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SEDOHEPTULOSE-7-PHOSPHATE KINASE ACTIVITY OF PHOSPHOFRUCTOKINASE FROM THE DIFFERENT TISSUES OF RABBIT

NAIF S. KARADSHEH, GOPI A. TEJWANI* AND ABBURI RAMAIAH
Department of Biochemistry, All-India Institute of Medical Sciences, New Delhi, 110016 (India)

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SUMMARY

1. Crystalline rabbit skeletal muscle phosphofructokinase and partially purified phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) from rabbit liver catalyze the phosphorylation of sedoheptulose 7-phosphate. The phosphorylated product of the reaction was shown to be sedoheptulose 1,7-diphosphate. Phosphofructokinases from heart, brain and erythrocytes of rabbit also appear to catalyze a similar reaction.

2. At optimal conditions, the rate of phosphorylation of sedoheptulose 7-phosphate by phosphofructokinase from both skeletal muscle and liver is about 100–120% of their rates with fructose 6-phosphate as substrate and the catalytic site for both the reactions appears to be the same.

3. The conclusion that sedoheptulose-7-phosphate kinase activity resides in phosphofructokinase is further supported on the following lines of evidence:

(a) There is a constant ratio of phosphofructokinase to sedoheptulose-7-phosphate kinase over a wide range of purification of phosphofructokinase from rabbit liver.

(b) Sedoheptulose 7-phosphate is a competitive inhibitor of fructose 6-phosphate phosphorylation.

(c) There is a similar inactivation and instability of phosphofructokinase and sedoheptulose-7-phosphate kinase as a result of passing the liver enzyme through Sephadex G-25 and there is a similar activation of the enzyme by the positive effectors of phosphofructokinase.

(d) There are similar kinetic properties of phosphofructokinase and sedoheptulose-7-phosphate kinase and their alteration with different treatments of the enzyme preparations of muscle or liver.

4. The stability and kinetic properties of liver phosphofructokinase are found to be different from the muscle phosphofructokinase.

* Present address: Department of Microbiology, Saint Louis University School of Medicine, Saint Louis, Mo. 63104 (U.S.A.).

INTRODUCTION

It was stated by Lardy[1] that the partially purified phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) from rabbit skeletal muscle catalyzed the phosphorylation of sedoheptulose 7-phosphate. The author did not identify the product nor provide evidence for the identity of phosphofructokinase with sedoheptulose-7-phosphate kinase activity and its extent of activity with sedoheptulose 7-phosphate as a substrate as compared with its natural substrate fructose 6-phosphate, because insufficient sedoheptulose 7-phosphate was then available. In addition, these studies were done at a time when the complex kinetics of phosphofructokinase and their importance in the regulation of glycolysis were not known.

Sedoheptulose 7-phosphate and crystalline rabbit skeletal muscle phosphofructokinase are now available and, therefore, it was thought of interest, to investigate whether the pure enzyme indeed catalyzes the phosphorylation of sedoheptulose 7-phosphate and if it does, to study its kinetic properties with sedoheptulose 7-phosphate as substrate. Similar studies were done with phosphofructokinase from liver of rabbit, with sedoheptulose 7-phosphate as substrate, to extend the comparative studies done earlier[2,3].

The results presented in this paper provide evidence that phosphofructokinase from liver and skeletal muscle of rabbit catalyzes the phosphorylation of sedoheptulose 7-phosphate to sedoheptulose 1,7-diphosphate with the same catalytic efficiency as with fructose 6-phosphate as substrate. The apparent K_m values for sedoheptulose 7-phosphate are much higher than the K_m values for fructose 6-phosphate. The kinetic properties of phosphofructokinase of muscle and liver with sedoheptulose 7-phosphate as substrate, are similar to their properties with fructose 6-phosphate as substrate. The results also show that phosphofructokinase from muscle and liver differ markedly in their stability and kinetic properties.

EXPERIMENTAL PROCEDURE

Chemicals and enzymes

The following chemicals and enzymes were obtained from Sigma Chemical Co., U.S.A.: sodium salt of fructose 6-phosphate, fructose 1,6-diphosphate, sedoheptulose 1,7-diphosphate, 5'-AMP, NADH, EDTA, Tris, bovine serum albumin and $(NH_4)_2SO_4$ suspensions of aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase and rabbit skeletal muscle phosphofructokinase type III (spec. act. 196 units). The barium salt of sedoheptulose 7-phosphate, obtained through the same source (85% pure) was converted to the sodium salt before use. The rabbit liver phosphofructokinase, partially purified as described earlier[4], was used. The partially purified phosphofructokinases from brain and heart of rabbit were a kind gift from Professor O.H. Lowry, and the homogeneous rabbit erythrocytes phosphofructokinase was a kind gift from Professor K. Uyeda. The yeast phosphofructokinase was purified to homogeneity according to the method of Lindell and Stellwagen[5] with a slight modification as described in ref. 6. All other chemicals used were of analytical reagent grade.

Determination of sedoheptulose-7-phosphate kinase activity

The sedoheptulose-7-phosphate kinase activity was measured in the same way

as that with fructose 6-phosphate, based on the fact that fructose-1,6-diphosphate aldolase also catalyzes the conversion of sedoheptulose 1,7-diphosphate to erythrose 4-phosphate and dihydroxyacetone phosphate[7]. The conversion of dihydroxyacetone phosphate to α-glycerophosphate, by the α-glycerophosphate dehydrogenase in presence of NADH was measured as described earlier[6] in a Perkin–Elmer spectrophotometer with attached recorder.

The enzyme was assayed at room temperature (25–27 °C). Unless otherwise mentioned the assay mixture, in a total volume of 1.0 ml, contained 50 mM Tris–HCl, pH 7.5, 3.0 mM $MgCl_2$, 0.15 mM NADH and excess of aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase, freed of $(NH_4)_2SO_4$ by dialysing them overnight against 2 l of 10 mM Tris–HCl, pH 8.0. In the assay system of muscle and yeast phosphofructokinase, 2.5 mM 2-mercaptoethanol was also present in addition to the above components. The auxiliary enzymes were present at concentrations so as to give at least ten times higher reaction rates than the maximum rate of phosphofructokinase reaction measured. Aldolase and α-glycerophosphate dehydrogenase are free of triosephosphate isomerase and all the auxiliary enzymes used for the estimation of phosphofructokinase activity are free of glucose-6-phosphate isomerase. The concentrations of ATP, fructose 6-phosphate and sedoheptulose 7-phosphate used are given in the legends to the tables and figures.

Oxidation of 1 mole of NADH corresponds to the utilization of 1 mole of sedoheptulose 7-phosphate (or 0.5 mole of fructose 6-phosphate). The sedoheptulose 7-phosphate, commercially purchased, was usually contaminated with about 2% of the triosephosphates which were always removed by preincubating the sedoheptulose 7-phosphate with auxiliary enzymes and excess of NADH before starting the sedoheptulose-7-phosphate kinase reaction. Our analysis of sedoheptulose 7-phosphate by glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase coupled enzymatic assay showed that, it contains 0.42% fructose 6-phosphate and 2.5% glucose 6-phosphate.

A unit of phosphofructokinase activity corresponds to the production of 1 μmole of fructose 1,6-diphosphate or sedoheptulose 1,7-diphosphate. The specific activity of phosphofructokinase is defined as the number of units per mg protein. Protein was determined by the method of Lowry *et al.*[8] with bovine serum albumin as a standard.

The reaction rates were not linear with enzyme concentrations for phosphofructokinase from liver and muscle of rabbit under certain conditions, therefore, for all the comparative studies, the same concentration of enzyme was used. The reaction rate with rabbit liver phosphofructokinase was variable depending on whether the substrate or the enzyme was used to initiate the reaction. When the reaction was initiated by adding the enzyme, the enzyme was applied on a small perspex spatula and immediately stirred into the assay mixture in the cuvette. The process of stirring and closing the cuvette chamber generally took 3–5 s. When the reaction was initiated by adding the substrate, the enzyme was always preincubated in an assay mixture in the cuvette for 2 min. The maximal initial linear portion of the enzymatic reaction was taken as the initial rate of the reaction.

RESULTS

Products of the phosphorylation of sedoheptulose 7-phosphate

The products of sedoheptulose-7-phosphate kinase reaction of rabbit liver and skeletal muscle phosphofructokinase preparations are ADP, as estimated by NADH oxidation in a coupled enzymatic assay[9] and sedoheptulose 1,7-diphosphate based on the evidence presented below.

The oxidation of NADH in the complete assay system is based on the following scheme of reactions:

$$\text{Sedoheptulose-7-}P + \text{ATP} \xrightarrow[\text{phosphofructokinase}]{Mg^{2+}} \text{Sedoheptulose-1,7-}P_2 + \text{ADP}$$

$$\text{Sedoheptulose-1,7-}P_2 \overset{\text{Aldolase}}{\rightleftharpoons} \text{Erythrose-4-}P + \text{Dihydroxyacetone phosphate}$$

$$\text{Dihydroxyacetone phosphate} + \text{NADH} + H^+ \overset{\text{Glycerophosphate dehydrogenase}}{\rightleftharpoons} \text{Glycerophosphate} + NAD^+$$

$$\text{Sum: Sedoheptulose-7-}P + \text{ATP} + \text{NADH} + H^+ \xrightarrow{Mg^{2+}} \text{Erythrose-4-}P + \text{Glycerophosphate} + \text{ADP} + NAD^+$$

Consistent with the above scheme of reactions, the oxidation of NADH did not occur when ATP, sedoheptulose 7-phosphate, Mg^{2+}, aldolase or α-glycerophosphate dehydrogenase was omitted. Thus, the only product which could be formed from sedoheptulose 7-phosphate and ATP and which could be the substrate for aldolase is sedoheptulose 1,7-diphosphate. The possibility that fructose 6-phosphate contamination (0.42%) in the sedoheptulose 7-phosphate preparation could have been phosphorylated to fructose 1,6-diphosphate was ruled out as the rate of NADH oxidation in the above set of reactions remained the same whether triosephosphate isomerase was present or absent in the coupled assay system, and sedoheptulose 1,7-diphosphate was identified in stoichiometric amounts as the product of the reaction.

The product formed from sedoheptulose 7-phosphate in the presence of muscle phosphofructokinase, ATP and Mg^{2+} was isolated by chromatography on a Dowex 1-formate column and eluted with a linear gradient of ammonium formate buffer according to the method of Bartlett[10], the major share of heptulose was found to be eluted much later than the sedoheptulose 7-phosphate, indicating the acquisition of much stronger acidic properties and coincides with the same elution position of standard sedoheptulose 1,7-diphosphate. Analysis of this elution volume, for heptulose[11], acid labile phosphorus[12], and total phosphorus[13], gave a ratio of 1:1:2 as did the standard sedoheptulose 1,7-diphosphate. Phosphorus was estimated according to the procedure of Taussky and Shorr[14]. The amount of sedoheptulose 1,7-diphosphate recovered in the elution volume, based on the content of heptulose, was 88.5% of the sedoheptulose 1,7-diphosphate formed in the reaction mixture as estimated by NADH oxidation. The amount of ADP formed in the reaction mixture as estimated by NADH oxidation[9], was equal to the amount of sedoheptulose 1,7-diphosphate. 1 μmole of standard sedoheptulose 1,7-diphosphate oxidized 0.9 μmole of NADH, using a molar extinction coefficient of NADH as 6270, which satisfies the stoichiometry of NADH

TABLE I

IDENTIFICATION OF PRODUCTS AND STOICHIOMETRY OF SEDOHEPTULOSE-7-PHOSPHATE KINASE REACTION

The complete reaction mixture contained: 90 μmoles Tris–HCl, pH 7.5, 40 μmoles $MgCl_2$, 26 μmoles $(NH_4)_2SO_4$, 20 μmoles ATP and 25 μmoles sedoheptulose 7-phosphate in a total volume of 1.8 ml. The reaction was initiated with addition of 12.7 units of muscle phosphofructokinase, and the mixture was incubated at 28 °C for 1 h, and then kept at 0 °C. An aliquot was estimated for ADP and sedoheptulose 1,7-diphosphate by NADH oxidation in a coupled enzymatic assay as mentioned in ref. 9 and in Experimental Procedrue, respectively. 19.7 μmoles of ADP and 20.08 μmoles of sedoheptulose 1,7-diphosphate were formed in the reaction mixture. The remaining was applied on a Dowex 1-formate column (12.8 cm × 1 cm) with a linear gradient elution. The gradient consisted of a 530-ml reservoir of double-distilled water connected with a reservoir containing an equal volume of 3.61 M formate buffer (2.665 M formic acid *plus* 0.945 M ammonium formate), pH 3.0. Fractions of 10 ml were collected and rate of flow was 10 ml/7.5 min. Unreacted sedoheptulose 7-phosphate was eluted between 35 and 125 ml and sedoheptulose 1,7-diphosphate was eluted between 305 and 415 ml, which coincided with the same elution positions of standard sedoheptulose 7-phosphate and sedoheptulose 1,7-diphosphate, respectively, when they were fractionated under similar conditions as mentioned above. An aliquot of the elution volume of the product sedoheptulose 1,7-diphosphate was analysed for heptulose, acid labile phosphorus and total phosphorus.

	NADH oxidation (μmole)	*Heptulose (μmole)*	*Acid labile phosphorus (μmole)*	*Total phosphorus (μmoles)*	*Heptulose : acid labile phosphorus : total phosphorus*
Standard sedoheptulose 1,7-diphosphate	0.425	0.485	0.553	1.075	1:1.14:2.22
Aliquot of elution volume of reaction product sedoheptulose 1,7-diphosphate	—	0.524	0.531	1.09	1:1.01:2.085
Substrate Sedoheptulose 7-phosphate	0.0	1	0.1	1.07	1:0.1:1.07

oxidation in the assay system of sedoheptulose-7-phosphate kinase. The details of these results are shown in Table I.

Competitive inhibition of fructose 6-phosphate phosphorylation by sedoheptulose 7-phosphate

The lack of phosphorylation of fructose 6-phosphate which was present as a contaminant of sedoheptulose 7-phosphate (0.42%) by the phosphofructokinase from both muscle and liver indicates perhaps that sedoheptulose 7-phosphate may competitively inhibit the phosphorylation of fructose 6-phosphate. That it is indeed so is shown in Fig. 1. These results suggest that perhaps the same enzyme protein is catalyzing both reactions at the same catalytic site.

The yeast phosphofructokinase (0.25 unit) did not catalyze the phosphorylation of sedoheptulose 7-phosphate (5 mM) or its contaminant fructose 6-phosphate suggesting that sedoheptulose 7-phosphate may be inhibitor of fructose 6-phosphate phosphorylation.

Ratio of phosphofructokinase to sedoheptulose-7-phosphate kinase activities of phosphofructokinase from skeletal muscle and various liver preparations of rabbit

The results of a typical partial purification of rabbit liver phosphofructokinase

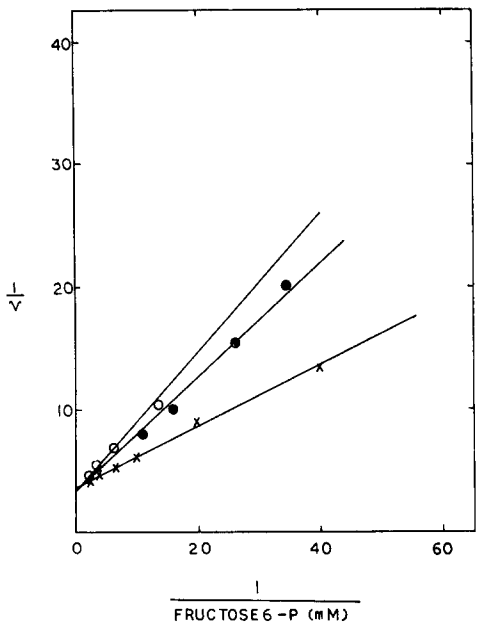

Fig. 1. Double reciprocal plot of reaction velocity *versus* fructose 6-phosphate concentration at two fixed sedoheptulose 7-phosphate concentrations. The concentrations of sedoheptulose 7-phosphate were 0 (×), 1 mM (●), and 4 mM (○). Rabbit muscle phosphofructokinase (spec. act. 196 units) was diluted 50-fold in 0.35 mM ATP, 100 mM Tris–HCl, pH 8.0, 4 μM fructose 1,6-diphosphate and 0.02% bovine serum albumin. The reaction was initiated by the addition of 3.87 μl of enzyme for all assays described here. v is the velocity of enzyme at any concentration of fructose 6-phosphate in presence of 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i, 0.185 mM 5′-AMP, 1 mM ATP and sedoheptulose 7-phosphate concentration was as mentioned above. Other conditions of assay are the same as described in the assay procedure. The rate of phosphorylation of fructose 6-phosphate in the presence of sedoheptulose 7-phopshate, was determined by subtracting the rate of reaction in absence of triosephosphate isomerase from the rate in its presence. The difference obtained in the rate of NADH oxidation is due to the amount of 3-phosphoglyceraldehyde formed from cleavage of fructose 1,6-diphosphate. This method is valid as shown by the fact that in absence of sedoheptulose 7-phosphate, the rate of NADH oxidation in presence of triosephosphate isomerase is exactly twice the rate in its absence.

and the rate of cleavage of both substrates at various stages of its purification are depicted in Table II. The ratio is about constant over a 160-fold range of its purification. The ratio is about 1.17 and was obtained when phosphofructokinase activity was determined at saturating concentrations of fructose 6-phosphate (5 mM) and at non-saturating concentration of sedoheptulose 7-phosphate (1.0 mM). The ratio was found to be 0.9 and 0.82 for the phosphofructokinase preparations of muscle and liver, respectively, when their activities were determined under optimal conditions at saturating concentrations of both substrates.

Inactivation of phosphofructokinase and sedoheptulose-7-phosphate kinase activities of phosphofructokinase preparation from rabbit liver

When the stock liver phosphofructokinase was applied on a Sephadex G-25 column (0.47 cm × 60 cm) equilibrated with 50 mM Tris–HCl, pH 7.5, containing 0.02% bovine serum albumin, and eluted with the same buffer, the enzyme was inactivated rapidly. The activity of phosphofructokinase determined immediately

TABLE II

RATIOS OF PHOSPHOFRUCTOKINASE AND SEDOHEPTULOSE-7-PHOSPHATE KINASE ACTIVITIES AT VARIOUS STAGES OF PARTIAL PURIFICATION OF RABBIT LIVER PHOSPHOFRUCTOKINASE

The reaction mixture contained 1 mM ATP, activators (5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP) and 5 mM fructose-6-*P* or 1 mM sedoheptulose-7-*P*, for determination of phosphofructokinase or sedoheptulose-7-phosphate kinase activities, respectively. The other conditions of assay are as described in the assay procedure. The partial purification of phosphofructokinase was done as described earlier[4].

Various stages of partial purification of phosphofructokinase	$\Delta A_{340\ nm}$/*min*		*Activity ratio phosphofructokinase/ sedoheptulose-7-P kinase*
	Phosphofructokinase	*Sedoheptulose-7-P kinase*	
20 000 × *g* supernatant	0.073	0.030	1.21
0.1 vol. isopropanol supernatant	0.069	0.030	1.15
0.15 vol. isopropanol precipitate	0.140	0.059	1.19
$(NH_4)_2SO_4$ precipitate	0.285	0.125	1.14

after elution, in the absence of positive effectors, is a small fraction of its steady rate in the presence of positive effectors. Similar results were obtained for sedoheptulose-7-phosphate kinase activity under the above conditions as shown in Table III. Such a marked effect of positive effectors was not found for either substrate on the stock liver enzyme when the reaction was initiated by adding enzyme.

In contrast when the stock enzyme was applied on a Sephadex G-25 column (0.75 cm × 8 cm) equilibrated with 0.35 mM ATP in addition to 50 mM Tris–HCl, pH 7.5, containing 0.02% bovine serum albumin and eluted with the same buffer, the enzyme was converted into a less active form[4]. This less active form when assayed in the presence of positive effectors of phosphofructokinase, showed a lag period of 2 min before it reached a steady rate. A similar lag period was also observed with

TABLE III

EFFECT OF PASSING THE STOCK LIVER ENZYME THROUGH SEPHADEX G-25 ON ITS ACTIVITY IN PRESENCE OR ABSENCE OF ACTIVATORS

1.7 units of rabbit liver stock phosphofructokinase preparation stored in 50 mM Tris–HCl, pH 7.5, 0.7 M $(NH_4)_2SO_4$, 10 mM K_2HPO_4, 0.33 mM EDTA and 10 mM ATP, was applied on a Sephadex G-25 column (0.47 cm × 60 cm), equilibrated and eluted with 50 mM Tris–HCl, pH 7.5, containing 0.02% bovine serum albumin. The rate of flow was 0.33 ml/min and fractions of 0.2 ml were collected. The fractions which were free of SO_4^{2-} were used for testing the enzyme activity under different conditions. The activators are: 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP.

Enzyme source	*Fructose-6-P (mM)*	*Sedoheptulose-7-P (mM)*	*ATP (mM)*	*Activators*	*Rate* ($\Delta A_{340\ nm}$/*min*)	*Maximum activity percentage*
1 µl stock enzyme	5.0	—	1.0	+	1.100	100
	5.0	—	0.078	—	0.630	57.3
	—	2.2	1.0	+	0.668	100.0
	—	6.0	0.078	—	0.324	48.6
50 µl Sephadex G-25 eluted fraction	5.0	—	1.0	+	0.315	100.0
	10.0	—	0.078	—	0.015	4.76
	—	5.0	1.0	+	0.160	100.0
	—	10.0	0.078	—	0.003	1.8

sedoheptulose-7-phosphate kinase activity (Table IV). The steady rate is about 2-fold higher than the rate initially observed. No such significant lag period was observed in the case of the muscle phosphofructokinase preparation with either fructose 6-phosphate or sedoheptulose 7-phosphate as substrate. These results further support the idea that sedoheptulose-7-phosphate kinase and phosphofructokinase activities may reside in the same protein.

TABLE IV

ACCELERATION OF THE RATE OF REACTION OF PHOSPHOFRUCTOKINASE AND SEDOHEPTULOSE-7-PHOSPHATE KINASE OF RABBIT LIVER BY POSITIVE EFFECTORS OF PHOSPHOFRUCTOKINASE

Rabbit liver stock phosphofructokinase preparation stored in 50 mM Tris–HCl, pH 7.5, 0.7 M $(NH_4)_2SO_4$, 10 mM K_2HPO_4, 0.33 mM EDTA and 10 mM ATP, was applied on a Sephadex G-25 column (0.75 cm × 8 cm), equilibrated and eluted with 50 mM Tris–HCl, pH 7.5, containing 0.02% bovine serum albumin and 0.35 mM ATP. 5 μl of the fraction which was free of SO_4^{2-} was used for the determination of enzyme activities. The reaction mixture contained either 5 mM fructose 6-phosphate or 1 mM sedoheptulose 7-phosphate and 1 mM ATP, 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP. The other conditions of assay are the same as described in the assay procedure.

Enzyme	$\Delta A_{340\ nm}/min$		*Fold difference*
	0–30 s	*Steady rate*	
Phosphofructokinase	0.064	0.120	2
Sedoheptulose-7-phosphate kinase	0.030	0.063	2

Kinetic properties of sedoheptulose-7-phosphate kinase

The effect of activators of phosphofructokinase on the activity of sedoheptulose-7-phosphate kinase. The positive effectors of phosphofructokinase like $(NH_4)_2SO_4$ or 5′-AMP, P_i and $(NH_4)_2SO_4$ in combination activate sedoheptulose-7-phosphate kinase similar to their effects on phosphofructokinase from muscle and liver and decrease the apparent K_m values of both enzymes for fructose 6-phosphate as well as sedoheptulose 7-phosphate. On preincubating the enzymes for 2 min there is a similar increase in the apparent K_m values of liver and muscle phosphofructokinase for fructose 6-phosphate and sedoheptulose 7-phosphate as shown in the Figs 2A, 2B, 3A and 3B and in Table V.

One of the effects of high concentrations of ATP was to increase the apparent K_m of phosphofructokinase for fructose 6-phosphate. For instance, for muscle phosphofructokinase, the apparent K_m value for fructose 6-phosphate, at low ATP concentrations was 0.063 mM which was increased to 0.21 mM at high concentration of ATP (Table V). Similarly the apparent K_m value for sedoheptulose 7-phosphate at low concentrations of ATP was 2.45 mM which was increased to 4.36 mM at high concentration of ATP (Table V). Liver phosphofructokinase exhibits normal Michaelis–Menten kinetics as shown by linear Lineweaver–Burk plots, when its activity was determined by adding enzyme last or in the presence of activators. The sedoheptulose-7-phosphate kinase also behaved similarly under these conditions (Table V). Under other conditions Lineweaver–Burk plots were non-linear and concave upward, for both of the substrates.

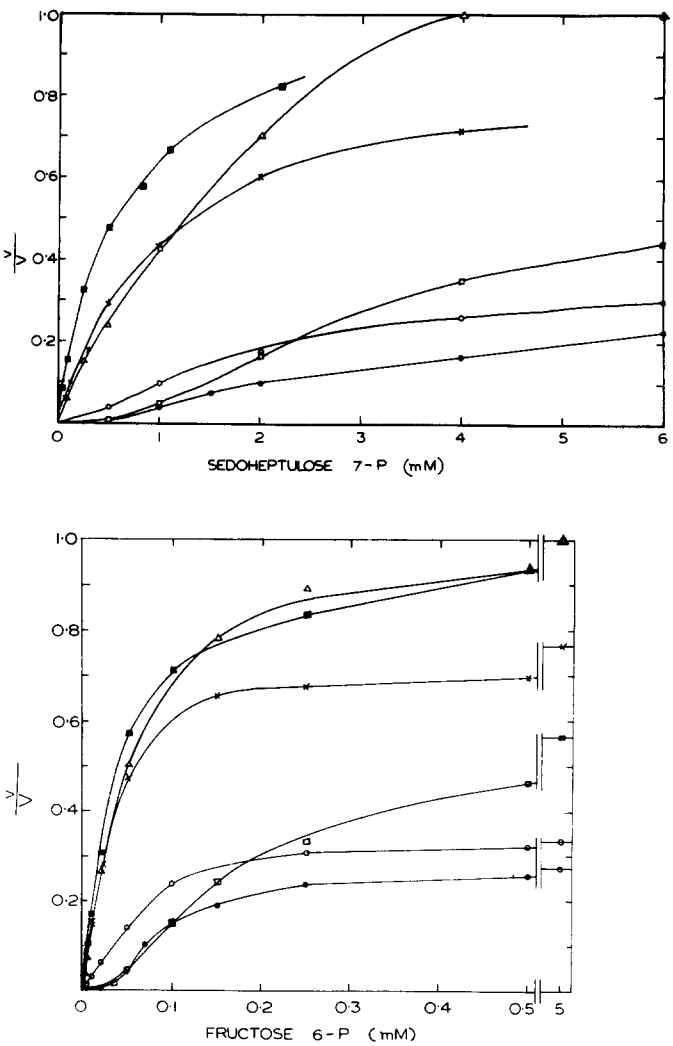

Fig. 2. (A) Effect of increasing concentrations of sedoheptulose 7-phosphate on the rate of sedoheptulose-7-phosphate kinase reaction of phosphofructokinase preparation of rabbit skeletal muscle, under various conditions. ○—○, 0.1 mM ATP; ●—●, 0.1 mM ATP + 2-min preincubation of enzyme in assay mixture; ×—×, 0.1 mM ATP + 2 mM $(NH_4)_2SO_4$; □—□, 1 mM ATP; △—△, 1 mM ATP + 2 mM $(NH_4)_2SO_4$; ■—■, 1 mM ATP + 5 mM $(NH_4)_2SO_4$ + 2.5 mM P_i + 0.185 mM 5′-AMP. Rabbit muscle phosphofructokinase (spec. act. 196 units) was diluted 20-fold as described in the legend to Fig. 1. 1 μl of this was used for all assays described here. The reaction was initiated by the addition of enzyme except in the case when it was preincubated in which case the reaction was initiated by the addition of ATP. v is the velocity of enzyme at any concentration of substrate. V is the velocity at 6 mM sedoheptulose 7-phosphate in presence of 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP and 1 mM ATP. Other conditions of assay are same as described in the assay procedure. (B) Effect of increasing concentrations of fructose 6-phosphate on the rate of reaction of phosphofructokinase of rabbit skeletal muscle under various conditions. ○—○, 0.1 mM ATP; ●—●, 0.1 mM ATP + enzyme preincubated for 2 min; ×—×, 0.1 mM ATP + 2 mM $(NH_4)_2SO_4$; □—□, 1 mM ATP; △—△, 1 mM ATP + 2 mM $(NH_4)_2SO_4$; ■—■, 1 mM ATP + 5 mM $(NH_4)_2SO_4$ + 2.5 mM P_i + 0.185 mM 5′-AMP. V is the activity of enzyme at 5 mM fructose 6-phosphate, 1 mM ATP in presence of 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP. All the other conditions are as described in (A).

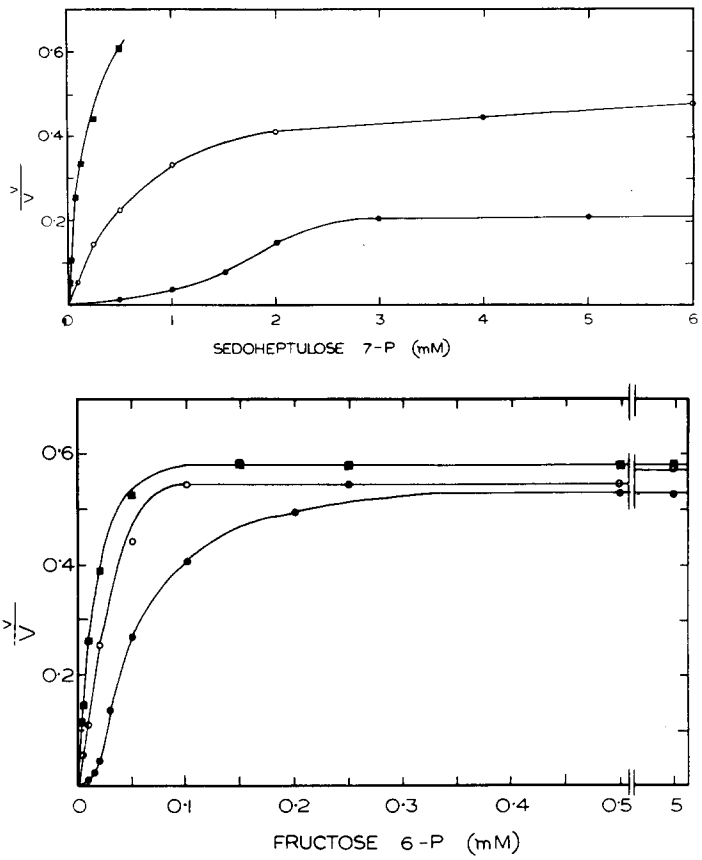

Fig. 3. (A) The effect of increasing concentrations of sedoheptulose 7-phosphate on the rate of sedoheptulose-7-phosphate kinase of phosphofructokinase preparation of rabbit liver under various conditions. 1 μl of stock rabbit liver phosphofructokinase (stored in presence of 50 mM Tris–HCl, pH 7.5, 0.7 M $(NH_4)_2SO_4$, 10 mM ATP, 10 mM K_2HPO_4 and 0.33 mM EDTA) was used. The concentration of ATP used in all cases was 0.088 mM, except in presence of activators (■—■) where 0.078 mM ATP was used. ○—○, reaction started by adding enzyme; ●—●, reaction started by adding ATP after preincubating the enzyme for 2 min; ■—■, reaction started by adding enzyme in presence of 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP. *v*, activity of enzyme at any substrate concentration. *V*, activity of enzyme at 2.2 mM sedoheptulose 7-phosphate in presence of 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP. All other conditions of assay were the same as described in the assay procedure. (B) Effect of increasing concentrations of fructose 6-phosphate on the rate of phosphofructokinase of rabbit liver. All the conditions of assay are the same as described in the legends for (A). ○—○, enzyme added last; ●—●, enzyme preincubated for 2 min, ATP added last; ■—■, enzyme added last in presence of 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP. *V*, activity of enzyme at 5 mM fructose 6-phosphate, 1 mM ATP in presence of 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP. *v*, activity of enzyme at any substrate concentration.

In the case of phosphofructokinase and sedoheptulose-7-phosphate kinase of muscle phosphofructokinase preparation the Lineweaver–Burk plots were linear only in the presence of activators of phosphofructokinase and non-linear, concave upward, under other conditions (Table V).

Inhibition of phosphofructokinase and sedoheptulose-7-phosphate kinase by excess concentrations of ATP. The muscle and liver phosphofructokinase and sedoheptulose-

TABLE V

EFFECT OF ACTIVATORS OF PHOSPHOFRUCTOKINASE AND PREINCUBATION OF MUSCLE AND LIVER PHOSPHOFRUCTOKINASE PREPARATIONS ON THE KINETIC CONSTANTS OF PHOSPHOFRUCTOKINASE AND SEDOHEPTULOSE-7-PHOSPHATE KINASE

The concentration of the sugar phosphates were varied at the indicated concentration of ATP. K_m values were determined either by Lineweaver–Burk plots when the plots were linear or by Hill plots[15]. The maximum velocity used in plotting the data according to Hill, was obtained by Lineweaver–Burk plots using those points at high concentration of substrate which fall on straight line.

Conditions of assay			*Muscle phosphofructokinase preparations*				*Liver phosphofructokinase preparations*			
ATP	*Preincubation of enzyme (min)*	*Activators**	*Fructose-6-P*		*Sedoheptulose-7-P*		*Fructose-6-P*		*Sedoheptulose-7-P*	
			K_m *(mM)*	*Lineweaver–Burk plot*	K_m *(mM)*	*Lineweaver–Burk plot*	K_m *(mM)*	*Lineweaver–Burk plot*	K_m *(mM)*	*Lineweaver–Burk plot*
Low ATP**	0.0	–	0.063	Non-linear (concave upward)	2.45	Non-linear (concave upward)	0.029	Linear	0.66	Linear
Low ATP**	2.0	–	0.097	Non-linear (concave upward)	7.08	Non-linear (concave upward)	0.05	Non-linear (concave upward)	1.76	Non-linear (concave upward)
0.078	0.0	+	—	—	—	—	0.0145	Linear	0.160	Linear
1.0	0.0	–	0.21	Non-linear (concave upward)	4.36	Non-linear (concave upward)				
1.0	0.0	+	0.055	Linear	0.60	Linear				

* Activators: 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5′-AMP.

** Low ATP: 0.088 mM ATP for studies with liver enzyme and 0.1 mM ATP for studies with muscle enzyme.

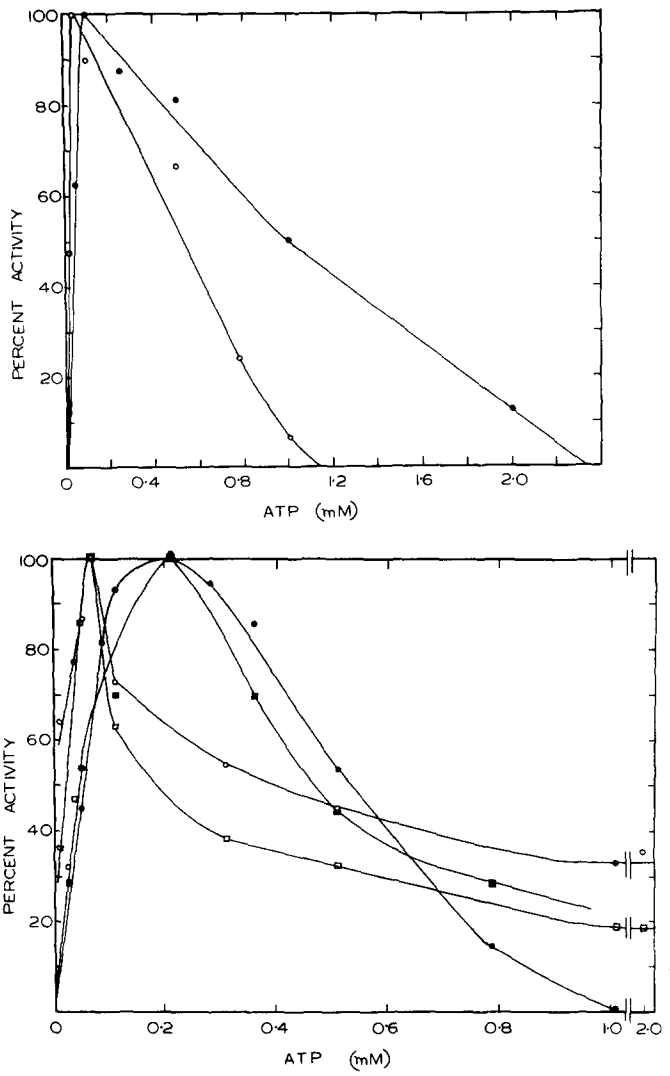

Fig. 4. (A) Effect of increasing concentrations of ATP on the rate of phosphofructokinase and sedoheptulose-7-phosphate kinase of phosphofructokinase preparation of rabbit muscle. ○—○, 0.025 mM fructose 6-phosphate corresponding to 1/2.52 times that of K_m value for fructose 6-phosphate at 0.10 mM ATP; ●—●, 1 mM sedoheptulose 7-phosphate corresponding to 1/2.45 times that of K_m value for sedoheptulose 7-phosphate at 0.1 mM ATP. 1 μl of 20-fold diluted (as described in legends to Fig. 1) rabbit muscle phosphofructokinase used in each case. Reaction in all cases was started by adding enzyme last. All other conditions of assay were the same as described in the assay procedure. (B) Effect of increasing concentrations of ATP on the rate of phosphofructokinase and sedoheptulose-7-phosphate kinase of phosphofructokinase preparation of rabbit liver. ○—○, 0.01 mM fructose 6-phosphate corresponding to 1/2.9 times that of K_m value for fructose 6-phosphate at 0.088 mM ATP. The reaction was initiated by adding 1 μl of the stock enzyme; □—□, 0.27 mM sedoheptulose 7-phosphate corresponding to 1/2.44 times that of K_m value for sedoheptulose 7-phosphate at 0.088 mM ATP. The reaction was initiated by adding 1 μl of the stock enzyme; ●—●, 0.055 mM fructose 6-phosphate, (corresponding to 1.1 times its K_m value under these conditions at 0.088 mM ATP); ■—■, 2 mM sedoheptulose 7-phosphate (corresponding to 1.1 times its K_m value at 0.088 mM ATP). The enzyme was preincubated for 2 min and the reaction was started by adding ATP last. All the other conditions of assay were same as described in the assay procedure.

7-phosphate kinase activities are inhibited by high concentrations of ATP as shown in Figs 4A and 4B. Muscle phosphofructokinase is inhibited by about 2-fold more than the sedoheptulose-7-phosphate kinase of this preparation, while in the case of liver, the inhibition by excess concentrations of ATP is more with sedoheptulose-7-phosphate kinase than with phosphofructokinase. 1 mM concentration of ATP almost completely inhibits phosphofructokinase of muscle while it inhibits phosphofructokinase of liver by only about 65% which could not be inhibited any further even by increasing the concentration of ATP to about 2 mM (Figs 4A and 4B). The liver phosphofructokinase as a result of its preincubation for 2 min in the assay mixture in the cuvette, becomes much more sensitive to inhibition by excess concentrations of ATP. Similar increase in inhibition of sedoheptulose-7-phosphate kinase of liver preparation by excess concentrations of ATP was observed as a result of its preincubation in the assay mixture in the cuvette.

Sedoheptulose-7-phosphate kinase activity of partially purified preparations of brain and heart and pure erythrocytes phosphofructokinases of rabbit. The preliminary results indicate that the enzyme from the above sources phosphorylate sedoheptulose 7-phosphate to sedoheptulose 1,7-diphosphate. Rabbit erythrocytes phosphofructokinase catalyzes the phosphorylation of sedoheptulose 7-phosphate at about the same rate as with fructose 6-phosphate. Whether sedoheptulose-7-phosphate kinase activity of these preparations is due to phosphofructokinase protein or not, is to be investigated.

DISCUSSION

From the data presented in Results, it is fair to conclude that phosphofructokinase from rabbit skeletal muscle and liver catalyzes the phosphorylation of sedoheptulose 7-phosphate to yield sedoheptulose 1,7-diphosphate. That this perhaps is a general property, is supported by the fact that in addition to phosphofructokinase of skeletal muscle and liver, the enzyme from heart, erythrocytes and brain of rabbit also appears to have sedoheptulose-7-phosphate kinase activity. In *Streptococcus faecalis*, it is shown that phosphofructokinase and sedoheptulose-7-phosphate kinase activities reside in the same protein[16] and their activities are about equal. Among the phosphofructokinases so far studied, only yeast enzyme does not phosphorylate sedoheptulose 7-phosphate.

Crystalline rabbit muscle phosphofructokinase has been shown to catalyze also the phosphorylation of glucose 1-phosphate to glucose 1,6-diphosphate[17] and fructose 1-phosphate to fructose 1,6-diphosphate[18] but its activity with these substrates under optimal conditions was about 0.67 and 5%, respectively, of its activity with fructose 6-phosphate as a substrate. The sedoheptulose-7-phosphate kinase activity of phosphofructokinase of muscle and liver on the other hand is about 100–120% of its activity with fructose 6-phosphate as substrate.

The kinetic properties of glucose-1-phosphate kinase activity of rabbit muscle phosphofructokinase have not been studied[17] but that of fructose-1-phosphate kinase have been studied[18]. The enzyme exhibits normal Michaelis–Menten kinetics with fructose 1-phosphate as substrate both at low and high concentrations of ATP. ATP at higher concentrations inhibits the phosphorylation of fructose 1-phosphate as it does with fructose 6-phosphate as substrate. But the mechanism of inhibition is

different in that the fructose-1-phosphate kinase inhibition is due to decrease in V while in the case of phosphofructokinase it is due to increase in the apparent K_m value of fructose 6-phosphate[18]. On the other hand the sedoheptulose-7-phosphate kinase activity of phosphofructokinase of skeletal muscle and liver are inhibited by excess concentrations of ATP in a similar way as with fructose 6-phosphate as a substrate, except that the inhibition of sedoheptulose-7-phosphate kinase activity of muscle phosphofructokinase with higher concentrations of ATP is about 2-fold less than that of the inhibition of phosphofructokinase under similar conditions (Figs 4A and 4B). The activators of phosphofructokinase also activate sedoheptulose-7-phosphate kinase (Figs 2A, 2B, 3A and 3B).

If the sedoheptulose-7-phosphate kinase activities of phosphofructokinase from skeletal muscle and liver of rabbit, observed under *in vitro* conditions are also present under *in vivo* conditions, it is expected that the concentration of sedoheptulose 1,7-diphosphate would increase similar to the increase in fructose 1,6-diphosphate in the cell under the conditions of phosphofructokinase activation as during anoxia[19] or muscular contraction[20]. So far as the authors are aware, there is only one report where the concentrations of sedoheptulose 1,7-diphosphate and fructose 1,6-diphosphate increased simultaneously and this increase may be ascribed to phosphofructokinase activation[12]. The authors of the article[12] however ascribed the increase in the concentrations of sedoheptulose 1,7-diphosphate and fructose 1,6-diphosphate to the reverse reaction of aldolase. But in view of the fact that the phosphofructokinase of red blood cells[12] would be activated by inorganic phosphate present in their incubation medium, and the homogeneous phosphofructokinase from the rabbit red blood cells appears to phosphorylate sedoheptulose 7-phosphate to sedoheptulose 1,7-diphosphate, the increase in the concentrations of sedoheptulose 1,7-diphosphate along with fructose 1,6-diphosphate could be due to the increase in the activity of phosphofructokinase.

Sedoheptulose 7-phosphate is a natural metabolite of many tissues of rabbit[21]. In mammalian cells it arises mainly through the operation of oxidative pentose phosphate pathway and transaldolase–transketolase reactions on fructose 6-phosphate[22]. The concentrations of sedoheptulose 7-phosphate would be quite considerable, since recycling of pentose phosphates to hexose phosphates, during which sedoheptulose 7-phosphate is used up, is a limited phenomenon[23]. For instance, the concentrations of sedoheptulose 7-phosphate in brain and liver were found to be 0.05 and 0.10 mM, respectively, which are higher, by about 2.5 times, than the concentration of fructose 6-phosphate in these tissues[24,25]. The concentration of sedoheptulose 7-phosphate can be even higher in mammary gland during lactation, because of the many-fold increase in the oxidative pentose phosphate pathway under these conditions[26–28]. Therefore, the phosphorylation of sedoheptulose 7-phosphate to sedoheptulose 1,7-diphosphate by phosphofructokinase or by a specific kinase may be one of the ways to prevent the accumulation of this compound in the cell. That sedoheptulose 1,7-diphosphate is found in a considerable concentration in red blood cells supports such a possibility[12].

Physiological significance of the sedoheptulose-7-phosphate kinase activity of phosphofructokinase

The physiological importance of sedoheptulose-7-phosphate kinase activity of

phosphofructokinase may lie in the production of erythrose 4-phosphate and dihydroxyacetone phosphate by the action of aldolase on sedoheptulose 1,7-diphosphate. During lactation in mammary gland the rate of synthesis of fatty acids is increased[29] and for their biosynthesis and esterification, NADPH and α-glycerophosphate are to be made at higher rate. The increased requirement for NADPH is met by the corresponding increase in the oxidative pentose phosphate pathway but a similar increase in glycolysis to provide dihydroxyacetone phosphate for the formation of α-glycerophosphate was not observed[27,28]. The increased demand for α-glycerophosphate could be met by the increased supply of dihydroxyacetone phosphate from sedoheptulose 1,7-diphosphate.

The increase in sedoheptulose 1,7-diphosphate results from the increased activity of sedoheptulose-7-phosphate kinase activity of phosphofructokinase which would be due to increased steady-state levels of sedoheptulose 7-phosphate under these conditions. This argument would be valid if the mammary gland phosphofructokinase catalyzes the phosphorylation of sedoheptulose 7-phosphate and the apparent K_m for sedoheptulose 7-phosphate is in the range of its concentration in the cell. Evidence so far obtained in our laboratory suggests that phosphofructokinase, which has been highly purified from goat mammary gland, also catalyzes the phosphorylation of sedoheptulose 7-phosphate to yield sedoheptulose 1,7-diphosphate (Karadsheh, N. S. and Ramaiah, A., unpublished).

In addition, sedoheptulose 1,7-diphosphate may function, as it does in microorganisms and plants, as a reservoir of erythrose 4-phosphate which is not very stable and appears to have important functions in animal and plant systems[30].

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Rabbit liver phosphofructokinase which has been purified to spec. act. of 75–100 units according to an unpublished procedure (Kaur, J. and Ramaiah, A.) was also shown to catalyze the phosphorylation of sedoheptulose 7-phosphate with a rate equal to about 108% of its rate with fructose 6-phosphate at optimal conditions, which is in the same range for the partially purified enzyme.

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