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REVIEW ARTICLE

Fructose 2,6-bisphosphate 2 years after its discovery

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Introduction

It was in 1909 that Young, in the laboratory of Harden, isolated from fermenting yeast the fructose bisphosphate (the Harden and Young ester) that was later identified by Levene & Raymond (1928) as fructose 1,6-bisphosphate. Some 71 years elapsed until a second fructose bisphosphate was isolated from living cells and identified as fructose 2,6-bisphosphate (Van Schaftingen *et al.*, 1980*c*). In contrast with its elder brother, Fru-2,6-P_2 is not an intermediary metabolite of glycolysis, but its most potent regulator. In the present review, we will summarize the work that led to its discovery and we will try to delineate its potential role in the control of metabolism in various organisms.

The discovery of Fru-2,6-P_2

The effect of glucagon on liver PFK

In 1979, four groups of investigators (Castano *et al.*, 1979; Clarke *et al.*, 1979; Kagimoto & Uyeda, 1979; Pilkis *et al.*, 1979) reported that the kinetic properties of PFK, measured in extracts of hepatocytes, were modified after incubation of the cells in the presence of glucagon. The hormonal action was to decrease the affinity of the enzyme for Fru-6-*P* and to increase the inhibition by ATP. These effects were reported by the first and the third groups of investigators to be stable upon gel filtration and purification of the enzymic preparation. They were believed to be the consequence of a phosphorylation of PFK by cyclic AMP-dependent protein kinase (Castano *et al.*, 1979; Kagimoto & Uyeda, 1979, 1980; Claus *et al.*, 1980*a*). In contrast, when the same problem was re-investigated in this laboratory (Van Schaftingen *et al.*, 1980*b*), it was found that the difference between PFK from control or from glucagon-treated hepatocytes disappeared upon gel filtration or purification of the enzyme. Furthermore, when a low-molecular-weight fraction, obtained by ultrafiltration, gel filtration or heat extraction of a control liver extract was added to the gel-filtered extract, it caused a remarkable stimulation of PFK when measured at low Fru-6-*P* concentration. The conclusion that the effect of glucagon was to remove a low-molecular-weight positive effector of PFK was first announced at the Symposium on Biological Cycles held in Dallas in the honour of Sir Hans Krebs' 80th birthday (Hers *et al.*, 1981*a*).

The explanation for the discrepancy between our data and those reported by other groups is not obvious. One possibility is that Castano *et al.* (1979) copurified PFK 2 (see below) with PFK 1 and that Fru-2,6-P_2 was formed from Fru-6-*P* and ATP, which were present in the elution mixture of the chromatographic step. Kagimoto & Uyeda (1980) perfused rat livers with [^{32}P]P_i and correlated the incorporation of ^{32}P into PFK with the kinetic changes measured in a crude $(NH_4)_2SO_4$ precipitate which, retrospectively, was likely to be contaminated with Fru-2,6-P_2.

Identification of the stimulator of PFK as Fru-2,6-P_2

The stimulator of PFK was identified as Fru-2,6-P_2 on the basis of the following observations: (1) gel filtration revealed that the molecular weight of the stimulator was very close to that of Fru-1,6-P_2; (2) the stimulator was destroyed within a few minutes in ice-cold diluted acid; (3) it was also destroyed by alkaline phosphatase and was barium-insoluble; (4) it was retained on anion-exchange columns and its elution pattern was similar to that of Fru-1,6-P_2; (5) it was not adsorbed on charcoal. All these properties indicated that the stimulator was an acid-labile, non-nucleotidic diphosphoric ester with a molecular weight similar to that of Fru-1,6-P_2 (Van Schaftingen *et al.*, 1980*b*).

The purification of the stimulator was achieved through the classical procedure described for the

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

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isolation of other sugar phosphates. Its structure was then revealed by analysis of the products formed during its acid hydrolysis. It was shown that, upon incubation at 0°C in the presence of 0.01 M-HCl, there was a strict parallelism between the disappearance of that stimulator and the formation of Fru-6-*P* in amounts equimolar to that of acid-labile phosphate present in the preparation. Furthermore, a reducing group was also revealed, which was therefore hidden in the native structure (Fig. 1). These data indicated that Fru-6-*P* was a component of the stimulator and was linked to phosphate by its anomeric carbon atom (Van Schaftingen *et al.*, 1980*c*). The identification of the stimulator as Fru-2,6-P_2 was, however, reported to be tentative because, strictly speaking, one could not rule out the possibility that another chemical group, which had not been assayed, was a constituent of the stimulator. This possibility was, however, soon discarded

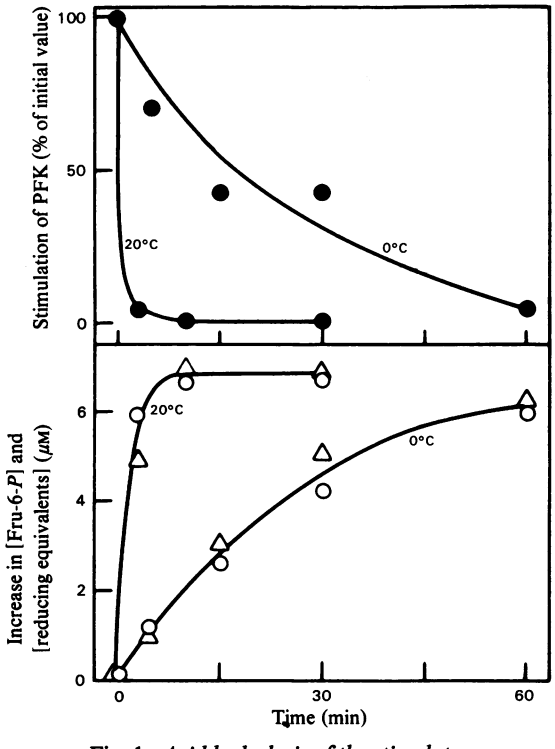

Fig. 1. *Acid hydrolysis of the stimulator*
The stimulator was incubated in the presence of 0.01 M-HCl either at 0°C or at 20°C. One reducing equivalent corresponds to the reducing power of one mol of Fru-6-*P*. At time zero, Fru-6-*P* was not detectable and the reducing equivalents were 3.5 μM. ○, Fru-6-*P*; △, reducing equivalents. (From Van Schaftingen *et al.*, 1980*c*).

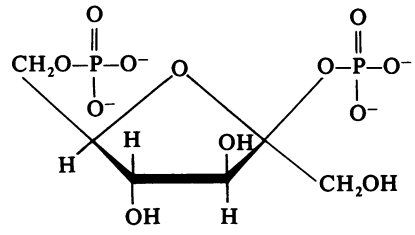

Fig. 2. *β-Fructose 2,6-bisphosphate*

when it was shown that a stimulator having all the properties of the natural one was formed upon incubation of Fru-6-*P* in the presence of concentrated phosphoric acid at 0°C, allowing the conclusion that no compounds other than Fru-6-*P* and phosphate enter into its structure (Van Schaftingen & Hers, 1980). Furthermore, the fact that the synthetic stimulator had exactly the same specific activity as the natural one ($K_a = 10^{-7}$ M) indicated that only one anomeric form was present. Knowing that natural fructosides are β-anomers (Pontis & Fischer, 1963), the most likely structure of the stimulator of PFK was therefore the β-fructofuranoside 2,6-bisphosphate shown in Fig. 2. (For a review, see Hers *et al.*, 1981*b*.)

Further evidence for an 'activation factor' of liver PFK

Important contributions along the line of research described above have also been made by other groups of investigators. Uyeda and his coworkers investigated the effect of purification and gel filtration on liver PFK and recognized the existence of an 'activation factor' which could be resolved by gel filtration into three components of different molecular weight; the second and predominant fraction had a molecular weight of 3000–4000 and appeared to be a polysaccharide (Furuya & Uyeda, 1980*a*). In a more recent publication, Uyeda *et al.* (1981*b*) stated that their factor was actually Fru-2,6-P_2 and that the discrepancy in molecular weight was due to an apparent anomalous behaviour of highly charged molecules on Sephadex chromatography.

Claus *et al.* (1980*b*) have reached the important conclusion that phosphorylation of PFK by cyclic AMP-dependent protein kinase had no effect on its activity. Furthermore, they obtained results which suggested that the inhibition of PFK by glucagon is due to changes in the level of (an) allosteric effector(s) which could be either a stimulator or an inhibitor. In a subsequent publication (Claus *et al.*, 1981*a*), the same group of investigators recognized

that the effector was a stimulator different from AMP and Fru-1,6-P_2.

The formation of Fru-2,6-P_2 from Fru-1,6-P_2 and n.m.r. analysis

A simple procedure for the conversion of Fru-1-*P* into Fru-2-*P* had been described by Pontis & Fischer (1963). It includes treatment of Fru-1-*P* with dicyclohexylcarbodi-imide in aqueous pyridine with formation of fructose cyclic 1,2-monophosphate, which can then be converted by alkaline hydrolysis to Fru-2-*P* in addition to Fru-1-*P*. This procedure was successfully adapted by three groups of investigators (Pilkis *et al.*, 1981*a*; Van Schaftingen & Hers, 1981*a*; Uyeda *et al.*, 1981*b*) for the conversion of Fru-1,6-P_2 into Fru-2,6-P_2 when the biological interest of the latter compound became obvious. Again, the synthetic compound had a stimulatory power equal to that of the natural one indicating that only one anomer was formed. N.m.r. analysis revealed that this anomer had the β configuration (Pilkis *et al.*, 1981*a*; Hesbain-Frisque *et al.*, 1981; Uyeda *et al.*, 1981*b*).

Concentration of Fru-2,6-P_2 in cells under various experimental conditions

Fru-2,6-P_2 can be assayed by its stimulatory action on mammalian PFK (Van Schaftingen *et al.*, 1980*b*) or on plant PP_i: fructose 6-phosphate 1-phosphotransferase (Sabularse & Anderson, 1981*b*). Alternatively, it can be measured, after separation from Fru-6-*P*, by the amount of Fru-6-*P* formed upon acid hydrolysis (Hue *et al.*, 1982).

Mammalian cells

The concentration of Fru-2,6-P_2 in the liver cell is in the micromolar range. In isolated hepatocytes from starved rats, this concentration is greatly increased upon incubation in the presence of large concentrations of glucose and is then rapidly decreased upon addition of glucagon (Van Schaftingen *et al.*, 1980*b*; Richards *et al.*, 1981; Hue *et al.*, 1981*b*). A similar effect of glucose and of glucagon in affecting the concentration of an unidentified activator of liver PFK (presumably Fru-2,6-P_2) has also been reported by Richards & Uyeda (1980) and by Claus *et al.* (1981*a*). Vasopressin, phenylephrine and ionophore A23187 can also increase the concentration of Fru-2,6-P_2 in isolated hepatocytes, but only when glycogen is present, and the effect of these agents could be correlated with their ability to stimulate glycogenolysis and to increase the concentration of Fru-6-*P* (Hue *et al.*, 1981*a*,*b*). The concentration of Fru-2,6-P_2 was low in the liver of diabetic rats (Neely *et al.*, 1981), a situation that is in agreement with the known high level of glucagon in the blood of these animals. Anoxia decreased the concentration of Fru-2,6-P_2 in isolated hepatocytes (Hue, 1982).

Fru-2,6-P_2 is also present at micromolar concentrations in skeletal muscle, heart and brain of the rat. In perfused rat hindlimb muscle, insulin and adrenaline respectively caused a 2- and 4-fold increase in Fru-2,6-P_2 and a stimulation of glycolysis. Electrical stimulation caused a 2.5-fold increase in lactate production, but decreased Fru-2,6-P_2. It also abolished the increase in this metabolite induced by adrenaline (Hue *et al.*, 1982). Fru-2,6-P_2 has also been detected in rat pancreatic islets and its concentration was reported to be higher in islets exposed to glucose than in islets deprived of glucose (Malaisse *et al.*, 1982*a*).

Yeast and plants

Fru-2,6-P_2 was found to be present at micromolar concentrations in *Saccharomyces cerevisiae* grown on glucose, but not in the same organism grown on glycerol. When *S. cerevisiae* had been deprived of glucose, Fru-2,6-P_2 was formed within 1 min after the addition of glucose to the culture (Lederer *et al.*, 1981). Fru-2,6-P_2 has been isolated from mung beans (Sabularse & Anderson, 1981*b*) and from spinach leaves (unpublished work from this laboratory).

Biosynthesis and biodegradation of Fru-2,6-P_2 and their control by hormones and metabolites

From the time-course of the effect of glucose and of glucagon on the concentration of Fru-2,6-P_2 in isolated hepatocytes (Van Schaftingen *et al.*, 1980*b*) one can estimate that the rate of synthesis and of degradation of Fru-2,6-P_2 in the liver is of the order of a few nmol/min per g of liver. The expected activity of the enzymes responsible for this metabolic conversion is therefore several orders of magnitude below that of most liver enzymes; their very low activity was a major hindrance in their characterization.

Liver phosphofructokinase 2

Four groups of investigators (Furuya & Uyeda, 1981; El-Maghrabi *et al.*, 1981; Van Schaftingen & Hers, 1981*c*; Hue *et al.*, 1981*b*) have independently observed that Fru-2,6-P_2 can be formed from Fru-6-*P* and ATP by a liver extract or by a partially purified preparation. This formation of Fru-2,6-P_2 could occur by various mechanisms, including the direct transfer of the γ-phosphoryl group of ATP onto the hydroxy group present on the anomeric carbon atom of Fru-6-*P*. Proof that the latter mechanism was actually operating was given by Van

Schaftingen & Hers (1981*c*), who demonstrated that, at all stages of the conversion, there was a stoichiometric amount of Fru-2,6-P_2 and of ADP formed for each Fru-6-*P* and ATP utilized. Furthermore, when [γ-^{32}P]ATP was used as substrate, [2-^{32}P]Fru-2,6-P_2 with the same specific activity as the precursor was formed. It could therefore be concluded that Fru-2,6-P_2 is formed in the liver by the following reaction:

$$\text{ATP} + \text{Fru-6-}P \rightarrow \text{ADP} + \text{Fru-2,6-}P_2$$

The enzyme catalysing this reaction is a 6-phosphofructo-2-kinase, which could be separated from the classical 6-phosphofructo-1-kinase by various means. It was called PFK 2 in order to distinguish it from the classical PFK, which should now be called PFK 1. This nomenclature takes into account both the order of discovery of the two enzymes and the carbon atom of Fru-6-*P* that is phosphorylated.

The apparent K_m values of PFK 2 for Fru-6-*P* and for ATP are approximately 0.4 and 0.5mM respectively. P_i and AMP, at physiological concentrations, increase the rate of reaction, whereas both phosphoenolpyruvate and citrate are inhibitory in the presence and in the absence of P_i. One major effect of P_i at physiological concentrations was to decrease to 50μM the K_m for Fru-6-*P* (Van Schaftingen & Hers, 1981*c*). Since the concentration of Fru-6-*P* in the liver is in that range, it might be expected to play a role in the control of the biosynthesis of Fru-2,6-P_2 in that tissue. Accordingly, Hue *et al.* (1981*b*) have observed a correlation between the concentrations of hexose-6-*P* and of Fru-2,6-P_2 in the liver under various experimental conditions, but not in the presence of glucagon.

Liver fructose-2,6-bisphosphatase

The purification and characterization of fructose-2,6-bisphosphatase was made possible when it was recognized that the enzyme is stimulated both by triphosphonucleotides and by α- or β-glycerophosphate (Van Schaftingen *et al.*, 1982). When incubated in the presence of these activators, a liver high speed supernatant caused the disappearance of Fru-2,6-P_2 at a rate of a few nmol/min per g of liver. The purified enzyme catalysed the stoichiometric conversion of Fru-2,6-P_2 into Fru-6-*P* and P_i; it is therefore a fructose-2,6-bisphosphatase which, by analogy with PFK 2, is now referred to as FBPase 2, whereas fructose 1,6-bisphosphatase becomes FBPase 1. Its K_m for Fru-2,6-P_2 is close to 10^{-7}M. It is strongly inhibited by Fru-6-*P*, the product of the reaction, of which the main effect is to decrease greatly the $V_{max.}$. Unlike FBPase 1, FBPase 2 is not Mg^{2+}-dependent is not inhibited by AMP and not stimulated by EDTA.

The effect of cyclic AMP-dependent protein kinase

As expected from the known action of glucagon to cause the disappearance of Fru-2,6-P_2, incubation of hepatocytes in the presence of the hormone decreased the activity of the enzyme that forms Fru-2,6-P_2 from Fru-6-*P* and ATP (Van Schaftingen & Hers, 1981*c*; Hue *et al.*, 1981*b*) and increased the activity of fructose 2,6-bisphosphatase (Van Schaftingen *et al.*, 1982) and these changes persisted after gel filtration. Hue *et al.* (1981*b*) obtained these modifications in the synthesis of Fru-2,6-P_2 with the same concentrations of glucagon that caused the activation of phosphorylase. In contrast, Richards *et al.* (1981) observed a similar effect at extremely low concentrations of glucagon (10^{-13}M) and concluded that at least part of the hormonal effect was not mediated by cyclic AMP.

Incubation of the purified PFK 2 (Van Schaftingen *et al.*, 1981*b*) or FBPase 2 (Van Schaftingen *et al.*, 1982) with ATP and the catalytic subunit of protein kinase from beef heart caused several changes in the kinetic properties of these enzymes. PFK 2 became less sensitive to its substrate, Fru-6-*P*, and to its positive effector, P_i, and more sensitive to its negative effectors, citrate and phosphoenolpyruvate. The decreased affinity for Fru-6-*P* would be compensated by the increased concentration of this substrate caused in the fed animal by glycogenolysis induced by glucagon, and the result would be little change in the activity of PFK 2; in contrast, the greater sensitivity to the inhibitory action of phosphoenolpyruvate would be reinforced by the increased concentration of that metabolite caused by glucagon, allowing therefore a rather large inhibition of PFK 2. The main change in the properties of FBPase 2 was an increase in $V_{max.}$, which was particularly apparent in the presence of ATP.

An overview

By its action on both PFK 1 and FBPase 1 (see the next section), Fru-2,6-P_2 controls the turning point of glycolysis and gluconeogenesis between the hexose monophosphates and Fru-1,6-P_2, which is itself in equilibrium with the triose phosphates and other C-3 derivatives. Its concentration in the liver integrates a series of effects summarized in Fig. 3, which affect either PFK 2 or FBPase 2 or both. The formation of Fru-2,6-P_2 is favoured by Fru-6-*P*, the substrate of PFK 2 and a potent inhibitor of FBPase 2. Because of its very low K_m, the latter enzyme is indeed regulated much more by product inhibition than by substrate concentration. AMP also favours the formation of Fru-2,6-P_2 and, in doing so, reinforces its positive effect on PFK 1 and negative effect on FBPase 1. Glucagon is the major effector which, by its symmetrical effect on PFK 2 and on FBPase 2,

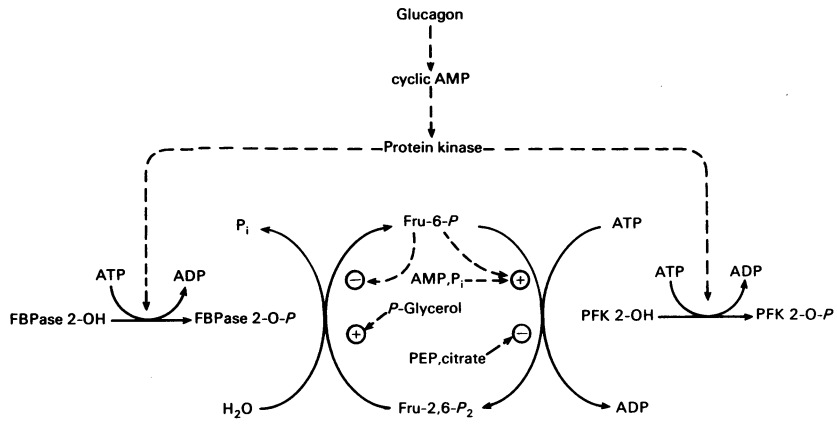

Fig. 3. *Biosynthesis and biodegradation of Fru-2,6-P_2 in the liver and their control by glucagon and metabolites*
Abbreviations: PEP, phosphoenolpyruvate; *P*-glycerol, glycerol phosphate.

causes the disappearance of Fru-2,6-P_2 despite the fact that it can also cause an increase in the concentration of Fru-6-*P* as a result of its well-known glycogenolytic action. C-3 derivatives and citrate reinforce the effect of glucagon.

PFK 2 activity has been found in a few tissues other than the liver. It was detected in pancreatic islets but was not decreased upon incubation of the islets in the presence of glucagon (Malaisse *et al.*, 1981*b*, 1982*b*). It was also found in a high-speed supernatant derived from pigeon or chicken skeletal muscle (unpublished work from this laboratory).

Biological effects of Fru-2,6-P_2

The stimulation of 6-phosphofructo-1-kinase

PFK is the prototype of a multimodulated enzyme. It has been the subject of numerous reviews, a recent and lucid one being that by Sols *et al.* (1981). Its control can be summarized by saying that one of its substrates, ATP, acts as a negative allosteric effector which induces a marked co-operativity for the second substrate, Fru-6-*P*; the latter acts as a positive effector which relieves the inhibition by ATP. Numerous other substances have an allosteric effect similar to and usually synergistic with those of ATP or Fru-6-*P*. Citrate and H^+ are negative effectors. The most important positive effectors are Fru-1,6-P_2, a product of the reaction, Glc-1,6-P_2, AMP, NH_4^+ and P_i.

The effects of Fru-2,6-P_2 on the kinetics of liver PFK have been investigated mostly by Van Schaftingen *et al.* (1981*a*) and by Uyeda *et al.* (1981*a*) with similar results. The data can be summarized by saying that Fru-2,6-P_2 is the most potent positive effector of PFK known at the present time; at concentrations lower than micromolar, it relieves inhibition by ATP and increases affinity for Fru-6-*P* (Fig. 4). Its effect is also clearly co-operative; however, because of the interaction of other effectors, the sensitivity of the enzyme to Fru-2,6-P_2 depends on the composition of the incubation medium. It can be estimated that, in the presence of physiological concentrations of ATP, Fru-6-*P*, AMP, P_i and NH_4^+, the K_a for Fru-2,6-P_2 is in the micromolar (and therefore physiological) range of concentration. Another characteristic of the effect of Fru-2,6-P_2 is its remarkable synergism with AMP (see Fig. 4 in Van Schaftingen *et al.*, 1981*a*). Fru-2,6-P_2 has also the property to protect PFK against inactivation by heat (Uyeda *et al.*, 1981*a*), by low pH and by 'PFK-inactivating enzyme' (Söling *et al.*, 1981; Söling & Brand, 1981).

An open question concerning the effect of Fru-2,6-P_2 on liver PFK is to know if phosphorylation of the enzyme affects its affinity for the stimulator. Furuya & Uyeda (1980*b*) have isolated from rat liver two fractions of PFK containing either 1.2 or 3.3 mol of phosphate/mol of enzyme. They reported that the low-phosphate form had a higher affinity for their 'activation factor' than did the high-phosphate form and they proposed that, through this mechanism, phosphorylation may be involved in modulating the activity of PFK in the liver. However, the low-phosphate fraction was likely to be contaminated with Fru-2,6-P_2 and for this reason would be much more sensitive to an additional amount of this co-operative effector. In this laboratory, we found that PFK extracted from control or glucagon-treated hepatocytes had the same affinity for Fru-2,6-P_2 (unpublished work from this laboratory).

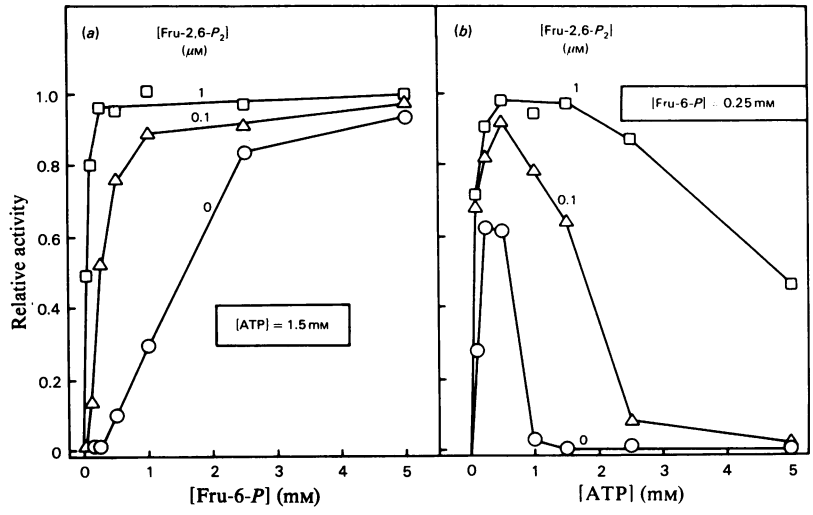

Fig. 4. *Effect of Fru-2,6-P_2 on (a) the affinity of liver PFK for Fru-6-P and (b) the inhibition of the enzyme by ATP* (From Van Schaftingen *et al.*, 1981*a*.)

Muscle PFK appears to be 2–3-fold more sensitive to Fru-2,6-P_2 than the liver enzyme (Uyeda *et al.*, 1981*a*). Other animal PFKs, including that from pancreatic islets (Malaisse *et al.*, 1981*a*) and from human erythrocytes (Heylen *et al.*, 1982) behave similarly to the liver enzyme with only minor differences. Yeast PFK is also highly stimulated by Fru-2,6-P_2 (Van Schaftingen *et al.*, 1981*a*; Avigad, 1981; Bartrons *et al.*, 1982). No effect of Fru-2,6-P_2 could be observed on PFK from mung beans (Sabularse & Anderson 1981*b*), spinach leaves or *Escherichia coli* (unpublished work from this laboratory).

One important spin-off of the investigation of the effect of Fru-2,6-P_2 on PFK was the near elimination of Fru-1,6-P_2, as well as Glc-1,6-P_2, as positive effectors of the enzyme under physiological conditions, at least as far as concerns the liver and yeast PFKs. Fru-1,6-P_2 can be both an inhibitor or a stimulator of PFK according to the experimental conditions. As described by Brand & Söling (1974), Fru-1,6-P_2 is an inhibitor competitive with Fru-6-P, showing typical product inhibition kinetics. This inhibitory effect is observed in the millimolar range of concentration. At lower concentration, Fru-1,6-P_2 is also a positive effector of PFK as was initially described by Passoneau & Lowry (1962) in the case of the rabbit muscle enzyme. The concentration of the effector at which this effect can be observed is, however, highly dependent upon the composition of the incubation medium. Fig. 5 shows that, under favourable conditions, Fru-1,6-P_2 and Glc-1,6-P_2

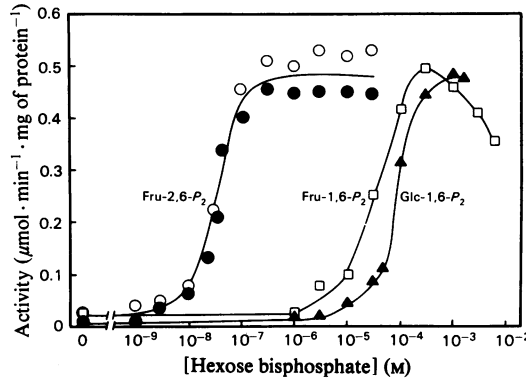

Fig. 5. *Stimulation of liver PFK by hexose bisphosphates*

The assays were performed in the presence of 3 mM-ATP, 5 mM-$MgCl_2$, 1 mM-AMP, 2 mM-Fru-6-P and 0.2 mM-P_i. Open symbols, ADP-coupled assay; Closed symbols, Fru-1,6-P_2-coupled assay. (From Van Schaftingen *et al.*, 1981*a*).

can cause a large activation of the liver enzyme but at concentrations 1000- and 2500-fold greater than that of Fru-2,6-P_2 required to obtain the same effect. When more physiological concentrations of substrates and effectors are used, the sensitivity to all hexose bisphosphates is decreased and all curves shown in Fig. 5. are shifted to the right. Under these

conditions, the inhibitory effect of Fru-1,6-P_2 predominates and overcomes its positive effect (Van Schaftingen *et al.*, 1981*a*). Yeast PFK is known not to be stimulated by Fru-1,6-P_2 (Vinuela *et al.*, 1963); in this laboratory it has been observed that Fru-1,6-P_2 counteracts the positive effect of Fru-2,6-P_2 on that enzyme, and therefore acts as an inhibitor under these conditions (Bartrons *et al.*, 1982).

The stimulation of PP_i: fructose 6-phosphate 1-phosphotransferase

In some types of cells, Fru-1,6-P_2 can be formed from Fru-6-P by transphosphorylation from inorganic pyrophosphate rather than from ATP. An enzyme responsible for this reaction was first described by Reeves *et al.* (1974) in *Entamoeba histolytica*. It was also found in a few bacteria (O'Brien *et al.*, 1975; Macy *et al.*, 1978; Sawyer *et al.*, 1977), in pineapple leaves (Carnal & Black, 1979) and in mung beans (Sabularse & Anderson, 1981*a*). Sabularse & Anderson (1981*b*) have made the remarkable observation that the activity of the mung bean enzyme is nearly dependent upon the presence of Fru-2,6-P_2. The effect of the stimulator (1 μM) was to decrease the K_m for Fru-6-P 67-fold and to increase the $V_{max.}$ 15-fold (Fig. 6). These two effects combined to give a 500-fold activation at 0.3 mM-Fru-6-P. According to the same authors, the enzyme appears to be widespread in plants.

The inhibition of fructose 1,6-bisphosphatase

It was found independently by Van Schaftingen & Hers (1981*b*) and Pilkis *et al.* (1981*a*) that Fru-2,6-P_2 is an inhibitor of FBPase 1 and that its action is synergistic with that of AMP. We reported that, although this inhibition was much stronger at low than at high substrate concentrations, it could not be a simple competition with substrate, not only because of its synergism with AMP (a non-competitive allosteric inhibitor) but also because it changed the saturation curve from almost hyperbolic to sigmoidal (Fig. 7). In contrast, Pilkis *et al.* (1981*b*) reported a strictly competitive type of inhibition with, however, an enzymic preparation which was largely desensitized toward inhibition by AMP ($K_I = 200\,\mu$M, compared with the more usual value of 30 μM reported by Pilkis *et al.*, 1981*c*) and which did not display the classical inhibition by excess substrate. In a subsequent publication Pilkis *et al.* (1981*c*) confirmed that Fru-2,6-P_2 transforms the substrate-saturation curve from hyperbolic to sigmoidal. Furthermore, they showed that Fru-2,6-P_2 protects both the catalytic site and the AMP allosteric site against inactivation by acetylation,

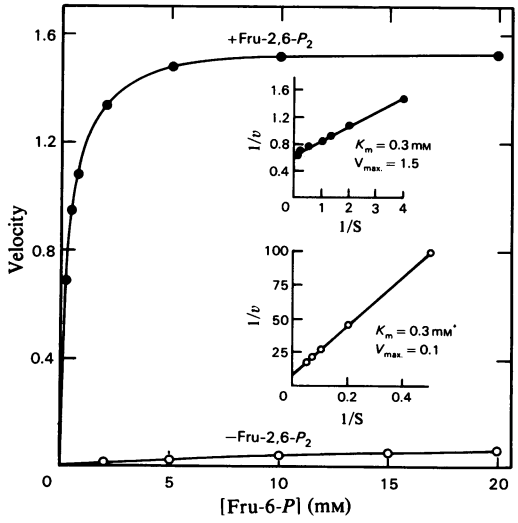

Fig. 6. *Effect of Fru-2,6-P_2 on the kinetic constants of PP_i:Fru-6-P 1-phosphotransferase*
(From Sabularse & Anderson, 1981*b*, with permission.)

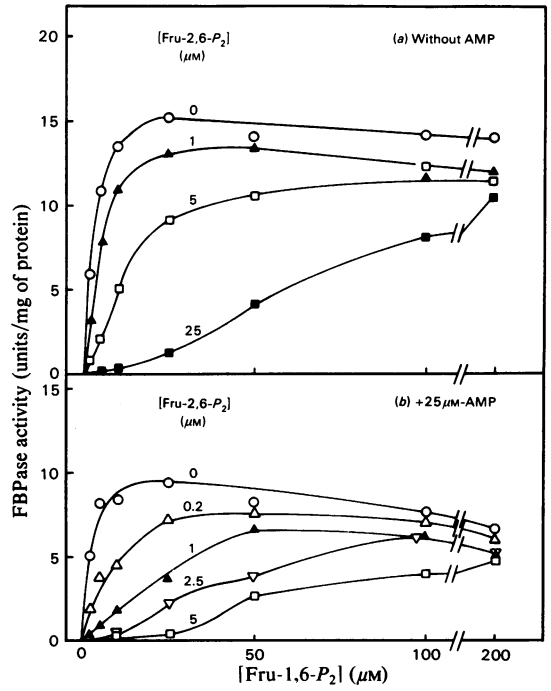

Fig. 7. *Inhibition of FBPase by Fru-2,6-P_2 at various concentrations of substrate in the absence (a) and in the presence (b) of 25 μM-AMP*
(From Van Schaftingen & Hers, 1981*b*.)

which indicates interaction of the inhibitor with both sites.

Liver FBPase 1 can be phosphorylated by cyclic AMP-dependent protein kinase with little change in enzymic activity (Riou *et al.*, 1977) and this phosphorylation was also observed in isolated hepatocytes incubated with glucagon (Claus *et al.*, 1981*b*). Mörikofer-Zwez *et al.* (1981) reported that, in a similar type of experiment, the glucagon treatment decreased the sensitivity of FBPase 1 to Fru-2,6-P_2, for which the K_a was approximately doubled by the presence of the hormone.

Commercial muscle FBPase 1 (Boehringer) was also inhibited by Fru-2,6-P_2. The kinetics of this inhibition were similar to those observed with the liver enzyme. In contrast, the commercial *Torula* yeast enzyme (Sigma) was inhibited by Fru-2,6-P_2 without introducing a positive co-operativity for the substrate. Spinach leaves contain two types of FBPase 1, which can be distinguished by their sensitivity to pH. Only the neutral enzyme was inhibited by low concentrations of Fru-2,6-P_2. The kinetics of these inhibitions have not been investigated in detail.

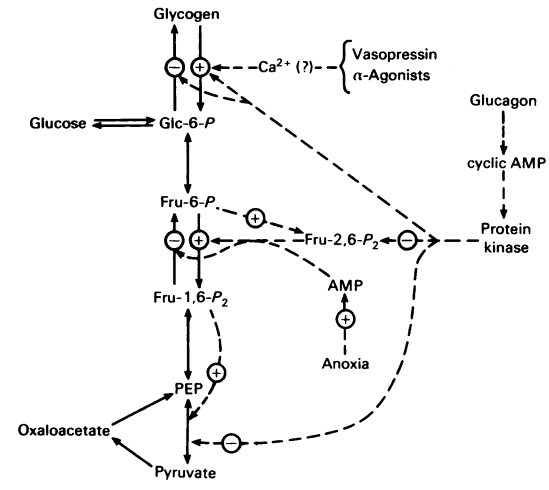

Fig. 8. *Regulation of glycolysis and gluconeogenesis in the liver*
Abbreviation: PEP, phosphoenolpyruvate.

The potential action of Fru-2,6-P_2 on other enzymic systems

In this laboratory, a series of enzymes has been assayed in crude liver preparations in the presence or absence of Fru-2,6-P_2 and no positive or negative effect of this compound could be demonstrated. These enzymes include glycogen phosphorylase, phosphorylase phosphatase, synthase phosphatase, glucokinase, glucose 6-phosphatase, pyruvate kinase, phosphoenolpyruvate carboxykinase and acetyl-CoA carboxylase.

The control of carbon metabolism: an integrated view

The three futile cycles of gluconeogenesis in the liver

Fig. 8 illustrates the paths of glycolysis and gluconeogenesis in the liver and their bidirectional control by cyclic AMP and Fru-2,6-P_2. At three levels, glycolysis and gluconeogenesis utilize different and antagonistic enzymes, the activity of which is determinant for the direction of the metabolic flux. At each of these levels, the simultaneous operation of the two antagonistic enzymes would be responsible for a so-called futile cycle, i.e. a transformation the net balance of which is the hydrolysis of ATP into ADP and P_i (for reviews, see Katz & Rognstad, 1976; Hue, 1981).

The first cycle is between glucose and Glc-6-*P*. There is good evidence that this futile cycle is always operative. This is because there is no on/off mechanism of control of glucokinase and glucose 6-phosphatase, which are high-K_m enzymes, regulated by the concentration of their substrate, glucose or Glc-6-*P*. The biological significance of this cycle has been extensively discussed by Hue & Hers (1974*b*).

The second cycle is between Fru-6-*P* and Fru-1,6-P_2. A recent isotopic investigation indicated that this cycle does not operate in the livers of starved rats *in vivo* unless a large amount of glucose is administered to the animal, but operates well in the livers of fed rats (Van Schaftingen *et al.*, 1980*a*). Similar results were obtained with isolated hepatocytes (Van Schaftingen *et al.*, 1980*b*). The effect of glucose was to increase greatly the concentration of Fru-2,6-P_2, the presence of which allowed PFK 1 to operate simultaneously with FBPase 1. The latter enzyme was inhibited by Fru-2,6-P_2 but could remain active because of a nearly 10-fold increase in the concentration of its substrate, Fru-1,6-P_2. The concentration of AMP, which is another potent effector of PFK 1 and FBPase 1, was not increased.

Glycolysis is also stimulated in the liver of fed animals by glycogenolytic agents, such as vasopressin, and phenylephrine, because of the increase in the concentration of Fru-6-*P* and secondarily of Fru-2,6-P_2 (Hue *et al.*, 1981*a*,*b*). In contrast, glucagon, in addition to its glycogenolytic action, causes the phosphorylation of PFK 2 and of FBPase 2 and consequently the disappearance of Fru-2,6-P_2. The latter effects are therefore required in order to allow an increase in gluconeogenesis despite the increase in the concentration of Fru-6-*P*. Anoxia is

another mechanism which increases glycolysis, but it is associated with a decrease in the concentration of Fru-2,6-P_2. We assume that the Pasteur effect is mediated by AMP which acts synergistically with Fru-2,6-P_2 both as stimulator of PFK 1 and as an inhibitor of FBPase 1.

A third cycle is between phosphoenolpyruvate and pyruvate. The best established mechanism of control of this cycle is the inactivation of pyruvate kinase by cyclic AMP-dependent protein kinase (Engström, 1978). Fru-2,6-P_2 has no known direct effect on the enzymes of this cycle but, as said above, causes a large increase in the concentration of Fru-1,6-P_2, which is a potent stimulator of pyruvate kinase and also counteracts its enzymic phosphorylation.

Fatty acid synthesis and ketogenesis

It is a well-established notion that carbohydrate and fat metabolism in liver are intimately coupled. The synthesis of fatty acid is maximal and ketogenesis is minimal in the livers of animals fed a high-carbohydrate diet; a reverse situation is rapidly obtained upon administration of glucagon. There is general agreement that the limiting step in the synthesis of fatty acid is at the level of acetyl-CoA carboxylase. It is therefore tempting to assume that Fru-2,6-P_2 could control the activity of that enzyme but, as said above, experiments performed to check this hypothesis have given negative results. On the other hand, a major factor that appears to regulate fatty acid synthesis is the availability of lactate and pyruvate, and there is a good indication that the effect of glucagon to inhibit fatty acid synthesis (and secondarily to favour ketogenesis) is linked to its ability to arrest glycolysis, most specifically at the level of phosphofructokinase (Harris, 1975; Lane & Mooney, 1980). It is therefore by this indirect mechanism that the concentration of Fru-2,6-P_2 appears to play a role in the control of lipogenesis and ketogenesis by glucagon in the liver.

Glycogen metabolism

As initially stressed by Soskin (1940), the biosynthesis and the biodegradation of glycogen in the liver are tightly controlled by the level of glycaemia. This is because glucose, being a ligand of phosphorylase *a*, changes its conformation and makes it a better substrate for phosphorylase phosphatase. Phosphorylase *a* is then converted into phosphorylase *b* and glycogenolysis is arrested. Because phosphorylase *a* is an inhibitor of synthase phosphatase, synthase *b* can now be converted into the active form, synthase *a*, and glycogen synthesis is initiated (for reviews see Hers, 1976; Stalmans, 1976; Fletterick & Madsen, 1980). As said above, we have been unable in this laboratory to detect an effect of Fru-2,6-P_2 on the activity or on the rate of interconversion of the two forms of both phosphorylase and synthase in a liver extract.

There are therefore two mechanisms by which glucose affects carbohydrate metabolism in the liver. One is by its direct binding to phosphorylase *a* and concerns glycogen metabolism, the other is by the formation of Fru-2,6-P_2 and concerns glycolysis, gluconeogenesis and lipogenesis. The advantage of this dual mechanism may be to allow two levels of sensitivity to glucose. One can indeed admit that the temporary disposal of glucose as glycogen is a primary function of the liver, which is initiated as soon as the level of glycaemia rises. Gluconeogenesis is another essential function of the liver, whereas the irreversible loss of glucose by glycolysis and lipogenesis would only occur at a relatively high level of glycaemia or when the glycogen stores have been filled. This implies that the concentration of Fru-6-*P*, which we believe is the signal for the formation of Fru-2,6-P_2, is not elevated in the first stage of hyperglycaemia. This was indeed observed by De Wulf & Hers (1967) in conscious mice, in the liver of which it could be demonstrated that the concentrations of Glc-6-*P* and UDP-Glc decrease soon after glycogen synthesis had been initiated by the intravenous administration of glucose. This can be explained by the intense activation of glycogen synthase, which operates now at a rate equal to or faster than that of glucose uptake. This decrease (or at least absence of increase) (Hue & Hers, 1974*a*) of Glc-6-*P* concentration was not observed in anaesthetized rats or in isolated liver preparations (Van Schaftingen *et al.*, 1980*a*,*b*) in which, for unknown reasons, the sensitivity of the whole system of glycogen metabolism to glucose is abnormally low.

Carbon metabolism in tissues and cells other than the liver

The presence of Fru-2,6-P_2 or of enzymes sensitive to its action suggests its participation in the control of metabolism in various tissues or cells.

It has been proposed by Malaisse *et al.* (1981*a*, 1982*b*) that, in the pancreatic islets, the effect of glucose to stimulate insulin secretion is mediated by an increased glycolytic rate, itself secondary to the formation of Fru-2,6-P_2. It is remarkable that glucagon has an effect synergistic with that of glucose on insulin secretion. It is therefore relevant that PFK 2 of the islets is not inactivated by cyclic AMP-dependent protein kinase. The effect of glucagon might then be similar to that of vasopressin in the liver (see above and Fig. 8).

In plants, FBPase is inhibited by Fru-2,6-P_2, but the activity of PFK is not affected. Instead, PP_i:Fru-6-*P* phosphotransferase is very sensitive to Fru-2,6-P_2. This enzyme appears widespread in plants but its discovery is too recent to allow a clear

appreciation of its role. It catalyses a reaction which is easily reversible *in vitro* and its participation either in glycolysis or gluconeogenesis depends on the relative concentrations of PP_i and P_i in its immediate surrounding. Its property to be stimulated by Fru-2,6-P_2 suggests that it could substitute for PFK in some subcellular compartment with favourable PP_i/P_i ratio. The obvious question related to Fru-2,6-P_2 in plants concerns the control of its concentration.

In yeast, glucose causes the arrest of gluconeogenesis by two mechanisms. Firstly, within about 1 min Fru-2,6-P_2 is formed (Lederer *et al.*, 1981), inhibits FBPase and also stimulates PFK. Secondly, in a similarly short time, glucose causes the phosphorylation of FBPase 1 (Müller & Holzer, 1981) followed by the proteolytic degradation of that enzyme which occurs over 1–2 h (Funayama *et al.*, 1980). The effect of glucose in this process, known as 'catabolite inactivation' appears to be mediated by one of its metabolites which is not beyond PFK and is not Glc-6-*P* (Ciriacy & Breitenbach, 1979; Gancedo & Gancedo, 1979). Fru-2,6-P_2 fulfils this requirement.

Fru-2,6-P_2, a signal of glucose

Glucose requirement and availability

Glucose is essential for the survival of individual cells and also of more complex organisms because of the following reasons. Firstly, in all types of cells, it is required for the biosynthesis of glycoproteins and glycolipids, which enter the composition of membranes. Secondly, being the substrate of glycolysis, it is required not only in anaerobiosis but also for the survival of cells which, like erythrocytes, are deprived of mitochondria. Thirdly, under normal circumstances, glucose is the only fuel of the brain. This is because this tissue is unable to utilize free fatty acids, which are bound to serum albumin and do not cross the haemato-encephalic barrier. Ketone bodies are an alternative fuel, but, except in prolonged fasting, their concentration in the blood is negligible. For these reasons, hypoglycaemia may cause irreversible damage to the brain and must be avoided at any cost.

Glucose is usually abundant in food and most cells rely on external sources for its supply. These cells are therefore normally not equipped for the biosynthesis of glucose. In contrast, the stores of glucose as glycogen are very limited and are rapidly exhausted during fasting. Gluconeogenesis becomes then essential for survival; in animals, it operates only in the liver and the kidney and its activity is controlled hormonally and also by glucose concentration. In yeast, gluconeogenic enzymes are usually formed only when glucose is not present in sufficient amount and are destroyed as soon as glucose is again available. This is a relatively cheap mechanism of control because glycolysis and gluconeogenesis, in contrast with other biosynthetic and biodegradative pathways, have most of their enzymes in common. Consequently, the control mechanisms, which operate by various means, including the concentration of substrates and effectors, the covalent modification of enzymes, as well as protein synthesis or degradation, are limited to a small number of enzymes. In bacteria, glucose elicits the repression of a series of enzymes related to the catabolism of other nutrients.

Fru-2,6-P_2 versus cyclic AMP

Fru-2,6-P_2 and cyclic AMP have in common several properties. Both are regulatory molecules with a long evolutionary history, since they are found in many types of cells, including bacteria (questionable for Fru-2,6-P_2), plants (questionable for cyclic AMP, as reviewed by Amrhein, 1977), yeast and animal cells. Both are metabolically active at micromolar concentrations and are regulatory signals, in the sense made explicit by Stryer (1981). Indeed, "although they are formed from ubiquitous molecules which are at the centre of metabolic transformations, they are themselves not part of a major metabolic pathway. They are used only as integrators of metabolism, not as biosynthetic precursors or intermediates in energy production. Hence, their concentration can be independently controlled".

Cyclic AMP was discovered 25 years ago by Sutherland & Rall (1958) as a heat-stable factor mediating the action of adrenaline and glucagon on the activation of liver phosphorylase. In 1965, Makman & Sutherland reported that cyclic AMP concentration decreased in *E. coli* soon after glucose addition, and since that time glucose repression of catabolic enzymes in bacteria has been associated with a deficiency of cyclic AMP. In bacteria, cyclic AMP binds to catabolic gene activator protein (CAP); in higher organisms it binds to a specific protein kinase which then phosphorylates several regulatory enzymes of glycogen metabolism and of gluconeogenesis with the main result that glucose is formed. For all these reasons, cyclic AMP has been called a hunger signal, which signifies the absence of glucose (Stryer, 1981). This is, however, an oversimplification, because cyclic AMP has numerous other effects which are unrelated to the presence or absence of glucose and the molecular mechanism of which is still badly understood. This utilization of the same chemical structure to fulfil very different functions may be considered as an example of 'molecular tinkering' which, according to Jacob (1977), is characteristic of evolution.

Fru-2,6-P_2 has effects antagonistic to those of cyclic AMP. It is the signal which signifies that

glucose is abundant and can be freely utilized and that gluconeogenesis can be stopped. In yeast it is formed in the presence of glucose and might be the signal responsible for the catabolite inactivation of gluconeogenic enzymes. Cyclic AMP prevents the formation and favours the destruction of Fru-2,6-P_2, at least in the liver, and it is tempting to speculate that Fru-2,6-P_2 could have a symmetric effect and counteract cyclic AMP formation.

Fru-2,6-P_2 was discovered less than 2 years ago. All its biological effects known at the present time are related to glucose metabolism, but one must consider the possibility that, as a result of 'molecular tinkering', it might also serve other purposes. Its role in many tissues or cells, including muscle, brain, plants and possibly bacteria, remains to be discovered. The main purpose of the present paper is to stimulate research along these various lines.

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