Biochem. J. (1987) 245, 313–324 (Printed in Great Britain)

REVIEW ARTICLE

Role of fructose 2,6-bisphosphate in the control of glycolysis in mammalian tissues

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Introduction

Glycolysis is an ubiquitous metabolic pathway. In addition to its catabolic role, glycolysis also serves an anabolic function by providing C_3 precursors for the synthesis of fatty acids, cholesterol and amino acids. It is therefore an amphibolic pathway. The difference in metabolic orientation is tissue-specific and depends on the hormonal and nutritional state. Therefore, it is not surprising that different and specific regulatory mechanisms exist to control glycolysis under these various conditions. Cancer cells are a special case in which glycolysis is abnormally high.

Qualitative and quantitative approaches have been developed to elucidate the control mechanisms of glycolysis. On the one hand, so-called 'rate-limiting' steps in the pathway have been identified by qualitative analysis. Reactions displaced far from equilibrium, particularly when catalysed by an allosterically regulated enzyme, have been assumed to qualify for control [1]. One such reaction is catalysed by phosphofructokinase (PFK-1). The experimental evidence supporting the key role of PFK-1 stems from the changes in the concentration of PFK-1 effectors observed when glycolysis is stimulated, e.g. following anoxia [2,3]. However, this information remains qualitative and may lead to an oversimplified view. On the other hand, the quantitative analysis of control, developed by Kacser & Burns [4] and Heinrich & Rapoport [5], allows the distribution of control among all steps in a pathway to be calculated. Such an approach has been applied to glycolysis in erythrocytes [6] and yeast [7], and it indicates that control is mainly distributed between hexokinase and PFK-1.

The study of the mechanism of action of glucagon on liver gluconeogenesis led to the discovery of fructose 2,6-bisphosphate (Fru-2,6-P_2) [8,9]. This sugar phosphate is a potent stimulator of PFK-1 [10–12] and is also an inhibitor of fructose 1,6-bisphosphatase (FBPase-1) [13,14]. Fru-2,6-P_2 has been detected in all mammalian tissues studied so far, as well as in fungi and plants, but not in prokaryotes [15].

Earlier reviews have dealt with the effect of Fru-2,6-P_2 on its two main targets, PFK-1 and FBPase-1, together with the regulation of its synthesis and breakdown in relation to the control of glycolysis/gluconeogenesis in liver [15–22].

Since Fru-2,6-P_2 is present in all mammalian tissues, it is tempting to suppose that it plays the major role in the control of glycolysis. The purpose of this article is to specify the conditions under which Fru-2,6-P_2 plays a role in the control of glycolysis, and to review recent studies dealing with Fru-2,6-P_2 metabolism in mammalian tissues other than liver.

Control of enzyme activity by Fru-2,6-P_2

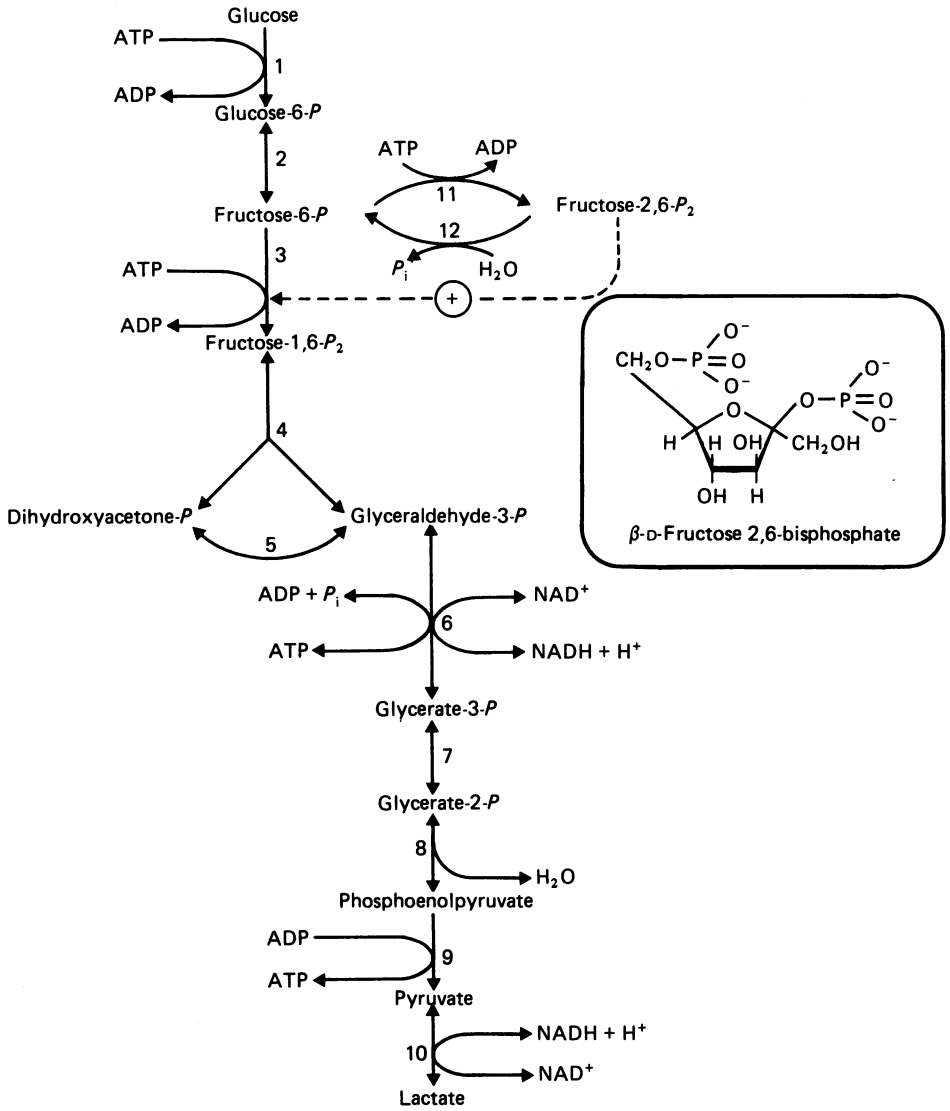
The two main targets of Fru-2,6-P_2 are PFK-1 and FBPase-1. Different isoenzyme forms of PFK-1 exist, all of which display similar kinetic properties [23,24]. ATP, one of the substrates of PFK-1, behaves as a negative allosteric effector inducing co-operativity for fructose 6-phosphate (Fru-6-P), the second substrate. This inhibition by ATP can be relieved by Fru-6-P acting as a positive effector. The most important positive effectors are AMP and Fru-2,6-P_2, which act in synergism. All PFK-1 isoenzymes studied so far are sensitive to Fru-2,6-P_2 [15]. In the presence of physiological concentrations of substrates and effectors, liver PFK-1 is almost completely inactive [25] unless micromolar concentrations of Fru-2,6-P_2 (within the physiological range) relieve the inhibition by ATP and allow glycolysis to proceed [10–12]. Thus, in liver and perhaps in other tissues, Fru-2,6-P_2 is a likely candidate to stimulate PFK-1 when the anabolic role of glycolysis prevails. In contrast, when glycolysis is the only source of ATP as, e.g., during anoxia, adenine nucleotides and P_i probably play the main regulatory role.

Fructose 1,6-bisphosphate (Fru-1,6-P_2), the product of the reaction catalysed by PFK-1, is yet another positive effector of this enzyme. Its concentration varies greatly in the cell and it binds to the same site as Fru-2,6-P_2 [26]. While the affinity of PFK-1 for Fru-2,6-P_2 is at least 100-fold greater than for Fru-1,6-P_2 in liver, it is only 10-fold greater in muscle [10,26,27]. Therefore, when the concentration of Fru-1,6-P_2 largely exceeds that of Fru-2,6-P_2 as, e.g., in contracting muscle [28,29], the effect of Fru-1,6-P_2 would prevail over that of Fru-2,6-P_2.

An interesting property of muscle PFK-1 is that its activity and response to regulatory ligands are influenced by its concentration and protein–protein interaction [30,31]. For example, binding of PFK-1 to membranes [32] or myofibrils [33] relieves inhibition by ATP, thereby stimulating enzyme activity. Therefore translocation of PFK-1 may be an important control mechanism *in vivo*. This translocation and the equilibrium between free and bound PFK-1, in turn, could be influenced by ligands and/or phosphorylation state of the enzyme [34]. It is hitherto unknown whether, and to what extent, such regulation exists *in vivo*.

Liver, muscle and adipose tissue PFK-1 are substrates

Abbreviations used: Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase; PFK-2, 6-phosphofructo-2-kinase; FBPase-1, fructose 1,6-bisphosphatase; FBPase-2, fructose 2,6-bisphosphatase; Fru-6-P, fructose 6-phosphate.



Scheme 1. Glycolytic pathway and structure of β-D-fructose 2,6-bisphosphate

The numbers in the Scheme refer to enzymes: 1, hexokinase; 2, phosphoglucose isomerase; 3, 6-phosphofructo-1-kinase; 4, aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, phosphoglycerate mutase; 8, enolase; 9, pyruvate kinase; 10, lactate dehydrogenase; 11, 6-phosphofructo-2-kinase; 12, fructose-2,6-bisphosphatase. The inset shows the structure of β-D-fructose 2,6-bisphosphate which is the natural anomer.

of the cyclic AMP-dependent protein kinase [35,36]. Phosphorylation affects the kinetic properties of muscle PFK-1 so that phosphorylated PFK-1 becomes less active (the affinity for positive effectors decreases and inhibition by ATP increases) [35,37–40]. However, the changes for the liver enzyme are small and have not always been observed [41]. Muscle PFK-1 is also a substrate of the Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C). Phosphorylation activates the enzyme by increasing the affinity for Fru-6-*P* [42]. Whether this effect is physiologically significant is questionable, since positive effectors such as Fru-2,6-P_2 inhibit the phosphorylation *in vitro* [43]. Muscle PFK-1 has also been reported to be a substrate of the tyrosine kinase activity of the purified epidermal growth factor receptor [44]; whether phosphorylation modifies the kinetic properties of PFK-1 and can occur in the intact cells is not known. The changes in activity of phosphorylated PFK-1 from adipose tissue are discussed below.

Fru-2,6-P_2 is an inhibitor of FBPase-1 [13,14]. The inhibition by Fru-2,6-P_2 is synergistic with that by AMP (except for muscle enzyme [45]), and greater at low than at high substrate concentration. Fru-2,6-P_2 changes the saturation curve for the substrate from a hyperbolic to a sigmoidal shape, suggesting an allosteric type of interaction. From kinetic and binding studies it appears that the action of Fru-2,6-P_2 is complex and involves its binding to a site which might be different from the Fru-1,6-P_2 site, although some overlapping between the two sites is not excluded (for a review see [15]). In livers of fed rats producing lactate, the tissue concentration of AMP (0.1–0.2 mM) and Fru-2,6-P_2 (10 μM) would be sufficient to completely inhibit FBPase-1 even at saturating concentrations of substrate. Yet, cycling of metabolites between glucose and triose phosphates occurs [8,46,47], indicating that not only PFK-1 but also FBPase-1 are active under these conditions. Actually, it has never been possible to demonstrate complete

Table 1. Properties of purified PFK-2/FBPase-2

Data were taken from references 63, 68, 71, 74, 75, 77, 79, 80, 82, 83 and 152. Abbreviations: N.D., not detectable; (B), (C), (P) and (R) indicate the source of enzyme: B, bovine; C, chicken; P, pigeon; R, rat.

	Heart	Muscle	Liver
PFK-2 activity			
Apparent K_m for Fru-6-*P* (μM)	50 (B)	48 (P)	50–140 (R) 40 (C)
$V_{max.}$ (munit/mg of protein)	180 (B)	11 (P)	70 (R) 112 (C)
Inhibition by 1 mM-*sn*-glycerol 3-phosphate (%)	15 (B)	–	75 (R)
Apparent K_i for citrate (μM)	79 (R)	93 (R) 83 (P)	250 (R) 490 (C)
Inactivation by cyclic AMP-dependent protein kinase	N.D. (B,R)	N.D. (R) Partial (P)	Yes (R) Yes (C)
FBPase-2 activity			
Apparent K_m for Fru-2,6-P_2 (nM)	1400 (B)	23 (P)	22 (C) 80 (R)
$V_{max.}$ (munit/mg of protein)	2.2 (B)	56 (P)	20–50 (R) 39 (C)
Apparent K_i for Fru-6-*P* (μM)	150 (B)†	140 (R) 50 (P)	26 (R) 8 (C)
Activation by cyclic AMP-dependent protein kinase	N.D. (B)	N.D. (P)	Yes (L) Yes (C)
E–P formation (mol/mol of holoenzyme)	0.05 (B)	0.7 (P)	0.7 (C) 0.3–0.6 (R)
Protein phosphorylation (mol of $^{32}P_i$ incorporated/mol of enzyme subunit)			
Cyclic AMP-dependent protein kinase	0.29 (B)	0.025 (P)	0.83–1.0 (R) 0.4 (C)
Protein kinase C	0.95 (B)	–	0.19 (R)
PFK-2/FBPase-2 activity ratio of non-phosphorylated form	80 (B) $\geqslant$ 29 (R)*	0.19 (P) 0.14 (R)*	1–4 (R) 3 (C)

* Partially purified preparations.
† Unpublished work from authors' laboratory.

inhibition of FBPase-1 *in vivo*. In contrast, PFK-1 is completely inactive during gluconeogenesis [47]. These observations suggest that FBPase-1 is less inhibited *in vivo* than would be expected from measurements *in vitro* and that the concentration of free AMP and Fru-2,6-P_2 are probably less than their total cytosolic concentration. In addition, FBPase-1 is less sensitive to the effect of Fru-2,6-P_2 than PFK-1. Moreover, kinetic and binding studies have shown that the affinity of FBPase-1 for Fru-2,6-P_2 is about 10-fold smaller than that of PFK-1 [10–14,27,48,49].

Liver FBPase-1 from rat, but not from mouse, rabbit or pig, is a substrate of the cyclic AMP-dependent protein kinase [50–54]. Phosphorylation decreases the K_m for Fru-1,6-P_2 but does not affect the response to AMP and Fru-2,6-P_2. The phosphorylation site is located in the *C*-terminal region of the rat enzyme. This site is not present in the other species, and the physiological relevance of this phosphorylation is obviously restricted.

Besides its effect on PFK-1 and FBPase-1, Fru-2,6-P_2 has been reported to affect several other enzymes. It stimulates pyrophosphate:fructose 6-phosphate phosphotransferase, an enzyme present in plants [55]. Because of its extreme sensitivity towards Fru-2,6-P_2, this enzyme from potato tubers is used for the microassay of Fru-2,6-P_2 [56,57]. Fru-2,6-P_2 has been reported to affect the activity of UDPGlc phosphorylase [58], trehalose phosphorylase [59], 6-phosphogluconate dehydrogenase [60] and phosphoglucomutase [61]. These effects have not always been confirmed, and some require high non-physiological concentrations of Fru-2,6-P_2. An unusual situation exists in *Trypanosoma brucei*, in which Fru-2,6-P_2 does not affect PFK-1 but stimulates pyruvate kinase activity [62].

Biosynthesis and degradation of Fru-2,6-P_2

The steady state concentration of Fru-2,6-P_2 depends on the balance between the activities of 6-phosphofructo-2-kinase (PFK-2), which synthesizes Fru-2,6-P_2, and fructose-2,6-bisphosphatase (FBPase-2) which catalyses its breakdown. The properties of this system have been extensively studied in rat liver and are summarized below for comparison with those in other tissues (for a review see also [63]).

PFK-2 catalyses the synthesis of Fru-2,6-P_2 from Fru-6-*P* and MgATP. The enzyme is saturated by MgATP, stimulated by P_i and inhibited by citrate, phosphoenolpyruvate and *sn*-glycerol 3-phosphate at physiological concentrations. In the presence of 5 mM-P_i, the K_m of PFK-2 for Fru-6-*P* is 50 μM, a concentration also in the physiological range [15].

The hydrolysis of Fru-2,6-P_2 to Fru-6-P and P_i is catalysed by a specific phosphatase, FBPase-2, which is stimulated by P_i, *sn*-glycerol 3-phosphate and nucleoside triphosphates. The K_m of this enzyme for Fru-2,6-P_2 is less than 0.1 μM, which is 100 times less than the total concentration of Fru-2,6-P_2 in the livers of fed rats. Fru-6-P, one of the reaction products, inhibits the enzyme which an apparent K_i of about 20 μM [15]. Therefore, since both the K_m of PFK-2 and the K_i of FBPase-2 for Fru-6-P are similar to the concentrations of Fru-6-P normally found in the liver, any change in Fru-6-P concentration should cause inverse changes in the two activities. A direct relation between the concentrations of Fru-6-P and Fru-2,6-P_2 has indeed been found in the liver under certain conditions [64].

The activities of PFK-2 and FBPase-2 copurify with a protein of M_r 110000, composed of two identical subunits of M_r 55000. Several lines of evidence suggest that this protein is a bifunctional enzyme with two distinct catalytic sites on the same polypeptide chain [65–68]. The liver bifunctional PFK-2/FBPase-2 is a substrate of cyclic AMP-dependent protein kinase which inactivates PFK-2 and activates FBPase-2 (see below).

The PFK-2 reaction appears to involve the formation of a ternary complex, whereas the FBPase-2 reaction follows a ping–pong mechanism involving a phosphoryl enzyme (E–P) intermediate. Indeed incubation of the bifunctional enzyme with Fru-2,6-[2-^{32}P]P_2 leads to the phosphorylation of a histidine residue and the rate of enzyme phosphorylation is 100 times faster than the overall rate of hydrolysis [69–72].

Sulphydryl groups appear to be important for both PFK-2 and FBPase-2 activities [73].

Evidence for the possible existence of isoenzymes of PFK-2/FBPase-2 was first obtained when the properties of purified bovine heart and rat liver enzymes were compared [74]. The study revealed that the two enzymes differ by their relative FBPase-2 content, kinetic properties, response to treatment by cyclic AMP-dependent protein kinase and protein kinase C and labelling from Fru-2,6-[2-^{32}P]P_2 [74–76] (Table 1). In addition, antiserum raised against rat liver PFK-2 does not cross-react with the heart enzyme [76]. A striking difference between type H (heart) and type L (liver) PFK-2/FBPase-2 is the low heart FBPase-2 activity, which is 80 times smaller than the PFK-2 activity [75]. One report has even claimed that heart PFK-2 is devoid of any FBPase-2 activity [76]. This has not been confirmed [75]. The fact that heart FBPase-2 is not activated by cyclic AMP-dependent protein kinase suggests that the low heart FBPase-2 activity is not due to contamination by type L isoenzyme [63]. Thus, the heart bifunctional enzyme is more a kinase than a phosphatase. Heart FBPase-2 activity is too small (0.01 nmol/min per g of tissue) to account for the changes in Fru-2,6-P_2 concentration observed *in vivo*. This suggests that either some hitherto unknown factor(s) could unmask the bisphosphatase activity *in vivo*, or that another FBPase-2 exists. Until now, however, all attempts to detect and purify another specific FBPase-2 have failed (M. H. Rider, A. M. Loiseau & L. Hue, unpublished results). On the other hand, it is not excluded that Fru-2,6-P_2 could act as a substrate for other reactions. Fru-2,6-P_2 is indeed a potential donor of either a phosphoryl or an osyl group, but no such reactions have yet been described.

By contrast with the type H, muscle PFK-2/FBPase-2 contains 5–10 times more phosphatase than kinase activity, and, in this respect, muscle PFK-2/FBPase-2 is more a phosphatase than a kinase and it resembles the phosphorylated form of liver PFK-2/FBPase-2 [63,77]. The enzyme has been purified to homogeneity from pigeon muscle [77]. However, unlike the type L but like the type H, muscle PFK-2 is not inactivated by treatment with cyclic AMP-dependent protein kinase [63,77]. Therefore, since muscle PFK-2/FBPase-2 differs from both the type L and H, the question is whether the muscle enzyme represents a third isoenzyme of PFK-2/FBPase-2. Immunological studies could help in answering this question. In spite of these different regulatory properties, the three enzymes have a similar molecular mass.

An interesting feature of rat skeletal muscle FBPase-2 activity is that its K_i (140 μM) for Fru-6-P is more than 5 times greater than that of liver FBPase-2 [63]. This may explain why muscle contraction, which results in a rise in Fru-6-P, does not always lead to a corresponding rise in Fru-2,6-P_2 [28,29].

In all mammalian tissues studied so far, it has been possible to measure PFK-2 activity as well as the formation of E–P from Fru-2,6-[2-^{32}P]P_2, suggesting the existence of a FBPase-2 activity. The PFK-2 activity (about 2–5 nmol/min per g in all mammalian tissues) is several orders of magnitude smaller than the activity of hexokinase, PFK-1 and pyruvate kinase, and than the overall glycolytic rate. This implies that the PFK-2/FBPase-2 dependent changes in Fru-2,6-P_2 concentration are too slow to qualify for instantaneous control of glycolysis as it is required, e.g. during muscle contraction. On the other hand, since both the activity per g of tissue and the specific activity of PFK-2/FBPase-2 are much smaller than those of other glycolytic enzymes, the intracellular concentration of PFK-2/FBPase-2 in liver is comparable with that of PFK-1 [78].

Control of PFK-2/FBPase-2 activity by phosphorylation

In liver, stimulation of gluconeogenesis by glucagon involves inhibition of glycolysis, which results not only from the inactivation of pyruvate kinase but also from the disappearance of Fru-2,6-P_2 [19]. This glucagon effect on Fru-2,6-P_2 probably results from the phosphorylation of PFK-2/FBPase-2. The liver bifunctional enzyme indeed belongs to the growing number of enzymes controlled by phosphorylation/dephosphorylation [65]. The cyclic AMP-dependent protein kinase catalyses the incorporation of 1 mol of phosphate/mol of 55 kDa subunit [79,80].

Partial proteolysis of the phosphorylated enzyme with thermolysin removes a single phosphorylated peptide which contains three adjacent arginine residues *N*-terminal to the phosphorylated serine [79]. This is more than the two basic residues required for site specificity, and may explain why liver PFK-2 is such a good substrate for cyclic AMP-dependent protein kinase.

The phosphorylation site of other liver glycolytic enzymes phosphorylated by the cyclic AMP-dependent protein kinase is likewise released by partial proteolysis on a low-M_r fragment. These enzymes include PFK-1 [37], FBPase-1 [52] and pyruvate kinase [81]. Thus the location of phosphorylation sites at the extremities of the protein structure may be a general feature by which the effects of phosphorylation are transmitted.

Table 2. Relationship between Fru-2,6-P_2 concentration and glycolytic flux in liver

The concentration of Fru-2,6-P_2 in liver is between 0.1 and 20 nmol/g wet wt. depending on the conditions. Three metabolic states are listed. In the first, Fru-2,6-P_2 is low, gluconeogenesis prevails and glycolysis is blocked. The last five conditions listed in the first group decrease Fru-2,6-P_2 concentrations but do not necessarily inhibit lactate production and/or stimulate gluconeogenesis. In the second metabolic state, Fru-2,6-P_2 is between 5 and 20 nmol/g, glycolysis prevails but gluconeogenesis is not stopped, therefore futile cycles occur. In the third state, Fru-2,6-P_2 is low and glycolysis is maximally stimulated by anoxia. For details, see the text.

Conditions	Mechanism for change in Fru-2,6-P_2 concentration	References
(*a*) Low Fru-2,6-P_2, gluconeogenic state		
Glucagon, β-adrenergic agonists, cyclic AMP	Cyclic AMP-dependent inactivation of PFK-2 and activation of FBPase-2	8,65,86,153
Starvation and diabetes	As above + decrease in PFK-2/FBPase-2 content	154–158
Muscular exercise	Cyclic AMP	159
Neonatal period	Cyclic AMP?	160
Regenerating liver	Cyclic AMP?	*
Fatty acids	Inhibition of PFK-2 by citrate	*
Adenosine	Cyclic AMP	101
Ethanol	Cyclic AMP + effect of *sn*-glycerol 3-phosphate on phosphorylation and activity of PFK-2/FBPase-2	85
Glycerol and large concentrations (> 5 mM) of lactate, pyruvate, alanine, fructose	PFK-2 inhibition/FBPase-2 stimulation by metabolites (phosphoenolpyruvate, *sn*-glycerol 3-phosphate)	85,99,102,161
2,5-Anhydromannitol	Inhibition of PFK-2?	162–164
Anaesthesia	?	165
(*b*) High Fru-2,6-P_2, glycolytic state		
Glucose load, refeeding	Increased Fru-6-*P* concentration	8,78,166–169
α-adrenergic agonists, vasopressin in fed animals	As above by stimulation of glycogen breakdown	64,91,113
Insulin	Anti-cyclic AMP	113,166,170,171
Genetically obese mice and rats	Low cyclic AMP	110–112
Low concentration (< 5 mM) of lactate, pyruvate, dihydroxyacetone, fructose	Increased Fru-6-*P* concentration	99
Sulphonylurea	?	172–174
(*c*) Low Fru-2,6-P_2 and maximal glycolysis		
Anoxia (N_2)	Interaction of metabolites with PFK-2/FBPase-2 activity?	121

* Unpublished experiments from authors' laboratory.

Phosphorylation by cyclic AMP-dependent protein kinase inactivates liver PFK-2. This inactivation results from a decrease in $V_{max.}$ (at physiological pH, but not at pH 8), an increase in K_m for Fru-6-*P* and an increased sensitivity towards inhibition by citrate and phosphoenolpyruvate [82]. On the other hand, phosphorylation by cyclic AMP-dependent protein kinase activates liver FBPase-2 by increasing the $V_{max.}$ without change in K_m. Moreover, the phosphorylated FBPase-2 is more sensitive to stimulation by nucleoside triphosphates and glycerol phosphate, and less sensitive to inhibition by Fru-6-*P* [15,83].

Inactivation and phosphorylation of liver PFK-2 is inhibited by Fru-2,6-P_2 [84] (half-maximal effect at 0.5 μM [85]). The physiological relevance of this observation is not clear since, in hepatocytes from fed rats, 10–20 μM-Fru-2,6-P_2 does not prevent glucagon from inactivating PFK-2 [86]. This suggests that other positive ligands could antagonize the effect of Fru-2,6-P_2, and *sn*-glycerol 3-phosphate is a potential candidate. Indeed, *sn*-glycerol 3-phosphate, which accumulates in hepatocytes treated with ethanol, favours PFK-2 inactivation and FBPase-2 activation *in vitro*, thereby offering an explanation for the effect of ethanol to decrease Fru-2,6-P_2 concentration in hepatocytes [85].

Incubation of intact hepatocytes with glucagon results in the phosphorylation of PFK-2/FBPase-2 [87]. However, phosphorylation of the bifunctional enzyme in intact hepatocytes has not been correlated with hormone concentration, cyclic AMP concentration and activities of PFK-2/FBPase-2. Therefore, the final criterion of Krebs & Beavo [88] to definitely establish control by phosphorylation/dephosphorylation has not been satisfied. According to Nimmo & Cohen's criteria [89] it also remains to be shown that the site phosphorylated in glucagon-treated hepatocytes is the same as that phosphorylated by cyclic AMP-dependent protein kinase *in vitro*.

Liver PFK-2 has been reported to be phosphorylated and inactivated by phosphorylase kinase [66]. This effect

is probably not relevant to the situation *in vivo*, because hormones like vasopressin and α-adrenergic agents, which stimulate phosphorylase kinase by mobilizing Ca^{2+}, do not cause phosphorylation of PFK-2 in hepatocytes [90]. Moreover, unlike glucagon, these agents increase the concentration of Fru-2,6-P_2 in hepatocytes [64,91] (see below).

Protein kinase C has been shown to phosphorylate heart, but not liver, PFK-2. Phosphorylation did not affect PFK-2/FBPase-2 activity [75]. It is not known whether PFK-2 isoenzymes from other tissues are also substrates for protein kinase C. This is particularly relevant to the stimulation of glycolysis and the activation of PFK-2 that are observed after treatment of fibroblasts with phorbol esters [92], which are known activators of protein kinase C.

Reversal of phosphorylation is catalysed by phosphoprotein phosphatase(s) which, in the classification system of Cohen and co-workers [93], correspond to type 2A and 2C phosphatase [94,95].

Regulation of PFK-2/FBPase-2 activity by cyclic AMP-dependent protein kinase has not been reported for tissues other than liver. Therefore, the latter appears as the exception rather than the rule. Interestingly, PFK-2 from yeast is a substrate of cyclic AMP-dependent protein kinase which, contrary to the case in liver, activates the enzyme [96].

Relationship between glycolytic flux and Fru-2,6-P_2 concentration in liver

Three metabolic states of the liver can be considered (Table 2). In the first, gluconeogenesis occurs and glycolysis is stopped so that futile cycling of metabolites is avoided. This situation is typical of fasting, diabetes, and glucagon treatment [19,47]. Under these conditions, the concentration of Fru-2,6-P_2 is very low (below 1 μM). This can be explained by the inactivation of PFK-2 and activation of FBPase-2 resulting from phosphorylation of the enzyme by cyclic AMP-dependent protein kinase [86]. Other agents acting through cyclic AMP have the same effect. The fall in Fru-2,6-P_2 concentration leads to inhibition of PFK-1 activity and, since glucagon also casues the inactivation of pyruvate kinase [97], the glycolytic flux is effectively blocked by this dual lock [19,47]. However, when glucagon is administered to fed animals, liver glycolysis is inhibited but not completely blocked, so that cycling between glucose and triose phosphate persists [8,46,47]. This is explained by the fact that glucagon stimulates glycogen breakdown, thereby producing a massive increase in hexose 6-phosphate concentration [98] which antagonizes the glucagon-induced inactivation of PFK-2 and activation of FBPase-2.

A decrease in Fru-2,6-P_2 seems to be required for the glucagon stimulation of gluconeogenesis from substrates entering at the level of triose phosphates. By contrast, with substrates entering at the level of pyruvate, the inactivation of pyruvate kinase by glucagon appears to be the major regulatory component [99]. The latter conclusion is in agreement with the results of the quantitative analysis of control performed by Groen and co-workers [100].

A fall in Fru-2,6-P_2 concentration is also obtained in regenerating liver (L. Hue, unpublished work) or in hepatocytes incubated with adenosine [101], ethanol [85], fatty acids (L. Hue, L. Maisin & M. H. Rider, unpublished work), or relatively large concentration of gluconeogenic precursors such as lactate, pyruvate, alanine or fructose [99,102]. The mechanisms responsible for the fall in Fru-2,6-P_2 under these conditions may involve changes in the activities of PFK-2/FBPase-2 induced by variations in the concentration of metabolites. Phosphoenolpyruvate, citrate and mainly *sn*-glycerol 3-phosphate are important in this respect, since they are known to exert a dual inhibitory action on PFK-2. Indeed, they are not only inhibitors of PFK-2 activity but they also promote the cyclic AMP-dependent phosphorylation of PFK-2/FBPase-2 [77,85].

The second metabolic state corresponds to the anabolic fed state when glucose is abundant as, e.g., after a carbohydrate meal. Under this condition the liver takes up glucose, some being stored as glycogen, while the rest is transformed into lactate and fatty acids. Glucose by itself is able to control both the synthesis of glycogen and glycolysis; it is not known whether it can also monitor the synthesis *de novo* of fatty acids. The stimulation of glycolysis by glucose can be explained by the increase in Fru-2,6-P_2 concentration [8,64]. Glycogen metabolism is also glucose-sensitive, with phosphorylase acting as a glucose sensor [103,104]. It appears that the glucose sensitivity of the liver for glycolysis is not the same as that for glycogen synthesis. In fasted animals, the activation of glycogen synthase requires less glucose than does the stimulation of glycolysis. The opposite situation is observed in livers of fed animals [78]. The physiological meaning of this observation is that in the fasted state, the glycogen stores are first replenished (from glucose or from gluconeogenic precursors), and it is only when an excess of glucose is given that gluconeogenesis is inhibited and lactate produced. This difference in sensitivity should be taken into consideration when discussing 'the glucose paradox' [105], namely that after glucose administration to fasted rats, gluconeogenesis persists and liver glycogen is synthesized mainly from gluconeogenic precursors [106–109].

An increase in liver Fru-2,6-P_2 is not only obtained after a glucose load in normal animals. It is also observed even during fasting in livers of genetically obese mice and rats [110–112]. This high glycolysis may contribute to obesity by keeping lipogenesis active even in the fasted state.

Studies with isolated hepatocytes from normal rats have shown that the relationship between Fru-2,6-P_2 concentration and lactate release holds only at relatively high Fru-2,6-P_2 concentration (above 5 nmol/g) [78]. This lack of correlation could result from the recycling of metabolites (PFK-1 and FBPase-1 both active and no net lactate production) which is actually observed [8]. It can also result from binding of Fru-2,6-P_2 to proteins. From the maximal binding capacity of liver cytosolic proteins one can calculate that, at 5 μM, more than 90% of Fru-2,6-P_2 is indeed sequestered to proteins [78]. Therefore the metabolic significance of Fru-2,6-P_2 is best expressed at relatively high concentrations when it allows the liver to switch from gluconeogenesis to glycolysis and vice versa.

An increase in Fru-2,6-P_2 is also observed in isolated hepatocytes incubated with low concentrations (below 5 mM) of gluconeogenic precursors such as fructose, dihydroxyacetone, lactate or pyruvate [99]. Similarly, incubation of hepatocytes from fed rats with vasopressin

or α-adrenergic agonists increase Fru-2,6-P_2 [64,91,113]. These agents promote glycogen breakdown by a mechanism independent of cyclic AMP. Glycogen breakdown leads to an increase in Fru-6-*P* and in Fru-2,6-P_2 concentration because the accumulation of hexose 6-phosphates is not counteracted, as is the case with glucagon, by an inactivation of PFK-2 and an activation of FBPase-2. Since vasopressin and α-agonists have little effect on pyruvate kinase [114–117] and since they stimulate pyruvate dehydrogenase [118,119], the increase in lactate output is less than expected from the changes in Fru-2,6-P_2.

In isolated hepatocytes, the mechanism responsible for the increase in Fru-2,6-P_2 is the increased supply of Fru-6-*P* either from glycogen, glucose or gluconeogenic precursors. The same mechanism applies in the livers of anaesthetized rats. In livers of conscious animals, however, the glucose-induced increase in Fru-2,6-P_2 concentration is not accompanied by an increase in hexose 6-phosphate concentration [78,98,120] or a stable change in the activity of PFK-2 [78]. The mechanism involved is unknown but one cannot exclude that an increase in Fru-6-*P* could have been masked artifactually. Indeed, in the time interval between killing of the animal and sampling of the liver, the hexose 6-phosphate pool may become depleted as a result of stimulation of glycolysis.

In the third situation, liver glycolysis is stimulated by anoxic conditions. This emergency situation probably does not occur under physiological conditions but can be readily obtained in isolated liver preparations. Under these conditions the concentration of Fru-2,6-P_2 remains low and therefore is not responsible for this burst of glycolytic activity [121]. The fall in ATP concentration and the increase in ADP and *sn*-glycerol 3-phosphate which are observed during anoxia may all contribute to decrease PFK-2 activity and hence Fru-2,6-P_2 concentration. By contrast with liver, hypoxic or anoxic conditions can occur in muscle under physiological conditions. As for the anoxic liver, it appears that the major stimulation of glycolysis in muscle during tetanic contraction is not due to an increase in Fru-2,6-P_2 concentrations [28,102].

Relationship between glycolytic flux and Fru-2,6-P_2 concentration in extra-hepatic tissues

Fru-2,6-P_2 has been detected in all mammalian tissues including red blood cells. Although a previous study failed to detect Fru-2,6-P_2 in erythrocytes [122], more recent studies [122a] established that 0.02–0.3 nmol of Fru-2,6-P_2/g could be detected depending on the conditions and that the previous failure could be explained by the presence of glycerate 2,3-bisphosphate, which inhibits both PFK-2 and the enzyme used to measure Fru-2,6-P_2 in extracts.

To identify the conditions in which Fru-2,6-P_2 could exert a control on glycolysis, the simplest approach is to search for conditions or treatments that change both glycolysis and Fru-2,6-P_2 concentration in the same direction. Table 3 summarizes data taken from the literature. In general, the rate of glycolysis was regulated by substrate supply (glucose), energy demand (e.g. anoxia), or hormones (e.g. adrenaline, insulin). There was a parallelism between the glucose-dependent increase in glycolysis and in Fru-2,6-P_2 in all tissues studied except in cultured astrocytes [123] and sperm cells [124]. Such a parallelism did not exist when glycolysis was stimulated by anoxia. Therefore, when energy provision by glycolysis becomes a major issue for the cell, the predominant control is probably exerted by adenine nucleotides, P_i and citrate rather than by Fru-2,6-P_2.

On the other hand, during aerobiosis, Fru-2,6-P_2 could play a regulatory role in the glucose-sparing effect of fatty acids and ketone bodies (the so-called glucose/fatty acids cycle). Indeed, the administration of fatty acids to hepatocytes or ketone bodies to perfused hearts decreases the glycolytic rate and the concentration of Fru-2,6-P_2 in parallel (L. Hue, L. Maisin & M. H. Rider, unpublished work). The latter is probably caused by an increased citrate concentration which inhibits PFK-2. Therefore, during aerobiosis, Fru-2,6-P_2 could be regarded as a glycolytic signal which is turned on by glucose availability and switched off by the presence of alternative fuels.

Hormones such as insulin and adrenaline increased both glycolysis and Fru-2,6-P_2 in skeletal muscle and heart [45,102,125,126]. This relationship does not mean that Fru-2,6-P_2 is the sole mechanism of control. More likely, the increase in Fru-2,6-P_2 is part of a concerted mechanism involving the stimulation of various glycolytic steps such as glucose transport, hexokinase, PFK-1, pyruvate kinase and pyruvate dehydrogenase. The description of the specific effects of adrenaline and insulin on these steps is outside the scope of this review.

Besides these general mechanisms of control, there are some tissue-specific regulations. In brain, renal medulla, tetanic muscle, erythrocytes and sperm cells, glycolysis is the sole, or at least the major, mechanism for energy provision [2]. In red blood cells [122a], sperm cells [124] and during tetanus in muscle [28,102], Fru-2,6-P_2 does not seem to be involved in glycolytic control because its concentration is either too low or unrelated to changes in the glycolytic rate. For brain and kidney, the limited information available does not allow any conclusion about the role of Fru-2,6-P_2 to be drawn. Interestingly, Fru-2,6-P_2 concentration in brain does not decrease upon fasting [15].

Fru-2,6-P_2 is expected to be the regulator *par excellence* when glycolysis serves an anabolic role since it is able to relieve the inhibition of PFK-1 by ATP. As discussed above, this is probably the case for the liver where lipogenesis is associated with glycolysis and high concentration of Fru-2,6-P_2. The same causal relationship probably holds true in mammary glands where the concentration of Fru-2,6-P_2 increases 3-fold during lactation [127]. For white adipose tissue, however, the situation is not clear since insulin, which is known to stimulate both glycolysis and lipogenesis, has been shown to decrease Fru-2,6-P_2 concentration [128] (see however [129] and below).

In all tissues, as in the liver, control of Fru-2,6-P_2 concentration depends on the relative activity of PFK-2/FBPase-2. A rise in Fru-6-*P* concentration favours the synthesis of Fru-2,6-P_2 provided that FBPase-2 is inhibited. The latter restriction might not hold for all tissues. Indeed, the K_i of FBPase-2 for Fru-6-*P* is relatively high in skeletal muscle [63,77]. This could also be the case for sperm cells and astrocytes and could explain why glucose does not increase Fru-2,6-P_2 concentration.

Regulation of PFK-2/FBPase-2 activity by cyclic

Table 3. Relationship between concentration of Fru-2,6-P_2 and glycolysis in various extrahepatic tissues

The values of Fru-2,6-P_2 content are the extremes recorded. The conditions that are underlined are those in which there is no relationship between Fru-2,6-P_2 and glycolysis. I, increase; D, decrease; 0, no change

Tissue and Fru-2,6-P_2 content (nmol/g wet wt.)	Conditions	Glycolysis	Fru-2,6-P_2	Reference
Skeletal muscle (0.2–0.8)	Adrenaline	I	I	45,102
	Insulin	I	I	45,102
	Low frequency electrical stimulation	0	I	28
	Tetanus	I	D	28,102
Heart (0.7–2)	Glucose	I	I	126
	Adrenaline	I	I	130, but 102,175
	Insulin	I	I	125
	Alloxan diabetes	D	D	176
	Anoxia	I	D	121
White adipose tissue (0.1–1)	Glucose, fructose	I	I	128
	Adrenaline	I	0 or D	128,129
	Insulin	I	D or I	128,129
	Alloxan-diabetes, starvation	D	D	129,176
Kidney (0.2–1)	Alloxan diabetes	I or 0	I	176
Adrenals (0.5–2)	Alloxan-diabetes	D	D	177
Brain (2–10)	Starvation	?	0	15
Cultured astrocytes (30)	Glucose	I	0	123
	Anoxia	I	0	123
Cultured neuroblastoma cells (4–10)	Glucose	I	I	123
	Anoxia	I	D	123
Enterocytes (0.5–1.2)	Glucose	I	I	178, see however 179
	Starvation	D	D	
Fibroblasts (3–8)	Glucose	I	I	146
	Insulin, EGF	I	I	92,144,145
	Confluency	D	D	180
Lens (1)	Alloxan-diabetes	0	0	176
Mammary gland (1–4)	Lactation	I	I	127
Pancreatic islets (1–20)	Glucose	I	I	181,182
Red blood cells (0.02–0.3)	pH 8	I	0 or D	122a
Sperm cells, testis (1–2)	Glucose	I	0	124
	Caffeine	I	0	124
	Alloxan-diabetes	D	D	176

AMP-dependent protein kinase seems to be restricted to the liver enzyme and therefore cannot explain the stimulatory effect of adrenaline on Fru-2,6-P_2 in muscle and heart. Alternatively, it is proposed that this effect of adrenaline on Fru-2,6-P_2 concentration [102,130] results from a stimulation of glycogen breakdown and an increased Fru-6-P concentration. Similarly, insulin increases Fru-6-P concentration by stimulating glucose transport. However, this increase is too small to explain the changes in Fru-2,6-P_2 concentration in muscle and heart [45,102,125]. The changes in Fru-2,6-P_2 brought about by insulin can be explained by an activation of PFK-2 (increase in $V_{max.}$, without change in K_m) that has been indeed observed in heart [125]. This insulin-induced activation of PFK-2 persists after partial purification of the enzyme, consistent with the possibility of covalent modification. Phosphorylation by cyclic AMP-dependent protein kinase or protein kinase C has been excluded and the mechanism responsible for the modification has still to be elucidated. A similar phenomenon may exist in skeletal muscle but has not yet been demonstrated. It would also be interesting to know the effect of insulin on the activity of FBPase-2, particularly in skeletal muscle, where the FBPase-2 activity is 10 times that of the kinase.

There is a discrepancy in the literature concerning the hormonal regulation of Fru-2,6-P_2 concentration in white adipose tissue. While adrenaline treatment was reported to decrease and insulin to increase Fru-2,6-P_2 concentration in epididymal fat pads [129], noradrenaline had little effect and insulin decreased Fru-2,6-P_2 in isolated adipocytes [128]. The reasons for this discrepancy are unknown. On the other hand, it is clear that adrenaline causes stable changes in PFK-1 activity (an increased apparent affinity for Fru-6-P and a small decrease in $V_{max.}$ [131,132]). Since these changes could not be interpreted in terms of Fru-2,6-P_2, it has been proposed that they could result from alterations in the concentration of other PFK-1 ligands, namely fatty acids [132]. Alternatively, these changes could be caused by phosphorylation of PFK-1 by cyclic AMP-dependent protein kinase. The latter has been demonstrated both *in vitro* and *in vivo* [36,133]. The phosphorylated enzyme is

less inhibited by ATP, more sensitive to stimulation by Fru-2,6-P_2, and thus is more active.

Fru-2,6-P_2 in tumour and fast-growing cells

Unlike normal cells, many tumour and fast-growing cells maintain a high glycolytic rate even under aerobic conditions [134]. The biochemical mechanism of this phenomenon, known for 60 years, is not yet fully understood. The difference can be accounted for, at least in part, by qualitative and quantitative changes in enzyme activity and hexose transport [135,136]. Recent work has shown that, on top of the differences already described, the Fru-2,6-P_2/PFK-2 system might play an important role in the maintenance of a high glycolytic rate in these cells. The evidence is based on the study of established lines of tumour cells and, in particular, on the study of the effect of growth factors, tumour promoters like phorbol esters, and oncogenic viruses on glycolysis in fibroblasts.

In established lines of cancer cells, the concentration of Fru-2,6-P_2 is either the same (rat hepatoma HTC cells [137], Ehrlich ascites tumour cells [138]) or higher (HeLa cells [139], adenocarcinoma [140]) than in the corresponding normal tissues. In HTC cells, the properties of PFK-2 are modified in such a way that the enzyme has lost the characteristic properties of the original liver type [141].

In quiescent fibroblasts stimulated by high mitogenic concentrations of insulin, growth factors or phorbol esters, there is a stimulation of glycolysis and a concomitant increase in Fru-2,6-P_2 concentration, activation of PFK-2 and stimulation of glucose transport [92,142–145].

A similar situation is obtained when chick embryo fibroblasts are transformed by retroviruses carrying either the v-*src* or v-*fps*, but not the v-*mil* and/or v-*myc*, oncogenes [146]. The glycolytic and Fru-2,6-P_2/PFK-2 changes induced by the Rous sarcoma virus carrying the v-*src* oncogene depend on the tyrosine-specific protein kinase activity of pp60v-*src*, the oncogene product. The comparison of the effects of v-*src* with those of phorbol esters on Fru-2,6-P_2 concentration suggests that both result from the stimulation of a step which is controlling PFK-2 activation and which is also controlled by protein kinase C. The time-course of the changes in PFK-2 activity argue against a direct activation of PFK-2 by protein kinase C or pp60v-*src*. The exact mechanism is not known.

The changes in the PFK-2/Fru-2,6-P_2 system occurring in transformed fibroblasts are not the only determinants of the 'glycolytic phenotype', because glucose transport is also stimulated [147]. The relative contribution of these two phenomena to the overall control of glycolysis is unknown. Nevertheless, a characteristic and intriguing feature of proliferating cells is their relatively high Fru-2,6-P_2 content. Similar changes in Fru-2,6-P_2 concentrations have also been observed during resumption of metabolic activities in plants [148] and in spores [149]. Moreover, the highest concentrations of Fru-2,6-P_2 ever recorded are found in fibroblasts treated with phorbol esters or transformed by v-*src*. These concentrations (more than 50 nmol/g [146]) are more than 10 times higher than that required for near-maximal stimulation of PFK-1. Therefore it is tempting to speculate that Fru-2,6-P_2 could either influence metabolic pathways other than glycolysis or be an intermediate in some other reactions (e.g. phosphorylation or osylation). It is not known whether these huge increases in Fru-2,6-P_2 concentration are required to keep the cells in a proliferative phase.

Metabolic detours from the glycolytic pathway

As discussed in a previous review [22], the similarity between Fru-2,6-P_2 and some other bisphosphoesters related to the glycolytic pathway is striking. Like Fru-2,6-P_2, glucose 1,6-bisphosphate and glycerate 2,3-bisphosphate are not true intermediates of glycolysis and their metabolisms involve detours from the glycolytic pathway. Neither are they direct precursors of the pathway. They are synthesized from glycolytic intermediates which are either substrates or products of the reactions they control. They all act as cosubstrates or effectors of reactions in the pathway.

The comparison can be extended to PFK-2/FBPase-2 and the glucose bisphosphatase family of enzymes [150]. These enzymes catalyse phosphoryl transfer reactions involving the formation of a phosphorylated enzyme intermediate from the bisphosphates. Similarly, the synthesis and degradation of glycerate 2,3-bisphosphate is catalysed by a multifunctional enzyme and involves the intermediate formation of a phosphorylated histidyl residue [151]. We tend to believe that this striking analogy is not fortuitous. It may indicate that metabolic detours are a general mechanism developed by the cell to synthesize effectors which control metabolic pathways. It also suggests that other enzymes could exist which use Fru-2,6-P_2 as a phosphoryl donor. In this context, it is worth mentioning that the mechanism of ribose 1,5-bisphosphate synthesis is not yet elucidated and that the understanding of glucose 1,6-bisphosphate hydrolysis in several tissues is far from complete [150].

Conclusion

The main role of Fru-2,6-P_2 is to control PFK-1 activity and hence glycolysis. Therefore, it is a glycolytic signal. It behaves as an integrator of information from various metabolisms on a single point of control, PFK-1. Cyclic AMP has the opposite effect by extending and spreading information from one molecule to various metabolisms. The difference in behaviour stems from the remarkable properties of cyclic AMP-dependent protein kinase, which is a multisubstrate enzyme, whereas PFK-2 and FBPase-2 are multimodulated enzymes and, thus, can act as metabolic integrators. Yet another difference is the hierarchy between the two signals: Fru-2,6-P_2 is indeed controlled by cyclic AMP but cannot control cyclic AMP. Therefore, Fru-2,6-P_2 can be regarded as a third messenger of hormone action.

Although Fru-2,6-P_2 is present in all tissues studied so far, it can not be regarded as *the* ubiquitous regulator of glycolysis under all conditions. It is suggested that its role is related to the metabolic significance of glycolysis which is an amphibolic pathway. Fru-2,6-P_2 probably plays no role in tissues that depend exclusively on glycolysis for their energy supply. In tissues that can use alternative fuels, a fall in Fru-2,6-P_2 concentration could signal the presence of non-glycolytic substrates and, by doing so, it can contribute to the glucose-sparing effect of these substrates. In skeletal muscle and heart, Fru-2,6-P_2 is also involved in the control of glycolysis by adrenaline and insulin. In liver, it is a major control element for switching from glycolysis to gluconeogenesis. In this

tissue, it is also probably important in the control of substrate provision for lipogenesis. Finally, one can speculate that the highest concentrations of Fru-2,6-P_2 which are found in transformed cells could not only keep PFK-1 and glycolysis active but also interfere with other pathways essential for cell proliferation.

We thank Professor G. G. Rousseau for his advice and criticisms, and M. Marchand and Th. Lambert for secretarial help. L.H. is Maître de Recherches of the Fonds National de la Recherche Scientifique (Belgium) and M.H.R. is a fellow of the International Institute of Cellular and Molecular Pathology. This work was supported in part by grants from the Caisse Générale d'Epargne et de Retraite (Belgium) and from the Fonds de la Recherche Scientifique Médicale (Belgium).

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