〔県立会津短大学報自然科学編第37号，21～36，1980〕

Purification of Pig Liver 6-Phosphogluconate Dehydrogenase and Charactarization of Mammalian 6-Phosphogluconate Dehydrogenase

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Introduction

The enzymatic reaction of 6-phosphogluconate dehydrogenase is shown in Fig. 1.

Reaction of 6-Phosphogluconate Dehydrogenase (6-PG DH)

$$NADP^+ + \underset{(6\text{-PG})}{\text{6-Phosphogluconate}} \rightleftharpoons CO_2 + \underset{(Ru\text{-}5\text{-}P)}{\text{Ribulose 5-Phosphate}} + NADPH + H^+$$

Fig. 1. The enzymatic reaction of 6-phosphogluconate dehydrogenase

The level of 6-phosphogluconate dehydrogenase in liver are regulated by dietary fatty acid(1,2,3). The evidence may indicates that the enzyme may play an important role in the regulation of fatty acid synthesis by means of NADPH supply. Rudack et al. have reported that the half-life for 6-phosphogluconate dehydrogenase *in vitro* was 13.5 hours(3). The rate seems to be relatively high but not enough to explain the rapid change of the rate of fatty acid synthesis.

Therefore the efficiency of catalytic activity of the enzyme in liver may be involved in short-term regulation of fatty acid synthesis.

Mammalian enzyme has recently been purified from sheep and rat liver(4,5). In the present work, however, pig liver 6-phosphogluconate dehydrogenase was purified for a further investigation of the properties of the enzyme. Dyson and D'Orazio(6) have reported that fructose 1,6-diphosphate is a competitive inhibitor with respect to 6-phosphogluconate for sheep liver enzyme. In contrast to this, Procsal and Holten(5) have failed to demonstrated such an inhibition with the rat liver enzyme.

We have examined the nature of the inhibition by some glycolytic intermediates.

The inhibitory effects of fructose 1, 6-diphosphate on pig and rat liver enzyme were rather feeble. But these enzymes were inhibited more strongly by phosphoenolpyruvate. The mechanism of the inhibition has been studied. It is not evident from the present work that the sensitivity of these enzymes to the metabolite may contribute the short-term regulation of the enzymatic activity in liver.

Materials

Pig and chicken liver were obtained at local slaughter house and stored in a frozen state at −20°C. Male rats of wister were watered and fed with low-fat high-carbohydrate diet (Clea, Tokyo) for 3 to 5 days before sacrifice in order to increase the level of the enzyme(5).

$NADP^+$, NADPH, ATP, 6-phosphogluconate, glucose 6-phosphate, glucose 1-phosphate, fructose 1, 6-diphosphate, 2,3-diphosphoglycerate, 3-phosphoglycerate, phosphoenolpyruvate, egg albumin, bovine serum albumin, lactate dehydrogenase (rabbit muscle), aldolase, catalase, glucose 6-phosphate dehydrogenase and triethanolamine hydrochloride were purchased from Boehringer Mannheim. Riburose 5-phosphate was prepared according to Pontremoli(7) and 5-phosphoribonate by bromide oxidation.

Human γ-globulin was obtained from Nutritional Biochemicals Corporation.

Trizma base was purchased from Sigma. DEAE-cellulose and CM-cellulose were obtained from Brown, Shephadex G-200 and blue dextran 2000 were from Pharmacia. Hydroxylapatite was prepared according to Tiselius et al. (8).

Enzyme Assays

The mixture used to standard assay for 6-phospogluconate dehydrogenase activity during the enzyme purification contained 50 μmol of Tris-HCl buffer, pH 7.5, 10 μmol of $MgCl_2$, 0.1 μmol of $NADP^+$, 0.5 μmol of 6-phosphogluconate, and enzyme in a final volume of 1.0 ml.

One unit of enzyme is that amount of enzyme which forms 1 μmol of NADPH in 1 minute at 25°C under the assay conditions described above.

Initial velocities were determined using Hitachi spectrophotometer 181 epuipped with recording apparatus.

In kinetic experiments, the mixture contained 130 μmol of triethanolamine•HCl-KOH buffer, pH 7.0, varied amounts of substrates, enzyme and other reagents. Rates were measured at 25° C with the recording spectrophotometer with expanded scale.

Estimation of Molecular Weight

The molecular weight of the native enzyme protein was determined by gel filtration on Sephadex G-200 (1.6x50cm) equilibrated with 10 mM potassium phosphate buffer pH 7.0. The sample (1ml) contained of 5 mg of protein markers, 1 mg of blue dextran and the enzyme. The elution profile was summarized by the following equation:

$$Kd = Ve - Vo \,/\, Vi - Vo$$

Kd, distribution coefficient; Ve, elution volume of the sample; Vo, void volume evaluated with blue dextran; Vi, total volume accessible to solvent.

The molecular weight of the subunit was determined by the method of Weber and Osborn(9). The gels were 10% cross-linked. A linear standard curve was obtained.

Electrophoresis

The method of electrophoresis on Cellogel was the slight modification of the method of Beitner and Naor(10). Electrophoresis was for 60 minutes. The buffer system used was Tris-citrate-EDTA (0.07 M Tris, 0.021 M citric acid and 0.005 M K_3EDTA) at pH 7.5. Cellogels were developed for 6-phosphogluconate dehydrogenase activity at 25°C for 30 minutes in an incubation medium consisting of 50 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.05 mM $NADP^+$, 0.05 mM 6-phosphogluconate, 0.1 mM phenazine methosulfate, and 0.4 mM nitro-blue tetrazolium. The protein was stained with Ponceaux 3R. Polyacrylamide gel electrophoresis was performed in 7 % polyacrylamide with Tris-glycine buffer (5 mM Tris-38.5 mM glycine, pH 8.3) according to Davis(11). The staining procedure for the enzyme activity was the same as that for Cellogels, and the protein was located with Amido black.

Purification of 6-phosphogluconate dehydrogenase

Frozen pig liver (1 kg) was thawed overnight at 4°C and homogenized with 3volumes of water in a Waring blender for 1 minute. The homogenate was adjusted to pH5.5～5.6 by addition 1 N acetic acid, and centrifuged at 10,000 × g for 15 minutes. The supernatant was adjusted to pH6.5～7.0 by addition of 5 N KOH. All subsequent steps were carried out at 4° C.

Solid ammonium sulfate (310 g per liter) was added, and the mixture was left for 30 minutes before centrifugation. To the supernatant solution, ammonium sulfate (90 g per liter)was added and centrifuged after 30 minutes. The resultant precipitate was taken in water, and the volume of the enzyme solution was adjusted with water to give 5 volumes of the precipitate. The enzyme solution was treated with two vol-

umes of bentonite suspention (30 mg per ml of water) and centrifuged. The supernatant fraction was treated with ammonium sulfate (400 g per liter). The precipitate was collected with water to a total volume of about 100ml. The enzyme was dialyzed against 1 liter of 5 mM potassium phosphate buffer, pH8.0 for 35～40 hours with 4 changes of same buffer. The dialyzed solution was applied on a DEAE-cellulose(400 ml) column equilibrated with the same buffer.

The column was eluted with a linear gradient from 10 (600 ml) to 80 mM (600 ml) potassium phosphate buffer, pH 8.0, 5 mM mercaptoethanol-1 mM EDTA 10%glycerol. To the fractions containing the enzyme was added ammonium sulfate (520 g per liter). The precipitate was taken about 20 ml of water and dialyzed against 500 ml of 5 mM mercaptoethanol-1mM EDTA for 24 hours with 3 changes.

The solution was applied to a CM-cellulose column (100 ml) previously equilibrated with 1 mM potassium phosphate butfer, pH 7.0. The enzyme was eluted using a linear gradient between 1 and 10 mM (300 ml of each) potassium phosphate buffer, pH 7.0-5 mM mercaptoethanol-1mM EDTA-20 % glycerol. Fractions containing the enzyme was pooled and placed on a hydroxylapatite column (30 ml). The linear gradient elution was from 10 to 60 mM (150 ml of each) potassium phosphate buffer, pH 7.0-5 mM mercaptoethanol-1 mM EDTA-20 % glycerol.

The enzyme thus eluted was pooled and dialyzed against 500 ml of 5 mM mercaptoethanol-1 mM EDTA overnight. The dialyzed enzyme solution was applied on a DEAE-cellulose column (30 ml), equilibrated with 3 mM potassium phosphate buffer, pH 7.0, and eluted with 3 to 30 mM (150 ml of each) potassium phosphate buffer, pH7.0-5 mM mercaptoethanol-1 mM EDTA 20 % glycerol. Fractions containing 6-phosphogluconate dehydrogenase were pooled and concentrated with Zeineh Microconcentrator Model UF-T.

Results

The pig enzyme was purified to homogeneity according to the modification of Villet and Dalziel (4) and Procsal and Holten (5).

Over-all yield of the purified enzyme was about 12～15 mg from 1 kg of liver with the specific activity of 16～19 units per mg at 25° C. An example of yield of the enzyme at various steps of purification is shown in Table I. However the omission of glycerol at column chromatography steps resulted a conciderable loss of activity.

TABLE I. Purification of Pig Liver 6-Phosphogluconate Dehydrogenase[a)]

Purification stop	Volume (ml)	Protein[b)] (mg)	Activity (units)	Sp. Act., (units/mg)	Yield (%)
1. Crude extract	2,460	116,000	1,070	0.0093	100
2. $(NH_4)_2SO_4$ fractionation	500	19,000	805	0.0424	75
3. Bentonite-$(NH_4)_2SO_4$fractionation	100	9,020	728	0.0807	68
4. First DEAE-cellulose chromatography	300	1,419	618	0.436	58
5. CM-cellulose chromatography	90	53	476	8.96	44.5
6. Hydroxylapatite chromatography	33	18	309	17.0	28.8
7. Second DEAE-cellulose chromatography	65	13	243	18.5	22.7

a) Starting from 1 kg of pig liver b) Protein determined by the methods of Lowry et al. (15)

The inactivations without glycerol at these steps were also observed for chicken liver enzyme. Rat liver enzyme was purified according to the modification of Procsal and Holten(5), but was inactivated without glycerol at CM-cellulose column chromatography.

The final preparation has no measurable activity of glucose 6-phosphate dehydrogenase, phosphoribose isomerase. The concentrated and purified enzyme was stable for about two months at 4°C and −20°C.

The stability was not markedly changed by addition or omission of glycerol.

Only one protein band was observed in disc gel and Cellogel electrophoresis for native and subunits protein. The molecular weight of the native enzyme and the subunits were 105,000 and 52,000 respectively as shown in Fig.2 and Fig.3.

The presense of two isozymes of 6-phosphogluconate dehydrogenase in rat adipose tissue has been reported. However, the particle-bound isozyme could not be confirmed with pig liver. In electrophoretic experiment with Cellogel, it was found that human erythrocyte, rat erythrocyte and liver contained one enzyme activity, and pig liver contained two activitity. However, purified pig liver enzyme migrated in one enzyme activity. On DEAE-cellulose column chromatography at pH7.0 and 8.0, the consistent evidence suggesting the presence of two isozyme could not obtained. In some experiment, one minor activity was eluted in front of the major peak. With the use of crude extract of chicken and pigeon liver, three or more activities were detected on electrophoresis with Cellogels.

The effect of heating the enzyme at 55°C for various time intervals on the enzyme activity is shown Fig.4. A linear relationship between time and the logarithm of activity was observed in both 0.01 M potassium phosphate buffer, pH 7.0 and 0.13 M trieth-

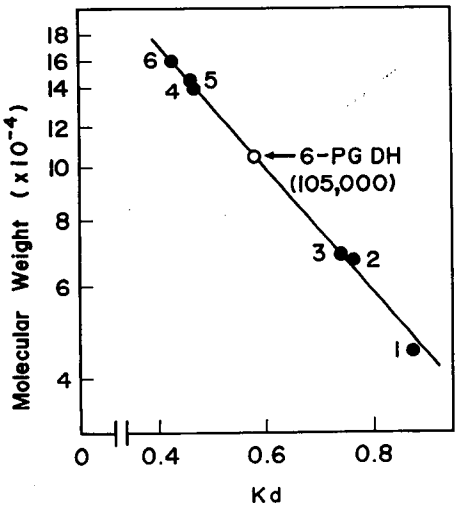

Fig.2.

Determination of molecular weight of pig liver 6-phosphogluconate dehydrogenase by gel filtration on Sephadex G-200.

The following marker protein (5 mg / ml) were used to calibrate the column: 1, ovalbumin; 2, bovine serum albumin; 3, hemoglobin; 4, lactate dehydrogenase; 5, aldolase; 6, γ-globulin. The open symbol represents the elution position of 6-phosphogluconate dehydrogenase.

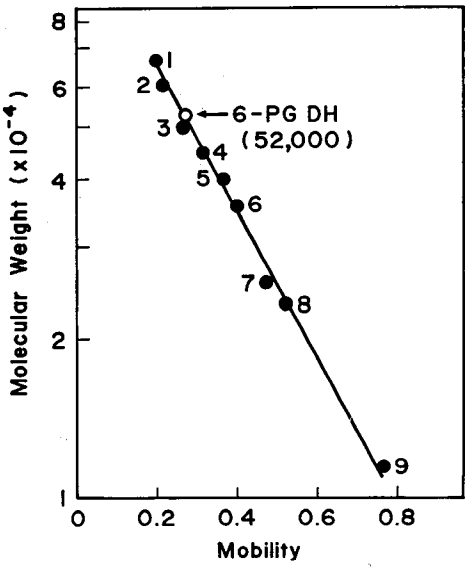

Fig.3.

Molecular weight determination of pig liver 6-phosphogluconate dehydrogenase by sodium dodesyl sulfate polyacrylamide gel electrophoresis. The following protein were used to calibrate the gels: 1, bovine serum albumin; 2, catalase; 3, globulin(H); 4, ovalbumin; 5, aldolase; 6, lactate dehydrogenase; 7, chymotrypsinogen; 8, γ-globulin (L); 9, cytochrome c. The open symbol represents the mobility of 6-phosphogluconate dehydrogenase.

anolamine • HCl-KOH buffer, pH 7.0. The enzyme was inactivated more rapidly in the triethanolamine buffer than the phosphate buffer. $NADP^+$ protected the enzyme from the heat inactivation. The rate of inactivation was decreased to a half in the presence of about 10 μM $NADP^+$.

The enzyme was inactivated in the presence of urea. The linear relationship between time and the logarithm of remaining activity was also obtained as summarized in Fig.5.

The variation of enzyme activity was measured at varied pHs.

The initial rates measured in the presence of 0.1 mM $NADP^+$ and 0.5 mM 6-phosphogluconate are summarized in Fig.6. The increase in enzyme activity by addition of $MgCl_2$ was at range of pH 7 to 9.

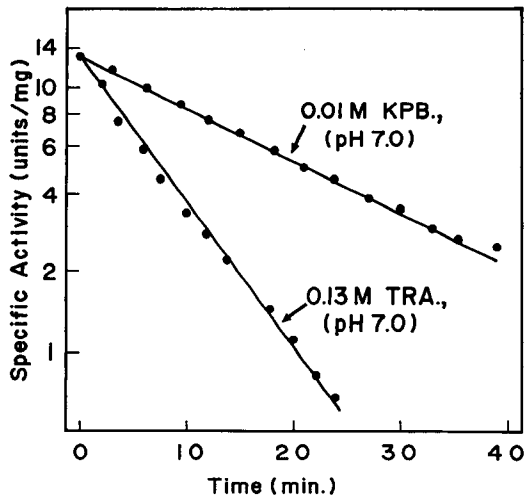

Fig.4.

The effect of heating the enzyme at 55°C for various time intervals on the enzyme activities.

The enzyme activities were determined at each time intervals with the method described in the text.

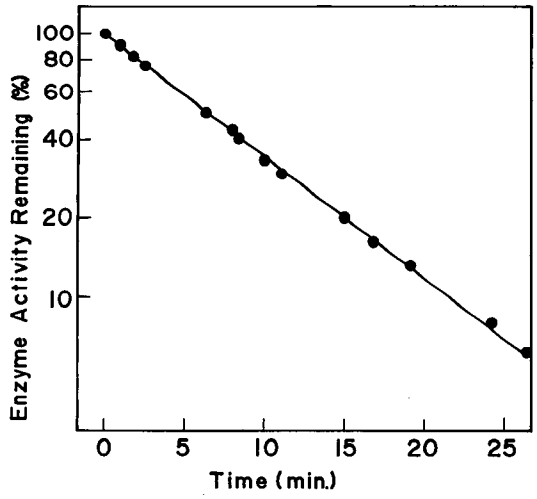

Fig.5.

The effect of 4 M urea on the enzyme activity.

The enzyme activities were determined at each time intervals with the method described in the text.

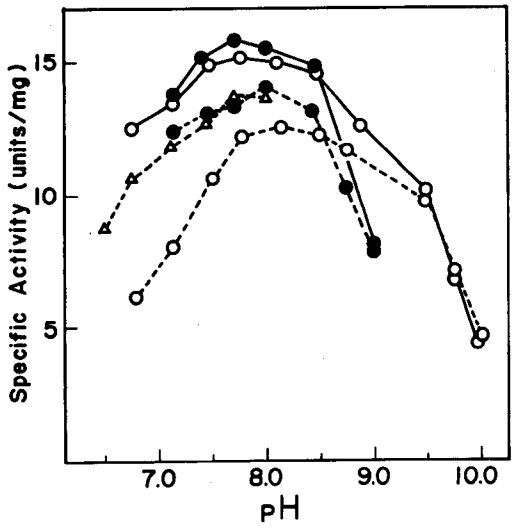

Fig.6.

The variation of the enzyme activity at varied pHs.
(●), 50 mM Tris-HCl buffer; (○), 50 mM triethanol amine·HCl-KOH buffer; (△), 50 mM potassium phosphate buffer. (---), no addition of $MgCl_2$; (—), addition of $MgCl_2$(final concn. was 5 mM).

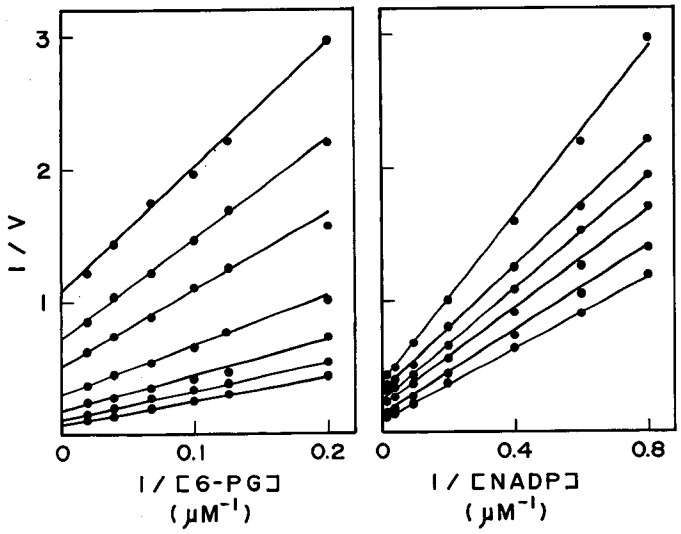

Fig.7.

Primary Lineweaver-Burk plots of initial rate measurements with the concentration of 6-phosphogluconate (or $NADP^+$) as variable and seven (or six) fixed concentrations of $NADP^+$ (or 6-phosphogluconate). Triethanolamine buffer (0.13 M, pH 7.0) was used.

At higher concentrations of $MgCl_2$(more than 10 mM)inhibited the enzyme. Pontremoli et al. (12) have reported that $MgCl_2$ increased the apparent affinity of the enzyme from *Candida utilis* for 6-phosphogluconate. However it was found that $MgCl_2$ lowered the affinity of pig liver enzyme for both 6-phosphogluconate and $NADP^+$ but increased maximal velocity.

The enzyme activity was substantially increased by addition of potassium acetate as reported by Procsal and Holten(5). The increase in activity was also confirmed at 25 to about 100 mM triethanolamine•HCl-KOH buffer, but the activity was decreased at the higher concentrations.

The inhibitory effect may be due to the increased concentration of Cl^-

The initial velocity studies are summarized in Fig.7. The data are consistent with a sequential mechanism according to the definition of Cleland(13). The lines in Lineweaver-Burk plots intersected not on the abscissa. Pontremoli et al.(12) and Procsal and Holten(5) proposed a random mechanism for the enzymes from *Candida utilis* and rat liver on the ground that the presence of one substrate did not change the affinity of other substrate. The relation was not confirmed and a random mechanism can not be accounted for pig liver enzyme.

The results on initial velocity studies were agreed with those of Villet and Dalziel(14).

Michaelis constants for 6-phosphogluconate and $NADP^+$ were 30 μM and 18 μM in the absence of $MgCl_2$, and 75 μM and 52 μM in the presence of 5 mM $MgCl_2$. Maximal velocity was increased from 16.6 units per mg protein to 25 units per mg by the addition of 5 mM $MgCl_2$.

Opposite data have been reported on the effect of fructose 1,6-diphosphate on mammalian enzyme. Dyson and D' Orazio(6) have reported that fructose 1,6-diphosphate is competitive with 6-phosphogluconate for sheep liver enzyme. In contrast to this, Procsal and Holten(5) have not confirmed the inhibition for rat liver enzyme. Pig liver enzyme was inhibited by high concentration of fructose 1,6-diphosphate.

But phosphoenolpyruvate was more effective inhibitor. The inhibitory effect phosphoenolpyruvate was infuluenced by the pH of the rection mixture. Higher inhibitory effect was observed around pH 7.

As shown Fig.8, 50% inhibition was obtained at 2 mM phosphoenolpyruvate and the inhibition was not linear but parabolic.

Phosphoenolpyruvate was reversible dead-end inhibitor at the concentrations of 5 mM or less, but higher concentrations (about 20 mM) of phosphoenolpyruvate inactivated the enzyme irreversibly.

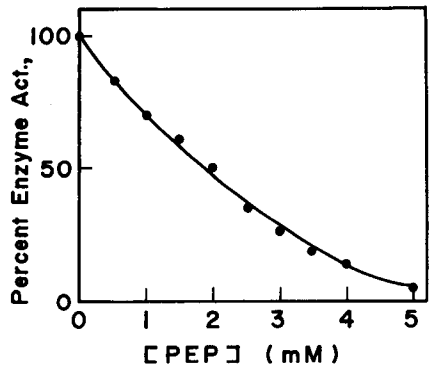

Fig. 8.

Kinetics of the enzyme activity inhibition at the various concentrations of phosphoenolpyruvate (PEP).

Reaction mixtures contained 1 μg/ml of the enzyme, phosphoenolpyruvate as indicated, $NADP^+$ at 100 μM, 6-phosphogluconate at 500 μM, $MgCl_2$ at 5 mM, and 0.13 M triethanolamine buffer at pH 7.0.

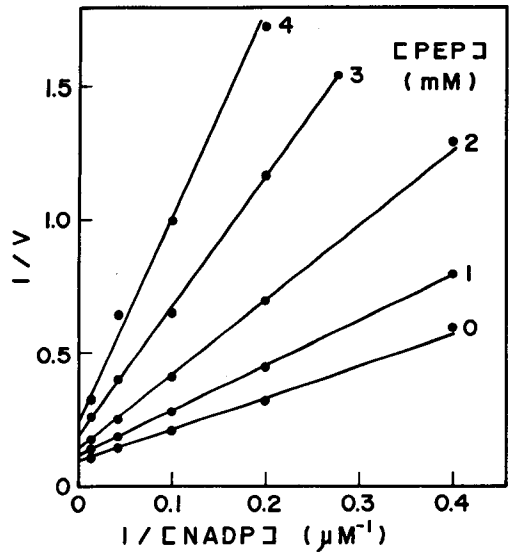

Fig. 9.

Inhibition of 6-phosphogluconate dehydrogenase by phosphoenolpyruvate (PEP).

Reaction mixtures contained 1 μg/ml of the enzyme, $NADP^+$ and phosphoenolpyruvate as indicated, 6-phosphogluconate at 500 μM, $MgCl_2$ at 5 mM, and 0.13 M triethanolamine buffer at pH 7.0.

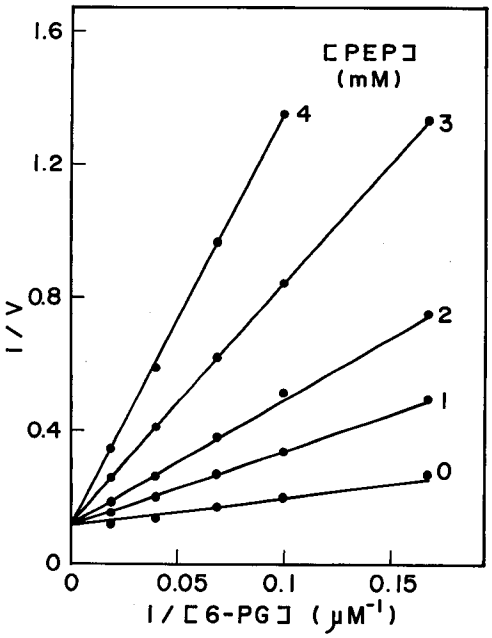

Fig.10.

Inhibition of 6-phosphogluconate dehydrogenase by phosphoenolpyruvate (PEP).

Reaction mixtures contained 1 μg / ml of the enzyme, 6-phosphogluconate and phosphoenolpyruvate as indicated, $NADP^+$ at 100 μM, $MgCl_2$ at 5 mM, and 0.13 M triethanolamine buffer at pH 7.0.

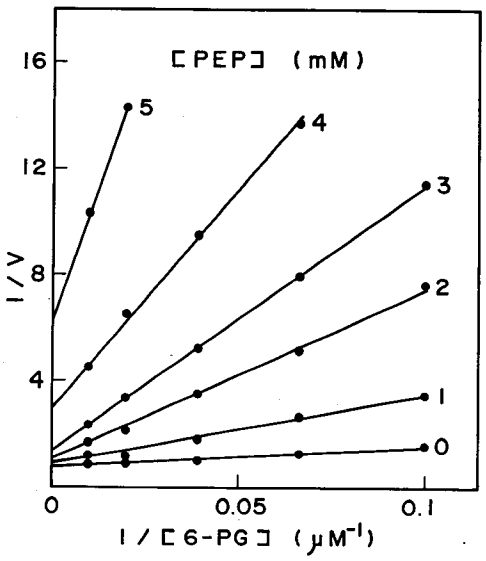

Fig.11.

Inhibition of 6-phosphogluconate dehydrogenase by phosphoenolpyruvate (PEP).

Reaction mixtures contained 1 μg / ml of the enzyme, 6-phosphogluconate and phosphoenolpyruvate as indicated, $NADP^+$ at 30 μM, $MgCl_2$ at 5 mM, and 0.13 M triethanolamine buffer at pH 7.0.

Phosphoenolpyruvate was S-parabolic I-linear noncompetitive inhibitor (according to the definition by Cleland(13)) with respect to $NADP^+$ (Fig. 9). Phosphoenolpyruvate was a parabolic competitive inhibitor with respect to 6-phosphogluconate at higher fixed concentrations of $NADP^+$ (Fig.10), whereas phosphoenolpyruvate was a parabolic noncompetitive one at lower concentrations of the fixed substrate (Fig.11).

The inhibitory effect of anions was summarized in Fig.12. The inhibition was apparently parabolic. The effect was not influenced by the pH in contrast to the inhibition by fructose 1,6-diphosphate or phosphoenolpyruvate (Fig.13). KCl was parabolic noncompetitive inhibitor with respect to $NADP^+$ (Fig.14) and 6-phosphogluconate (Fig. 15).

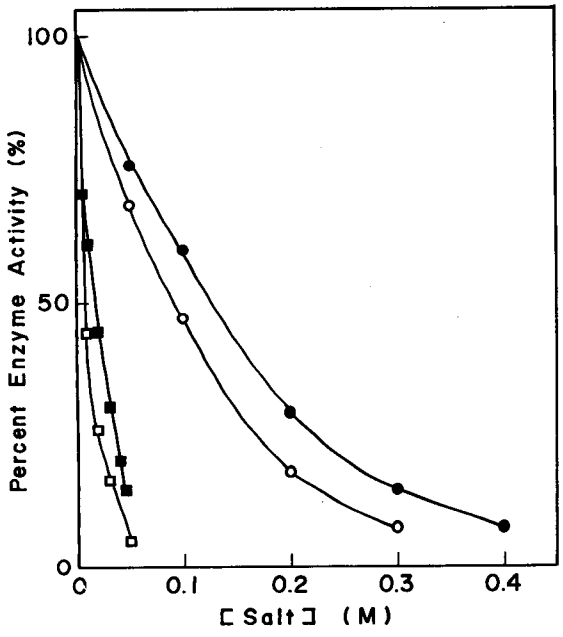

Fig.12.

Inhibition of 6-phosphogluconate dehydrogenase by anions.

Reaction mixtures contained 1 μg/ml of the enzyme, 6-phosphogluconate and $NADP^+$ at 25 μM, K_2SO_4(□), K_3PO_4(■), potassium formate (○) or KCl (●) as indicated, and 0.13 M triethanolamine buffer at pH 7.0.

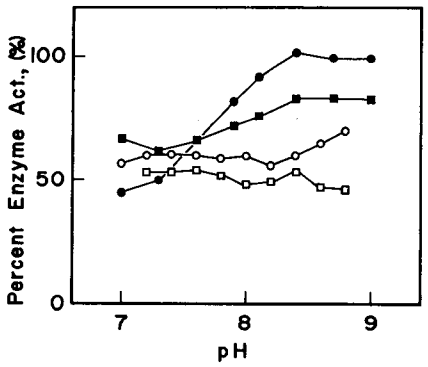

Fig.13.

Inhibition of 6-phosphogluconate dehydrogenase by anions, fructose 1,6-diphosphate or phosphoenolpyruvate.

Reaction mixtures contained 1 μg/ml of the enzyme, 6-phosphogluconate and $NADP^+$ at 25 uM, K_2SO_4 (○) at 10 mM, KCl (□) at 100 mM, fructose 1,6-diphosphate (■) at 2 mM or phosphoenolpyruvate (●) at 2 mM, and 0.13 M triethanolamine buffur at various pHs.

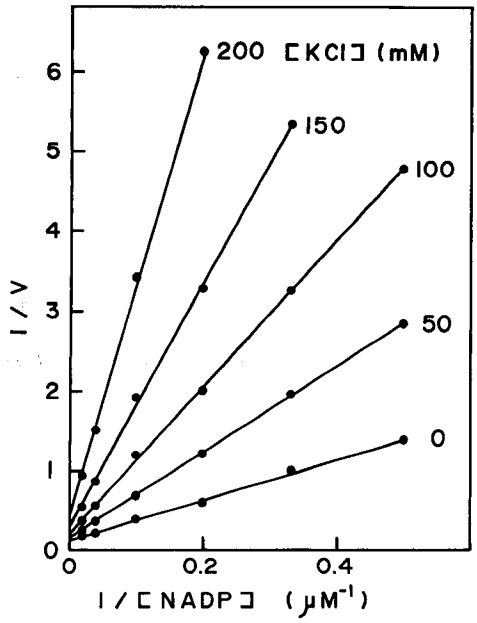

Fig.14.

Inhibition of 6-phosphogluconate dehydrogenase by KCl.

Reaction mixtures contained 1 μg/ml of the enzyme, $NADP^+$ and KCl as indicated, 6-phosphogluconate at 500 μM, and 0.13 M triethanolamine buffer at pH 7.0.

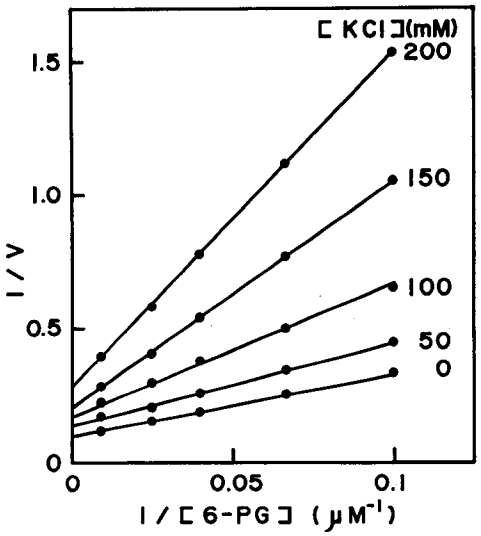

Fig.15.
Inhibition of 6-phosphogluconate dehydrogenase by KCl.
Reaction mixtures contained 1 μg / ml of the enzyme, 6-phosphogluconate and KCl as indicated, $NADP^+$ at 100 μM, and 0.13 M triethanolamine buffer at pH 7.0.

Discussion

6-Phosphogluconate dehydrogenase from pig liver was purified to homogeneity. The presence of the isozyme was not confirmed by electrophoretic experiments and inactivation procedures. The molecular weight for native enzyme and the subunit was estimated to be 105,000 and 52,000 respectively.

The values agree well with those values for rat and sheep liver enzyme. Hence, pig liver enzyme is a dimer of two subunits of identical molecular weight.

The results on initial velocity studies were agreed with those of Villet and Dalziel (14). They suggested that the mechanism of the enzyme was ordered addition of two substrates. The enzyme binds first with $NADP^+$ and then the complex reacts with 6-phosphogluconate.

Preliminary results on the mechanism of pig liver enzyme was consistent with the ordered mechanism but not with random mechanism (unpablished data). Dyson and D'Orazio reperted that fructose 1,6-diphosphate was competitive with respect to 6-phosphogluconate and discussed the structural similarity of 6-phosphogluconate and the inhibitory phosphate compound. If fructose 1,6-diphosphate is an dead-end inhi-

bitor bound to enzyme • $NADP^+$ complex to be bound next with 6-phosphogluconate, the mode of inhibitory effect of $NADP^+$ can not be explained.

Their results on the inhibition by fructose 1,6-diphosphate was apparently parabolic, (Fig. 1A. of reference). Pig and rat liver enzymes were inhibited by both fructose 1, 6-diphosphate and phosphoenolpyruvate. Phosphoenolpyruvate was competitive with respect to 6-phosphogluconate at high level of $NADP^+$ (Fig.10) but noncompetitive at low level of the coenzyme (Fig.11). Very similar inhibition pattern was obtained by the anion, Cl^- The data suggested that the inhibition by fructose 1, 6-diphosphate and phosphoenolpyruvate is not due to competition with 6-phosphogluconate.

The inhibition constants for fructose 1,6-diphosphate and phosphoenolpyruvate were high compared with the concentrations of these compounds in liver. Thus, it is not evident that the sensitivity of pig liver enzyme to the metabolites may contribute the short-term regulation of the catalytic activity.

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