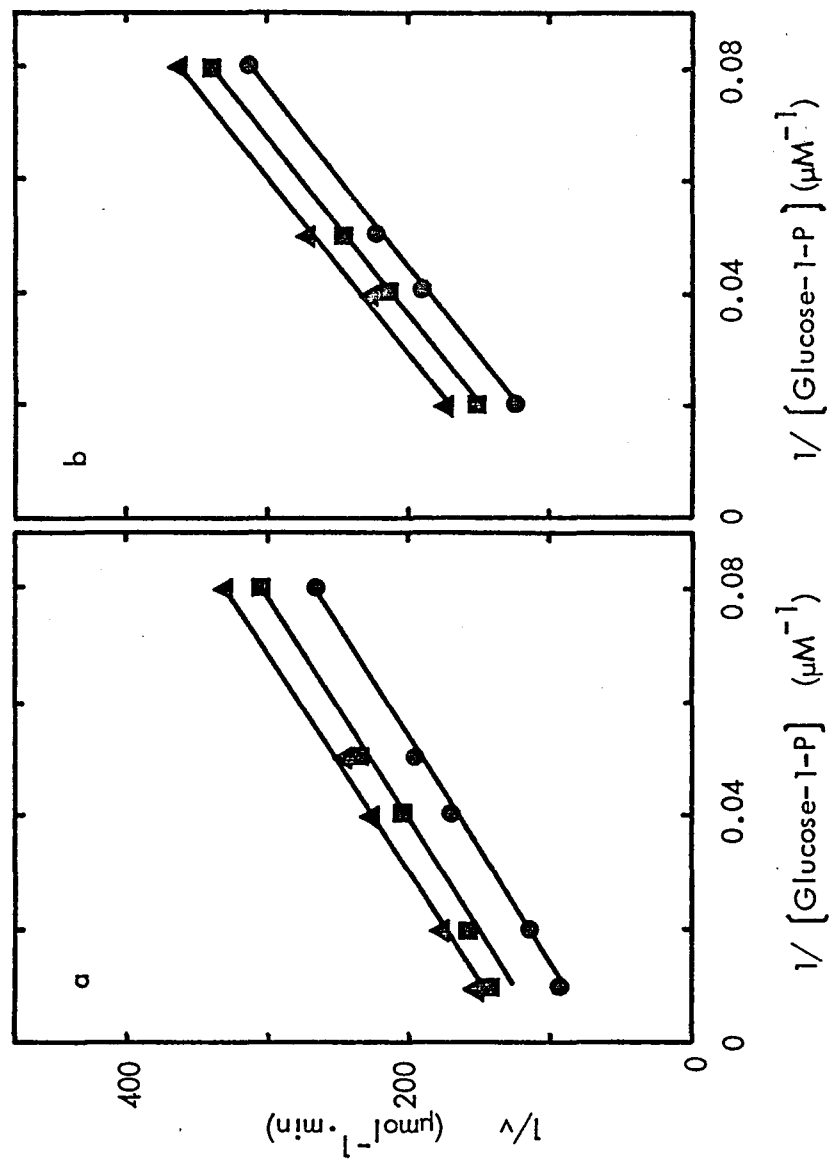
Fig. 16

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.