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The involvement of fructose 2,6-bisphosphate in substrate cycle control in the nonoxidative stage of the pentose phosphate pathway. A phosphorus magnetic resonance spectroscopy study

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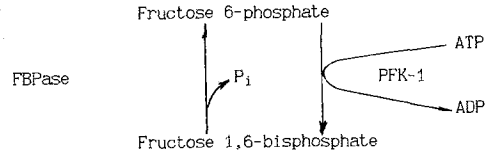
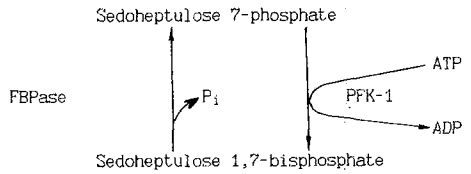
Abstract. The role of fructose 2,6-bisphosphate in the interconversion of sedoheptulose 7-phosphate and sedoheptulose 1,7-bisphosphate in rat liver cytosol fractions was studied by means of phosphorus magnetic resonance spectroscopy. When the activity of 6-phosphofructo-1-kinase was inhibited by a high concentration of ATP, the addition of fructose 2,6-bisphosphate led to a marked decrease in sedoheptulose 7-phosphate levels, accompanied by an increased concentration of ADP. Fructose 2,6-bisphosphate essentially inhibited both the decrease in sedoheptulose 1,7-bisphosphate concentration and the accumulation of P_i in the incubation mixture. The data provided evidence that fructose 2,6-bisphosphate can regulate the substrate cycle: sedoheptulose 7-phosphate $\rightleftharpoons$ sedoheptulose 1,7-bisphosphate in the liver, and thus control the flux through the nonoxidative stage of the pentose phosphate pathway.
Key words. Fructose 2,6-bisphosphate; NMR-spectroscopy; 6-phosphofructo-1-kinase; rat liver; sedoheptulose 7-phosphate; sedoheptulose 1,7-bisphosphate.

Fructose 2,6-bisphosphate (Fru 2,6-P_2) is a powerful regulator of carbohydrate metabolism, controlling the enzymes that catalyse the formation and hydrolysis of fructose 1,6-bisphosphate (Fru 1,6-P_2). Fru 2,6-P_2 activates 6-phosphofructo-1-kinase (EC 2.7.1.11.; PFK-1) and simultaneously inhibits fructose 1,6-bisphosphatase (EC 3.1.3.11.; FBPase) regulating the carbohydrate flux through the substrate cycle[1-3]:



The action of Fru 2,6-P_2 on PFK-1 can be attributed to its ability to enhance the enzyme's affinity for fructose 6-phosphate (Fru 6-P) and to remove inhibition due to a high ATP concentration. In doing so, Fru 2,6-P_2 is a synergist with AMP[4,5]. The mechanism of Fru 2,6-P_2 action on FBPase probably involves the interaction of this inhibitor with two sites, catalytic and allosteric, on the enzyme[6,7].

Since sedoheptulose 7-phosphate (Sed 7-P) and sedoheptulose 1,7-bisphosphate (Sed 1,7-P_2) are, along with Fru 6-P and Fru 1,6-P_2, subtrates for PFK-1 and FBPase[8-10], it is reasonable to suggest that Fru 2,6-P_2 participates in the control of the pentose pathway substrate cycle in its nonoxidative stage[11]:



To verify this hypothesis, Blackmore and Schuman[11] studied the effect of glucose and glucagon on the levels of heptulose phosphates in hepatocytes, since these agents are known to increase (glucose) and decrease (glucagon) the concentration of Fru 2,6-P_2 in the liver[3,12]. It has also been shown that the hepatic Fru 2,6-P_2 concentration is controlled by a bifunctional enzyme (6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase), which is involved in both the synthesis of Fru 2,6-P_2 from Fru 6-P and ATP and in the hydrolysis of Fru 2,6-P_2 to Fru 6-P and P_i[13]. The action of glucose on the Fru 2,6-P_2 concentration is explained by the fact that the increase in glucose concentration parallels an increase in Fru 6-P concentration, which consequently stimulates the activity of 6-phosphofructo-2-kinase. Glucagon evokes an increase in the concentration of cyclic AMP in the cell, which leads to the activation of the cyclic AMP-dependent protein kinase and to the phosphorylation of the bifunctional enzyme, resulting in reduced kinase and enhanced phosphatase activity[13,14]. Blackmore and Schuman[11] have shown that perfusion of the liver with 50 mM glucose causes an appreciable rise of the Sed 1,7-P_2 concentration, whereas addition of glucagon produces a fourfold increase in the concentra-

tion of Sed 7-P. Heptulose phosphates were extracted from the liver and purified by Dowex-1 ion exchange chromatography, and the resolved heptulose phosphates were measured using the cystein-H_2SO_4 method. The results provided evidence for the participation of glucose and glucagon in the regulation of the nonoxidative stage of pentose pathway. Presumably, the effect of glucose and glucagon on the heptulose phosphate concentrations was mediated through the Fru 2,6-P_2 system.

The aim of the present work was to establish whether Fru 2,6-P_2 participates in the conversion of Sed 7-P and Sed 1,7-P_2 in the soluble fraction of rat liver. For this purpose we used ^{31}P-NMR-spectroscopy which enabled us to follow simultaneously changes in the concentration of substrates (heptulose phosphates, ATP) and products (ADP, P_i) of the enzymatic reactions.

Materials and methods

Male rats (Wistar) weighing 250–300 g were used in the experiments. Sedoheptulose 7-phosphate barium salt, sedoheptulose 1,7-bisphosphate sodium salt, fructose 2,6-bisphosphate, Tris and EDTA were purchased from Sigma Chemical Company (St. Louis, USA); ATP and ADP were purchased from Serva GMBH (Heidelberg, Germany).

Sedoheptulose 7-phosphate barium salt was converted to sodium salt by treating with an equimolar concentration of Na_2SO_4. The $BaSO_4$ precipitate was removed by centrifugation.

To obtain a soluble fraction the rat liver was cut with scissors and homogenized in a teflon Potter homogenizer for 40 s in five volumes of 30 mM Tris-HCl buffer pH 7.6, containing 0.25 M sucrose and 0.01 mM EDTA. The homogenate was centrifuged at $50000 \times g$ for 2 h. The precipitate was discarded and the supernatant used for the studies.

In experiments on Sed 7-P phosphorylation the incubation mixture (in a total volume of 1.6 ml) contained the following components: 50 mM Tris-HCl buffer pH 7.3; 0.65 mM Sed 7-P; 0.9 mM ATP; 0.13 mM AMP; 5 mM $MgCl_2$ and 0.1 mM EDTA. Fru 2,6-P_2 was added to a final concentration of 5 µM. The reaction was started by adding 250 µl of liver sytosol. The samples were incubated at 37 °C for 10 min and 20 min and the reaction stopped by heating in a boiling water bath for 1 min. The denatured protein was removed by centrifugation, and the supernatant (with 5 mM EDTA added to bind Mg^{2+}) was used to determine the loss of Sed 7-P and ATP. A sample boiled immediately after the addition of the enzyme solution served as control.

Dephosphorylation of Sed 1,7-P_2 was carried out in a medium made up of 50 mM Tris-HCl buffer pH 7.3; 1 mM Sed 1,7-P_2; 2 mM $MgCl_2$; and 0.01 mM EDTA. The concentration of added Fru 2,6-P_2 was 10 µM. The reaction was started by adding 150 µl of liver cytosol preparation. The samples were incubated at 37 °C for 10 and 15 min, and the protein-free supernatant (with 5 mM EDTA added) was used to measure the loss of Sed 1,7-P_2.

^{31}P-NMR was applied to investigate changes in the concentrations of heptulose phosphates, ATP, ADP, and P_i. ^{31}P-NMR spectra were taken using a Bruker AM-400 spectrometer (resonance frequency 162 MHz on phosphorus nuclei, $B_o = 9.4$T). The spectra were a collection of 5000 scans with 45°RF pulses and a relaxation delay of 2 s. Spectra were collected with 8K data points at 40 ppm spectral width. The scale of chemical shift was calibrated using ethylene diamine tetraphosphonic acid as external standard. The NMR signal were identified using the spectra of standard solutions of Sed 7-P and Sed 1,7-P_2. Changes in relative concentrations of Sed 7-P and Sed 1,7-P_2 were estimated by the changes in the signal intensities of the spectra. The decrease in ATP and increase in ADP concentrations were calculated by the signal intensities of alpha phosphate groups; pH was adjusted to 6.9 ± 0.1 prior to NMR spectrum registration.

Results and discussion

Figure 1 shows the NMR spectra from samples in which the phosphorylation of Sed 7-P was studied. Within 10 and 20 min after the initiation of the enzymatic reaction a significant decrease in Sed 7-P and ATP concentrations was observed in the presence of Fru 2,6-P_2.

The changes in relative Sed 7-P concentrations and ADP/ATP ratio are shown in figure 2. In the absence of Fru 2,6-P_2 no appreciable loss of Sed 7-P was observed during a 20 min incubation, which may be explained by the inhibition of PFK-1 by the high concentration (0.9 mM) of ATP. An inhibited phosphorylation of Sed 7-P at ATP concentrations higher than 0.4 mM was observed by Karadshen et al.[8] in their experiments with partly purified PFK-1 preparations from rabbit liver.

Shown in figure 3 are the ^{31}P-NMR spectra of samples in which dephosphorylation of Sed 1,7-P_2 was tested. During incubation (10 min) a loss of Sed 1,7-P_2 was observed. The addition of Fru 2,6-P_2 appreciably slowed the depletion of Sed 1,7-P_2 and almost completely inhibited the accumulation of P_i. It may be inferred that the disappearance of Sed 1,7-P_2 in the presence of Fru 2,6-P_2 was due to the presence of aldolase in the cytosol preparation. The changes in the relative concentration of Sed 1,7-P_2 during a 15 min incubation in the presence of cytosol are shown in figure 4.

It will be noted that the products of kinase or phosphatase action in figure 1 and figure 3 (i.e. Sed 1,7-P_2

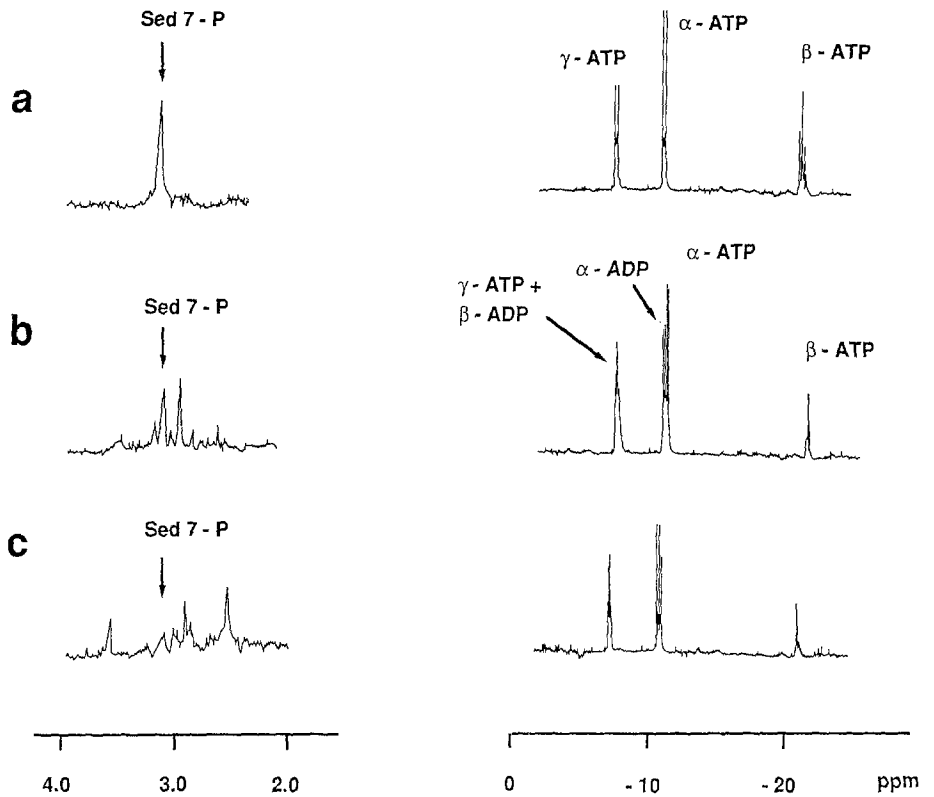


Figure 1. ^{31}P-NMR spectra of the reaction mixture in the 6-phosphofructo-1-kinase test *a* before, *b* after 10 min and *c* 20 min of incubation in the presence of liver cytosol and fructose 2,6-bisphosphate. Sed 7-P = sedoheptulose 7-phosphate.

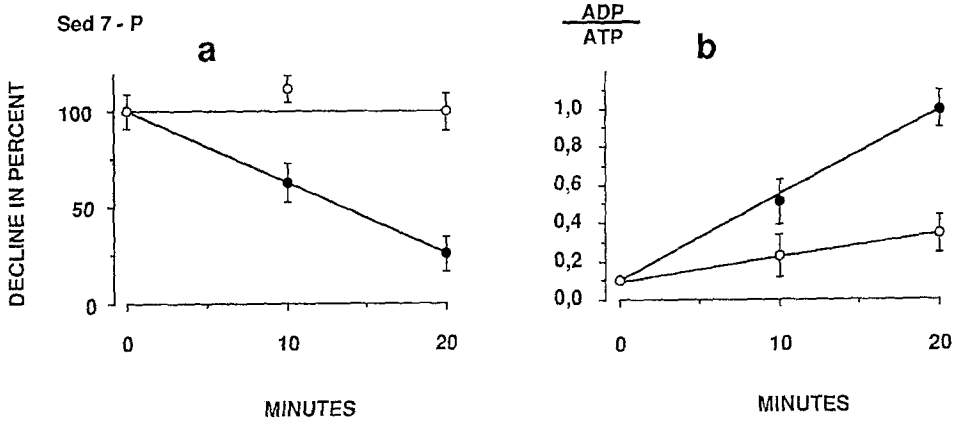


Figure 2. Changes in the relative concentration of *a* sedoheptulose 7-phosphate and *b* ADP/ATP ratio during incubation in liver cytosol without fructose 2,6-bisphosphate (○) and in the presence of 5 μM fructose 2,6-bisphosphate (●).

and Sed 7-P) have been subsequently converted in aldolase and transketolase reactions into other metabolites. Thus, dihydroxyacetonephosphate (DOAP) will be formed from Sed 1,7-P_2 in the reaction catalysed by aldolase, and DOAP formed can be converted to glyceraldehyde 3-P(GAP). The latter in turn can enter the transaldolase reaction with Fru 6-P and erythrose 4-P formation, and then Fru 6-P can be phosphorylated by PFK-1. However, on the basis of the data of Karashen et al.[8], who found that Sed 7-P was a competitive inhibitor of liver PFK-1 with respect to Fru 6-P, we believe that Sed 7-P must prevent Fru 6-P phosphorylation since its concentration (at least in the initial period of incubation) significantly exceeds that of Fru 6-P, the formation of which requires the participation of two molecules of Sed 7-P:

$$\begin{array}{c}\text{Sed 7-P} + \text{ATP} \rightarrow \text{DOAP} + \text{Erythrose 4-P} \\ \uparrow\downarrow \\ \text{Sed 7-P} + \text{GAP} \rightleftharpoons \text{Fru 6-P} + \text{Erythrose 4-P}\end{array}$$

Though Fru 6-P phosphorylation in the presence of Fru 2,6-P_2 cannot be entirely excluded, we nevertheless

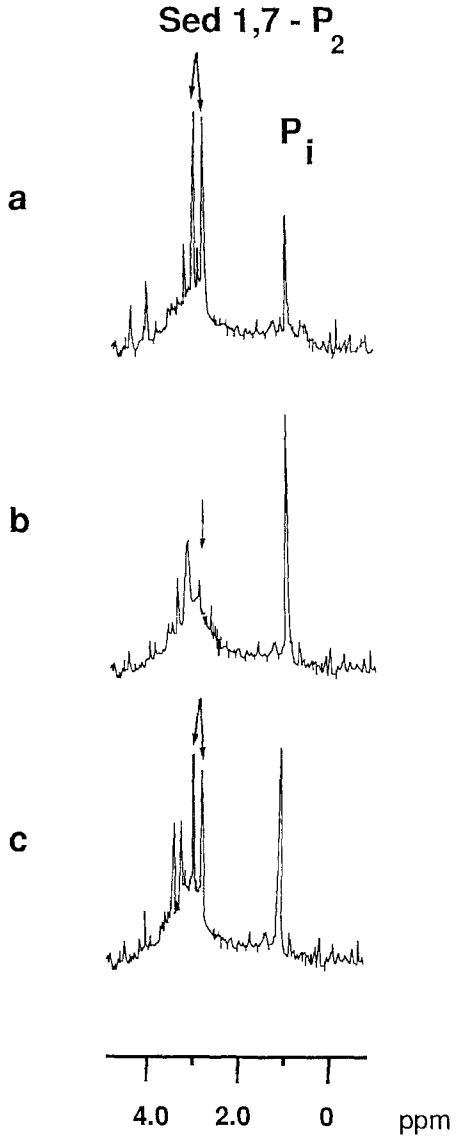


Figure 3. ^{31}P-NMR spectra of reaction mixture in the fructose 1,6-bisphosphatase test *a* before and *b* after 10 min incubation in the absence and *c* in the presence of fructose 2,6-bisphosphate. Sed 1,7-P_2 = sedoheptulose 1,7-bisphosphate.

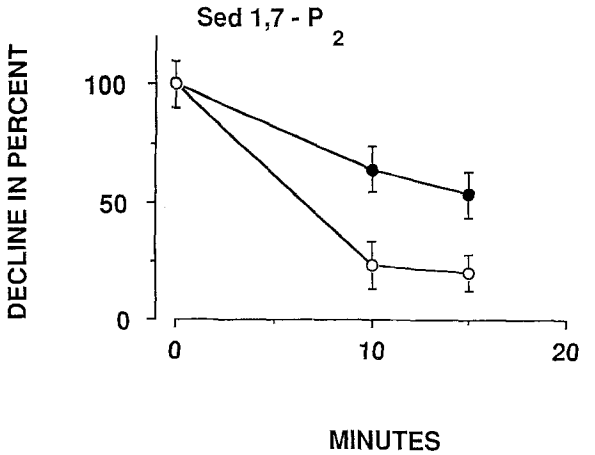


Figure 4. The decline of the relative concentration of sedoheptulose 1,7-bisphosphate during incubation in liver cytosol without fructose 2,6-bisphosphate (○) and in the presence of 10 μM fructose 2,6-bisphosphate (●).

believe that our results demonstrate that Fru 2,6-P_2 activates Sed 7-P phosphorylation, since under our experimental conditions no loss of Sed 7-P occurred in the absence of Fru 2,6-P_2 (See fig. 2). The absence of Sed 7-P phosphorylation at an ATP concentration of 0.9 mM corroborates the early published data that this reaction is inhibited by high ATP concentrations. Besides that our results demonstrate the activating effect of Fru 2,6-P_2 due to the removal of the high ATP inhibition of PFK-1.

The phosphorylating activity of liver PKF-1 on Fru 6-P and Sed 7-P as substrates was found to be roughly the same[8]. At the same time the Sed 7-P concentration in the liver (0.1 mM) exceeds the Fru 6-P level by 2-2.5 fold[15]. Interestingly, the value of V_{max} for Sed 7-P phosphorylation was found to increase appreciably in the mammary gland of mice during the lactation period, finally becoming higher than the V_{max} of Fru 6-P phosphorylation[15]. These data indicate that Sed 7-P is a physiological substrate for PFK-1.

The dephosphorylation of Sed 1,7-P_2 can be effected by FBPase[9,10]. Mizunuma and Tashima[9] have shown that the activity of FBPase from the mouse liver using 1 mM Sed 1,7-P_2 as substrate was only a half of the dephosphorylation activity when a similar concentration of Fru 1,6-P_2 was used as substrate.

The results of the present study enable us to suggest that Fru 2,6-P_2 activates the phosphorylation of Sed 7-P and inhibits the dephosphorylation of Sed 1,7-P_2, and thus participates in the control of the sedoheptulose substrate cycle in the nonoxidative stage of the pentose pathway.

Many studies have postulated that metabolic intermediates can switch between the pentose pathway (its anaerobic part) and glycolysis, which both occur in cytosol, depending on the concentration of intermediates formed in the cells[11,16–18]. At least four possible sites of switching between glycolysis and the nonoxidative stage of pentose pathway are known to exist: three transketolase, transaldolase and aldolase reactions. The scheme depicting the relationship of the pentose pathway of carbohydrate metabolism with glycolysis is shown in figure 5.

Blackmore and Schuman[11] suggest that an increase in the liver Fru 2,6-P_2 level will stimulate flux through the following pathway:

$$\text{Ribose 5-P} + \text{Xylulose 5-P} \xrightarrow{TK} \text{GAP} + \text{Sed 7-P};$$

$$\text{Sed 7-P} + \text{ATP} \xrightarrow{PFK} \text{Sed 1,7-P}_2 + \text{ADP};$$

$$\text{Sed 1,7-P}_2 \xrightarrow{ALD} \text{GAP} + \text{Erythrose 4-P};$$

$$\text{Erythrose 4-P}_2 + \text{Xylulose 5-P} \xrightarrow{TK} \text{GAP} + \text{Fru 6-P}$$

(the latter reaction is not shown in fig. 5).

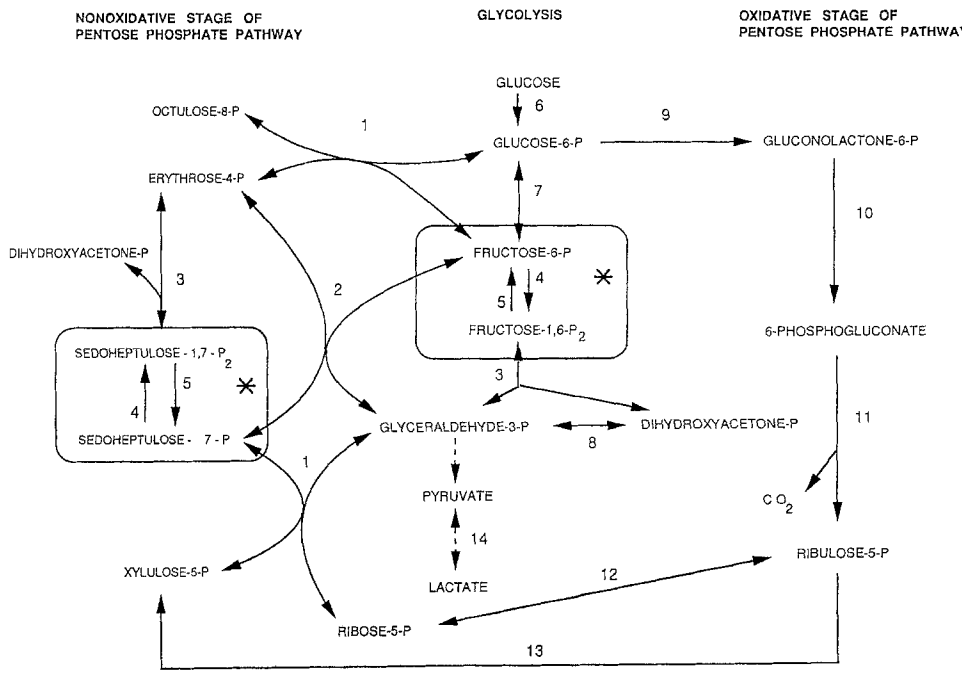


Figure 5. Interrelationship between the pentose phosphate pathway and glycolyis. *1*, transketolase; *2*, transaldolase; *3*, aldolase; *4*, 6-phosphofructo-1-kinase; *5*, fructose 1,6-bisphosphatase; *6*, hexokinase; *7*, glucose 6-phosphate isomerase; *8*, triose phosphate isomerase; *9*, glucose 6-phosphate dehydrogenase; *10*, 6-phosphogluconolactonase; *11*, 6-phospho-D-gluconate: $NADP^+$ 2-oxidoreductase (decarboxylating); *12*, ribose 5-phosphate isomerase; *13*, ribulose phosphate 3-epimerase; *14*, L-lactate dehydrogenase. * Sites of fructose 2,6-bisphosphate interaction.

Sum: 3 Pentose 5-P $+ ATP \rightarrow$ Fru 6-P + 3 Triose $+ ADP$.

The reversal of the nonoxidative stage of pentose phosphate pathway, yielding ribose 5-P from Fru 6-P and GAP, may be necessary when the requirement for ribose 5-P is decreased but the energy demand is increased. Then additional Fru 6-P and GAP will enhance ATP formation in glycolysis. Though the conversion of ribose 5-P excess into glycolytic intermediators can occur without Sed 7-P phosphorylation, operation of the sedoheptulose cycle will allow Fru 2,6-P_2 to regulate this process and thus to provide hormonal control.

Thus the increase in cellular Fru 2,6-P_2 concentration will lead not only to activation of glycolysis, but also to an augmented carbohydrate flux via nonoxidative stage of the pentose phosphate pathway towards the formation of hexose phosphates.

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