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Control of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in isolated hepatocytes by glucose and glucagon

Role of a low-molecular-weight stimulator of phosphofructokinase

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1. Recycling of metabolites between fructose 6-phosphate and triose phosphates has been investigated in isolated hepatocytes by the randomization of carbon between $C_{(1)}$ and $C_{(6)}$ of glucose formed from [1-^{14}C]galactose. 2. Randomization of carbon atoms was regularly observed with hepatocytes isolated from fed rats and was then little influenced by the concentration of glucose in the incubation medium. It was decreased by about 50% in the presence of glucagon. 3. Randomization of carbon atoms by hepatocytes isolated from starved rats was barely detectable at physiological concentrations of glucose in the incubation medium, but was greatly increased with increasing glucose concentrations. It was nearly completely suppressed by glucagon. These large changes can be attributed to parallel variations in the activity of phosphofructokinase. 4. The main factors that appear to control the activity of phosphofructokinase under these experimental conditions are the concentration of fructose 6-phosphate, the concentration of fructose 1,6-bisphosphate and also the affinity of the enzyme for fructose 6-phosphate. 5. The affinity of phosphofructokinase for fructose 6-phosphate was diminished by incubation of the cells in the presence of glucagon and also by filtration of an extract of hepatocytes through Sephadex G-25 and by purification of the enzyme. When assayed at 0.25 or 0.5mM-fructose 6-phosphate, the activity of phosphofructokinase present in a liver Sephadex filtrate was increased by a low-molecular-weight effector, which could be isolated from a liver extract by ultrafiltration, gel filtration or heat treatment, but was rapidly destroyed in trichloroacetic acid, even in the cold. This effector appears to be a highly acid-labile phosphoric ester. Its concentration was greatly increased in hepatocytes incubated in the presence of glucose and was decreased in the presence of glucagon.

In a previous publication (Van Schaftingen *et al.*, 1980*a*), we reported that recycling between fructose 6-phosphate and fructose 1,6-bisphosphate occurs *in vivo* in the livers of fed rats and mice, although not in the livers of starved animals. Recycling was also observed within a few minutes after administration of glucose to starved rats and this effect was abolished by glucagon. We came to the conclusion that these large differences in the rate of recycling reflected similar changes in the activity of phosphofructokinase. The main factors which appear to control this activity are the concentration of fructose 6-phosphate, the concentration of fructose 1,6-bisphosphate, and the affinity of the enzyme for fructose 6-phosphate. It has indeed been reported that glucagon decreases this affinity (Castaño *et al.*, 1979; Pilkis *et al.*, 1979) and also causes the phosphorylation of the enzyme and a reinforcement of its inhibition by ATP (Kagimoto & Uyeda, 1979). The changes in kinetics were reported to be stable after gel filtration or partial purification of the enzyme and, as reviewed by Claus & Pilkis (1980), are believed to result from a cyclic AMP-dependent phosphorylation of liver phosphofructokinase.

In the present paper we report a study of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in isolated hepatocytes. This preparation is

Abbreviation used: Hepes 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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indeed suitable for parallel investigation of recycling and of several parameters that are expected to influence the activity of phosphofructokinase. As was found for studies *in vivo* (Van Schaftingen *et al.*, 1980*a*), the randomization of carbon between $C_{(1)}$ and $C_{(6)}$ of glucose formed from [1-^{14}C]galactose (Rognstad & Katz, 1976) appears to be the best available method to measure recycling of metabolites between fructose 6-phosphate and triose phosphates in isolated hepatocytes.

In the course of this investigation, we were able to confirm that glucagon causes a large decrease both in the randomization of carbon (Rognstad & Katz, 1976) and in the affinity of phosphofructokinase for fructose 6-phosphate (Castaño *et al.*, 1979; Pilkis *et al.*, 1979) in isolated hepatocytes. However, we have also observed that similar changes in the kinetic properties of phosphofructokinase could be obtained by gel filtration of an extract of hepatocytes and could be reversed by the addition of a low-molecular-weight factor present in the liver of normal fed rats. We have investigated the concentration of this factor in hepatocytes in relation to the effect of glucose and glucagon on the fructose 6-phosphate/fructose 1,6-bisphosphate cycle.

Materials and methods

Chemicals and enzymes

Biogel P-2 was obtained from BioRad Laboratories (Richmond, CA, U.S.A.), ATP–agarose from Sigma, and *o*-phthalaldehyde from Aldrich Europe Division (Beerse, Belgium). Calf intestine alkaline phosphatase (grade I) was purchased from Boehringer (Mannheim, Germany) and was desalted on Sephadex G-25. The source of other chemicals, hormones and enzymes is as previously reported (Hue *et al.*, 1978; Van Schaftingen *et al.*, 1980*a*).

Isolation and manipulation of hepatocytes

All experiments were performed with male Wistar rats weighing 200–250g, either fed *ad libitum* or starved overnight. Hepatocytes were isolated as previously described (Hue *et al.*, 1978). A portion (2ml) of the cell suspension [corresponding to about 20mg of protein, as measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard] was shaken (120strokes/min) in stoppered 20ml vials at 37°C for 30min in the presence of 5mM-glucose (unless otherwise indicated). After this preincubation, the experiment was started by addition of either a larger amount of glucose, glucagon or [1-^{14}C]galactose.

In experiments with tracers, the cells were incubated for 15min in the presence of 250000c.p.m. of [1-^{14}C]galactose/ml. The cells were then separated from the incubation medium and fixed in perchloric acid by centrifugation of the suspension in bulb tubes as described by Hems *et al.* (1975). The perchlorate extract was used for the determination of intracellular metabolites, and the supernatant for the isolation of [^{14}C]glucose.

Phosphofructokinase activity was measured in cells previously incubated for 15min in the presence of various concentrations of glucose or glucagon. The cell suspension was briefly centrifuged in a table centrifuge and the pellet was frozen in acetone/solid CO_2. This separation procedure took about 15s. The frozen pellets of cells were thawed in 0.9ml of 50mM-Hepes/100mM-KF/15mM-EGTA (sodium salt), adjusted to pH7.1 at 20°C with KOH (homogenization buffer). After centrifugation for 10min at 2000*g*, the supernatant [a 10% (w/v) cellular extract] was collected and kept frozen until further processing. When the cellular extract had to be filtered through Sephadex, the frozen pellets were initially thawed in 0.4ml of homogenization buffer to make a 20% (w/v) cellular extract. This extract was diluted 2-fold either in the course of gel filtration or by addition of an equal volume of homogenization buffer. It has been checked that freezing and thawing the hepatocytes allows a complete extraction of phosphofructokinase.

Other liver preparations

For the preparation of a liver extract, the tissue was homogenized in a Potter–Elvehjem tube with 4vol. of homogenization buffer and the homogenate was centrifuged for 15min at 60000*g* at 0–4°C. Fractions (1ml) of the supernatant were kept frozen. They were thawed and filtered through Sephadex G-25 before being used for the assay of the stimulator.

Livers were perfused with a Krebs–Henseleit (1932) bicarbonate buffer containing 20mM-glucose, in the apparatus described by Hems *et al.* (1966). The first 100ml of perfusate was discarded and the perfusion was continued with 200ml of recirculating medium for 25min. Glucagon (10^{-6}M) was added to the perfusion medium 10min before the end of the experiment. The livers were then freeze-clamped (Wollenberger *et al.*, 1960).

Gel filtration of the extracts and purification of phosphofructokinase

A portion (1ml) of a 20% cellular or liver extract was passed through a column (1.4cm × 23cm) of Sephadex G-25 (medium size particles) equilibrated with the ice-cold homogenization buffer, which also contained 0.1mM-fructose 6-phosphate and 0.35mM-glucose 6-phosphate. The protein fraction (2ml) was recovered, without loss of phosphofructokinase activity. In contrast, when the filtration was performed in the absence of hexose 6-phosphates as stabilizing agent, the activity of phosphofructokinase measured at substrate saturation

decreased by more than 50% in 30min at 0°C and was not measurable after 2h.

Phosphofructokinase was purified by affinity chromatography as described by Castaño *et al.* (1979) except that the enzyme was eluted from agarose–ATP with the equilibration buffer containing 1mM-ATP and 1mM-$MgCl_2$; the purification was 40-fold with a yield of 90%.

Measurement of phosphofructokinase and of its stimulator

The enzyme was assayed as described previously (Van Schaftingen *et al.*, 1980*a*) unless otherwise indicated. A portion (50μl) of a 10% cellular or liver extract or gel filtrate was used in each assay performed in a total volume of 1ml. The activity ratio is the ratio of enzyme activity measured at low (0.25 or 0.5mM) and saturating (5mM) concentration of fructose 6-phosphate. For the measurement of the stimulatory effect, the source of enzyme was a Sephadex filtrate (see above) prepared from the whole liver of a fed animal. The samples to be analysed were added to the assay mixture at the beginning of the preincubation; 25mM-KF and 3.8mM-EGTA were present in the assay. In the experiment described in Fig. 5, the stimulator was separated from the high-molecular-weight fraction by centrifugation (800*g* for 40min) at 0°C in an ultrafiltration membrane cone (Centriflo 25, Amicon, Lexington, MA, U.S.A., theoretical mol.wt. cut-off 25000). It has been checked that this ultrafiltrate was devoid of phosphofructokinase activity. In all other experiments, the separation was performed by heating the extracts at 80°C for 5min and elimination of the denatured proteins by centrifugation.

The activity of phosphofructokinase is influenced by many factors, including small changes in pH and in the concentration of reactants or products of the reaction. It has been stressed by Emerk & Frieden (1975) that, as a consequence of this, the study of the regulatory kinetic properties of phosphofructokinase is beset with uncertainties. Indeed, minor changes in the composition of the assay may be responsible for differences in experimental data obtained from one day to the other. This restriction applies to a rigorous quantitative analysis of the results but does not affect the general significance of the work.

Destruction of the stimulator of phosphofructokinase by alkaline phosphatase

To test the action of alkaline phosphatase on the stimulator of phosphofructokinase, the following procedure was used. A frozen pellet of hepatocytes, previously incubated for 10min in the presence of 20mM-glucose, was thawed in 0.9ml of 10mM-Hepes, pH7.1, and immediately heated at 80°C for 5min. The mixture was then centrifuged and 0.25ml of the supernatant was incubated for 15min in the presence of 0.25mM-$ZnCl_2$/2.5mM-$MgCl_2$/0.1M-Tris/HCl, pH8, with and without 1 unit of calf intestine alkaline phosphatase in a total volume of 1ml. The reaction was stopped by again heating the mixture at 80°C for 5min and centrifuging. The stimulator was assayed in the supernatants.

Analysis of radioactive glucose and metabolites

The isolation of glucose and the determination of the radioactivity of $C_{(6)}$ were performed as previously described (Van Schaftingen *et al.*, 1980*a*). $C_{(2)}$–$C_{(5)}$ were isolated as formate after reduction of glucose to sorbitol followed by periodate oxidation. Samples containing about 50μg of glucose were incubated with 5mg of $NaBH_4$ in the presence of 0.02M-NaOH at room temperature for 24h in a final volume of 0.25ml. The unreacted $NaBH_4$ was destroyed by the addition of 0.20ml of 1M-HCl. Periodate oxidation was performed in the dark by addition of 0.4ml of 0.5M-potassium phosphate buffer, pH7.5, 0.1ml of methanol and 0.2ml of 8% (w/v) sodium periodate. After 30min, the samples were passed through a column (0.5cm × 6cm) of AG1 (X8, formate form). The column was washed with 20ml of water and formate was eluted with 3ml of 1M-NaCl. A sample of the eluate was mixed with 1vol. of water and 20vol. of scintillation mixture (Bio-Fluor, New England Nuclear) and the mixture was counted in a liquid-scintillation counter. When this procedure was applied to [1-^{14}C] and [U-^{14}C]glucose, 0.2 and 66% of the radioactivity was recovered in the formate fraction respectively. Metabolites were measured as previously described (Van Schaftingen *et al.*, 1980*a*).

Results

Randomization of carbon atoms in isolated hepatocytes

Time course of the carbon-atom exchange. Fig. 1 shows the results of an experiment in which a suspension of hepatocytes isolated from a fed animal was incubated in the presence of 20mM-glucose and a tracer amount of [1-^{14}C]galactose. The half-life of this labelled precursor was a few minutes. [^{14}C]-Glucose was formed for 20–30min and then its concentration reached a plateau. It contained a rapidly increasing proportion of its ^{14}C in $C_{(6)}$ and only a negligible amount in $C_{(2)}$–$C_{(5)}$. The greatest changes in the distribution of ^{14}C within the molecule occurred during the first 5–10min. Later, the radioactivity present in $C_{(2)}$–$C_{(6)}$ continued to increase slowly.

Effect of glucose and of glucagon. When the hepatocytes were isolated from fed rats (Fig. 2*a*), the randomization of ^{14}C between $C_{(1)}$ and $C_{(6)}$ of the

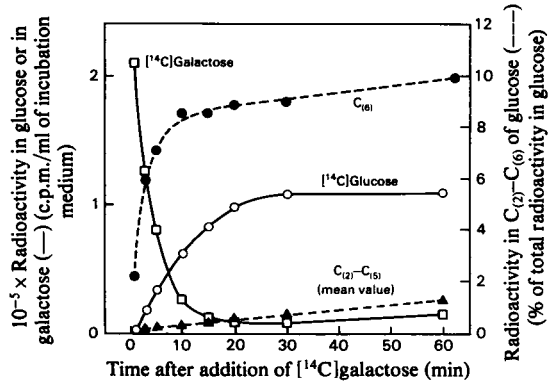

Fig. 1. *Time-course of the conversion of [1-^{14}C]galactose to [^{14}C]glucose by isolated hepatocytes*

Hepatocytes were isolated from the liver of a fed rat and preincubated for 30min in the presence of 20mM-glucose. A tracer amount of [1-^{14}C]galactose was then added. $C_{(2)}$–$C_{(5)}$ have been counted together and the average value is shown.

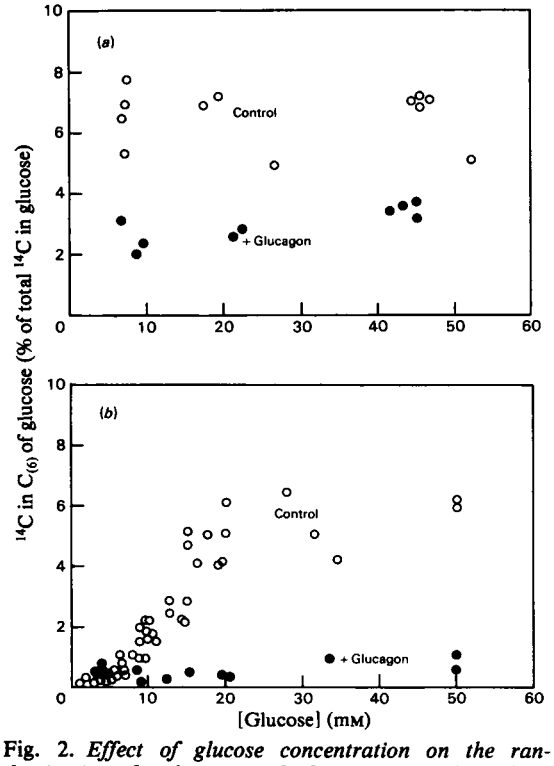

Fig. 2. *Effect of glucose concentration on the randomization of carbon atoms by hepatocytes isolated from the livers of fed (a) or starved (b) rats*

The cells were incubated for 20min at the concentration of glucose indicated, in the presence (●) or absence (○) of 10^{-6}M-glucagon. [^{14}C]Galactose was added 5min after the addition of glucose or glucagon. These data were obtained with three (*a*) or five (*b*) preparations of cells.

newly formed glucose was easily measurable at all glucose concentrations, including the physiological range, and was not influenced by large changes in the concentration of glucose. Glucagon decreased this randomization by about 50%. When the hepatocytes were isolated from starved rats (Fig. 2*b*), there was a negligible randomization of carbon between $C_{(1)}$ and $C_{(6)}$ at or below the physiological concentration of glucose (5mM). The randomization became much greater at higher glucose concentrations and reached a plateau at about 20mM-glucose. At all glucose concentrations, glucagon caused an almost complete abolition of the randomization. The randomization of carbon that occurred within hepatocytes obtained from both fed (Fig. 3*a*) and starved (Fig. 3*b*) rats was also expressed as a function of the concentration of glucose 6-phosphate within these cells.

The effect of glucagon and of increasing concentrations of glucose in the incubation medium on the intracellular concentration of fructose 1,6-bisphosphate and on the activity ratio of phosphofructokinase was investigated with hepatocytes obtained from fed (Fig. 4*a*) and starved (Fig. 4*b*) rats. Glucose greatly increased the intracellular

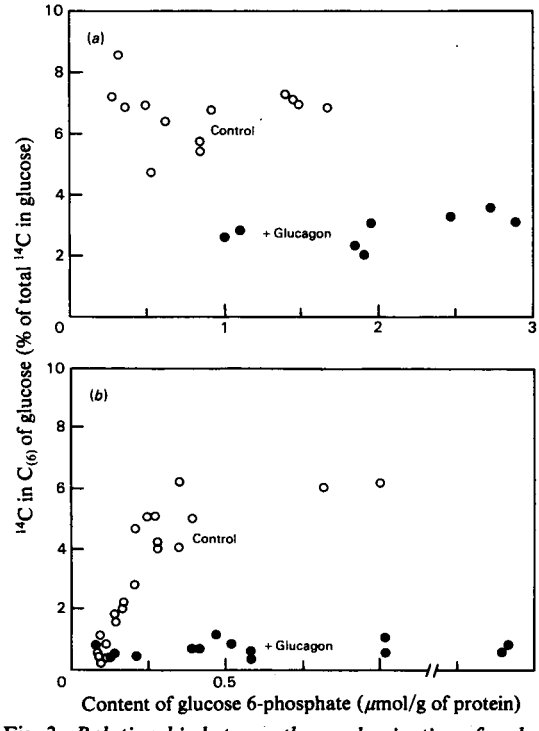

Fig. 3. *Relationship between the randomization of carbon atoms by isolated hepatocytes and the intracellular content of glucose 6-phosphate*

The same experiments as for Fig. 2 are shown.

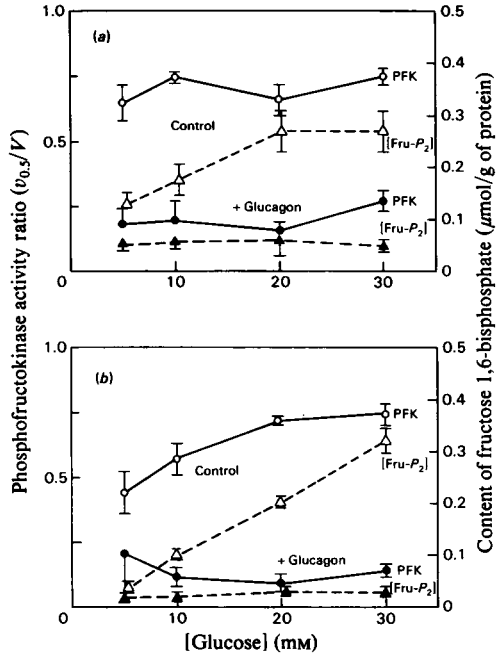

Fig. 4. *Effect of glucose concentration on the activity ratio of phosphofructokinase (PFK) and on the content of fructose 1,6-bisphosphate (Fru-P_2) in hepatocytes isolated from the liver of fed (a) and starved (b) rats*

Values shown are means ± S.E.M. for three different cell preparations. The concentration of glucagon was 10^{-6} M.

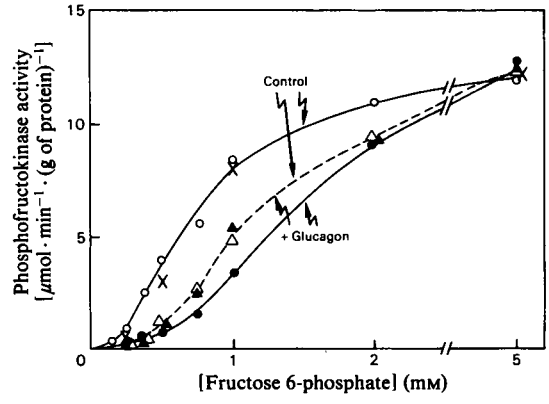

Fig. 5. *Saturation curve of phosphofructokinase for fructose 6-phosphate*

Enzyme activity was assayed in an extract of hepatocytes obtained from the liver of a fed rat and incubated for 15 min in the presence of 10 mM-glucose with (filled symbols) or without (open symbols) 10^{-6} M-glucagon. The enzyme activity was also measured after filtration of the extract through a column of Sephadex G-25 (broken line). The latter assay was also performed in the presence of 50 μl of ultrafiltrate (×) prepared from a homogenate of control cells.

concentration of fructose 1,6-bisphosphate under both experimental conditions. It also increased the activity ratio of phosphofructokinase in the hepatocytes from starved rats but this effect reached saturation at lower glucose concentrations than did the effect on fructose 1,6-bisphosphate. Glucose was without effect on the activity ratio of phosphofructokinase in hepatocytes obtained from fed rats. In the presence of glucagon, the activity ratio of phosphofructokinase and the concentration of fructose 1,6-bisphosphate were greatly decreased, particularly in the hepatocytes from starved rats, and glucose was without effect. It has been verified that the effect of glucagon to decrease the randomization of carbon and the activity ratio of phosphofructokinase could be obtained within the physiological range of concentrations (10^{-9}–10^{-10} M) of the hormone (results not shown).

Effect of gel filtration and partial purification on the kinetics of phosphofructokinase

Fig. 5 shows the saturation curves for fructose 6-phosphate of phosphofructokinase present in extracts of hepatocytes isolated from the liver of a fed rat and incubated in the absence or in the presence of glucagon. It is apparent that, after treatment with glucagon, the preparation had a markedly decreased affinity for fructose 6-phosphate. However, when the cellular extracts were filtered through Sephadex G-25, the saturation curves of the two preparations were superimposable. Indeed, the affinity of the enzyme in the control preparation was decreased whereas that for the enzyme in glucagon-treated preparation was slightly increased.

We have also been able to purify, by affinity chromatography on agarose–ATP, the phosphofructokinase present in hepatocytes obtained from a fed rat and incubated in the absence or in the presence of glucagon. The kinetic properties of the two purified preparations were identical, having an affinity for fructose 6-phosphate similar to that of the enzyme from the glucagon-treated cellular extract (results not shown). These experiments indicate that partial purification, as well as gel filtration, can mimic the effect of glucagon to decrease the affinity of phosphofructokinase for fructose 6-phosphate. They suggest that this effect is due to the removal of a low-molecular-weight stimulator of the enzyme.

Some properties of the low-molecular-weight stimulator of phosphofructokinase

The existence of such a stimulator was confirmed

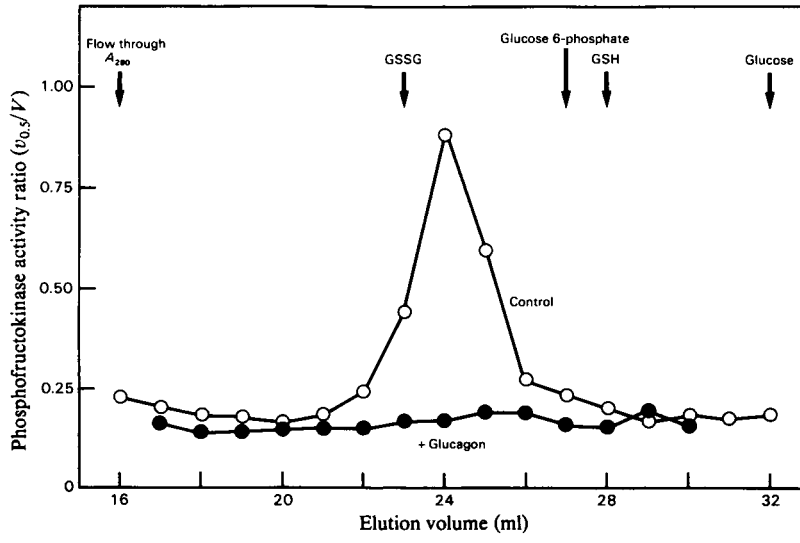

Fig. 6. *Elution profile of the stimulator of phosphofructokinase from Biogel P-2*

A 20% extract of cells obtained from a starved rat and incubated for 15 min in the presence of 20 mM-glucose, with or without 10^{-6} M-glucagon, was heated for 5 min at 80°C and centrifuged. A portion (1 ml) of the supernatant was passed through a column (1.4 cm × 30 cm) of Biogel P-2 equilibrated with the homogenization buffer. The same buffer was used for elution; 0.2 ml of each fraction was added to the assay mixture. Reduced (GSH) and oxidized (GSSG) glutathione were measured as described by Hissin & Hilf (1976).

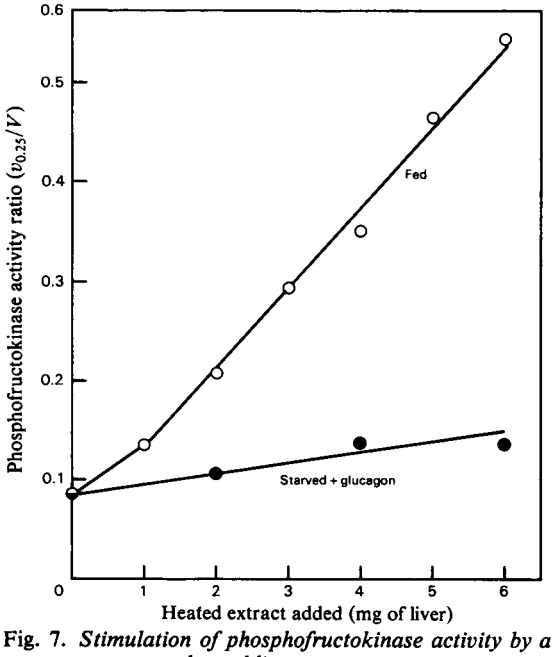

Fig. 7. *Stimulation of phosphofructokinase activity by a heated liver extract*

The assay was performed with 25 μl of a 10% liver Sephadex filtrate in the presence of various amounts of a liver extract previously heated at 80°C for 5 min; this extract was made either from the liver of a fed rat perfused in the presence of 20 mM-glucose or from the liver of a starved rat perfused in the presence of 20 mM-glucose and 10^{-6} M-glucagon.

by the fact that, in the experiment shown in Fig. 5, the addition to the assay system of an ultrafiltrate obtained from the untreated cells increased the activity of the gel filtrates (obtained either from the control or from the glucagon-treated cells) up to the level of that of the control extract. An identical effect was obtained by the addition of a cellular extract heated at 80°C, but more than 50% of the stimulator was lost in 5 min at 100°C. Fructose 1,6-bisphosphate (5 μM) was without effect.

The property to stimulate phosphofructokinase activity was lost within a few minutes upon incubation of the liver extract at 0°C in the presence of 0.3 M-trichloroacetic acid, and it was also lost upon incubation of the extract in the presence of purified alkaline phosphatase. These properties suggest that the stimulatory factor is a highly acid-labile phosphoric ester. Upon filtration through Bio-Gel P-2 (Fig. 6), the factor was eluted between oxidized (mol.wt. 613) and reduced (mol.wt. 307) glutathione and before glucose 6-phosphate (mol.wt. 260). The fact that the latter compound was eluted before reduced glutathione indicates that the order of elution from Bio-Gel P-2 allows only a rough estimate of the molecular weight of phosphoric esters. In another experiment, the extract obtained by heating at 80°C was mixed with a tracer amount of [^{14}C]fructose 1,6-bisphosphate; the mixture was passed through a column of Sephadex G-15, from which the stimulatory factor was eluted together with fructose 1,6-bisphosphate (results not shown).

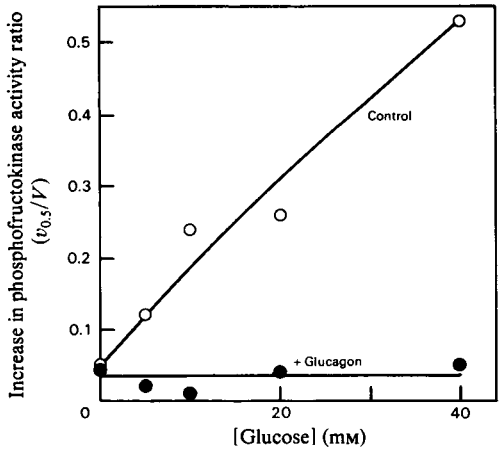

Fig. 8. *Effect of the concentration of glucose in the incubation medium and of glucagon on the concentration of the stimulator of phosphofructokinase in hepatocytes*

Hepatocytes obtained from the liver of a starved rat were incubated for 10 min in the presence of glucose at the concentration indicated with or without 10^{-6} M-glucagon. The amount of heated cellular extract added to the assay and the amount of liver filtrate used as a source of enzyme both corresponded to 5 mg of liver. The activity ratio measured without addition of heated extract was 0.18.

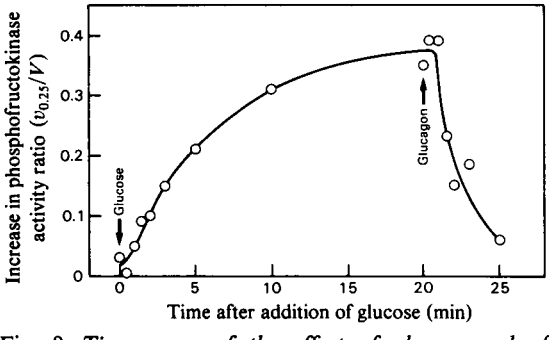

Fig. 9. *Time-course of the effect of glucose and of glucagon on the concentration of the stimulator of phosphofructokinase in isolated hepatocytes*

Hepatocytes isolated from the liver of a starved rat were preincubated for 30 min in the absence of glucose. Glucose was then added at a final concentration of 20 mM; glucagon was added 20 min later at a final concentration of 10^{-6} M. Other procedures are as described in the legend to Fig. 8. The activity ratio measured without addition of heated extract was 0.08.

Effect of glucose and of glucagon on the concentration of the stimulator of phosphofructokinase in isolated hepatocytes

When phosphofructokinase was assayed at a low concentration of fructose 6-phosphate (0.25 or 0.5 mM), its activity was increased in an almost linear fashion by increasing amounts of an 80°C extract originating from the liver of a fed rat perfused with 20 mM-glucose; no or little stimulation was observed with a similar preparation originating from a starved rat and perfused with glucagon (Fig. 7). This procedure appears therefore useable as a bioassay of the stimulator. The results of such an analysis performed with hepatocytes obtained from a starved rat are shown in Fig. 8. It is apparent that increasing glucose concentrations in the incubation medium greatly increased the concentration of the stimulator in the cells. Similar results (not shown) were also observed with hepatocytes obtained from fed rats. Under both conditions, glucagon greatly decreased the concentration of the effector.

The time course of the effects of glucose and of glucagon is shown in Fig. 9. The effect of glucose occurred without any clear latency and was sustained for at least 20 min. The effect of glucagon started after a latency of about 1 min and was nearly complete within 5 min. We have also checked that the effect of glucagon could be obtained at physiological (10^{-9}–10^{-10} M) concentrations of the hormone (results not shown).

Discussion

Fructose 6-phosphate/fructose 1,6-bisphosphate cycle in isolated hepatocytes

The randomization of carbon atoms between the upper and the lower 'halves' of glucose formed from [1-^{14}C]galactose appears to be a reliable semi-quantitative method for the estimation of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in isolated hepatocytes (Rognstad & Katz, 1976) as well as *in vivo* (see Van Schaftingen *et al.*, 1980*a*). The low radioactivity found in $C_{(2)}$–$C_{(5)}$ of glucose indicates that the exchange between $C_{(1)}$ and $C_{(6)}$ cannot be explained by a 'Cori cycle' which would operate in a mixture of two populations of cells, one of them performing glycolysis, the other synthesizing glucose. Such a heterogeneity of liver parenchymal cells has indeed been postulated by Jungermann & Sasse (1978). If this had been the case, the radioactivity would have been distributed in nearly all the carbon atoms of the newly formed glucose, because oxaloacetate is in rapid equilibrium with succinate (Reichard *et al.*, 1963).

As a rule, the results obtained concerning the activity of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in isolated hepatocytes are in agreement with those previously published for the rat *in vivo* (Van Schaftingen *et al.*, 1980*a*). Indeed, the cycle was operative at physiological glucose concentrations in cells obtained from fed rats, although not from starved rats. In the latter preparation, it

could be induced by high glucose concentrations and was then abolished by glucagon. This effect, obtained in a condition in which gluconeogenesis, and therefore fructose bisphosphatase, are expected to be most active, indicates that the intensity of recycling in hepatocytes, as *in vivo*, is controlled by the activity of phosphofructokinase rather than by that of fructose bisphosphatase. In both conditions, an increased randomization of carbon atoms was accompanied by an increased concentration of hexose 6-phosphate and of fructose 1,6-bisphosphate and also by an increased affinity of phosphofructokinase for fructose 6-phosphate. Each of these factors can affect the activity of phosphofructokinase, and therefore the intensity of recycling, and their relative importance will be further discussed below.

Glucagon was also able to decrease, although not to suppress, the randomization of carbon atoms in hepatocytes obtained from fed rats, an effect that was not apparent *in vivo* (Van Schaftingen *et al.*, 1980*a*). This discrepancy could be explained by multiple differences between the two systems and particularly by the presence of other hormones and interfering substances *in vivo*. It is also difficult to compare our results with those of Rognstad & Katz (1976) who have worked exclusively with hepatocytes obtained from starved rats and incubated in the presence of dihydroxyacetone. These authors were the first to describe the effect of glucagon to decrease the randomization of carbon atoms.

An advantage of the use of isolated cells as compared with the experiments *in vivo* is that they allow us to establish dose–response curves for the two main effectors of the system, glucose and glucagon. Both of them were effective at physiological concentrations (5–15 mM for glucose; 10^{-10}–10^{-9} M for glucagon).

Control of phosphofructokinase activity by glucagon and glucose

A major advance in our knowledge of the control of phosphofructokinase activity has been the discovery that the activity of the enzyme measured at low concentrations of fructose 6-phosphate (Castaño *et al.*, 1979; Pilkis *et al.*, 1979) or in the presence of an excess of ATP (Kagimoto & Uyeda, 1979) is markedly diminished in an extract of hepatocytes that has been incubated in the presence of glucagon. These changes were reported to persist upon gel filtration and upon purification and were believed to be caused by a cyclic AMP-dependent phosphorylation of the enzyme. It is remarkable, however, that purification by affinity chromatography (Castaño *et al.*, 1979) resulted in a decrease in the affinity of phosphofructokinase for fructose 6-phosphate to a value intermediate between that of the control and of the glucagon-treated preparation. These data are therefore in partial agreement with our finding that purification of the enzyme, as well as gel filtration, mimics the effect of glucagon. The effect of gel filtration was counteracted by the addition of a low-molecular-weight fraction obtained from control livers. These observations allow the conclusion that the effect of glucagon is not to induce a stable change in the kinetic properties of phosphofructokinase, but to remove a low-molecular-weight stimulator of the enzyme. Remarkably, the concentration of this stimulator was greatly increased by incubation of the cells in, or perfusion of the liver with, a high concentration of glucose. The fact, reported by other groups of workers (Castaño *et al.*, 1979; Kagimoto & Uyeda, 1979), that phosphofructokinase from control and glucagon-treated hepatocytes maintained different kinetic properties after partial purification indicates that the stimulator has a great affinity for the enzyme and may accompany it during purification.

Its sensitivity to purified alkaline phosphatase and to trichloroacetic acid indicates that the low-molecular-weight stimulator of phosphofructokinase is an acid-labile phosphoric ester. Gel filtration indicates that it has a molecular weight similar to that of fructose 1,6-bisphosphate. It is, however, not fructose 1,6-bisphosphate, which had no effect and was indeed removed by aldolase under our assay conditions; fructose 1,6-bisphosphate is also not destroyed by trichloroacetic acid. Other properties of the stimulatory factor of phosphofructokinase, which are reported elsewhere (Van Schaftingen *et al.*, 1980*b*), indicate that it is fructose 2,6-bisphosphate.

It is also noticeable that gel filtration of an extract of cells previously treated with glucagon slightly increased the activity of phosphofructokinase, presumably by the removal of an inhibitor. No inhibitor of phosphofructokinase was, however, detected in the heated extract of glucagon-treated cells.

Role of the stimulator of phosphofructokinase in the control of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle

The concentration of the stimulator in hepatocytes varied greatly according to the concentration of glucose and of glucagon in the incubation medium. As a rule, it was high in conditions in which the randomization of carbon atoms was elevated and vice versa. It must be recognized, however, that, in certain conditions, changes in the concentration of the stimulator were much greater than those in randomization (compare Fig. 8 with Fig. 2) or than the variation in the activity ratio of phosphofructokinase (compare Fig. 8 with Fig. 4). This discrepancy can be explained by the high affinity of the stimulator for phosphofructokinase, which may be rapidly saturated. Furthermore, changes in

glycolytic flux may be larger than indicated by the randomization of carbon atoms, which is only a semi-quantitative measurement of metabolite recycling. Thus, in contrast with the data shown in Fig. 2(*a*), Woods & Krebs (1971) have observed a large effect of the concentration of glucose on the production of lactate by perfused livers of well-fed rats. The newly recognized stimulator can therefore be qualified as a regulator of phosphofructokinase and of glycolysis in the liver, and its potential role must be considered in comparison with that of other effectors such as fructose 6-phosphate and fructose 1,6-bisphosphate.

As already noted *in vivo* (Van Schaftingen *et al.*, 1980*a*), and again apparent in Fig. 3, there is no clear correlation between the randomization of carbon atoms and the concentration of hexose 6-phosphate in the cells, indicating that the fructose 6-phosphate concentration is not the only factor that controls phosphofructokinase activity. The changes in fructose 1,6-bisphosphate concentration usually follow the variation in phosphofructokinase activity, and it is therefore difficult to decide if they are the cause or the result of these modifications. Furthermore, the major part of fructose 1,6-bisphosphate is presumably bound to various proteins in the liver (Sols & Marco, 1970; Feliú *et al.*, 1977) so that the concentration of free fructose 1,6-bisphosphate is probably very low. The newly discovered stimulator of phosphofructokinase appears therefore as an adequate substitute for fructose 1,6-bisphosphate in the control of glycolysis in the liver.

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