

Role of fructose 2,6-bisphosphate in the control by glucagon of gluconeogenesis from various precursors in isolated rat hepatocytes

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1. Hepatocytes from overnight-starved rats were incubated with 1–20 mM-fructose, -dihydroxyacetone, -glycerol, -alanine or -lactate and -pyruvate with or without 0.1 μ M-glucagon. The production of glucose and lactate was measured, as was the content of fructose 2,6-bisphosphate. 2. The concentrations of fructose (below 5 mM) and dihydroxyacetone (above 1 mM) that gave rise to an increase in fructose 2,6-bisphosphate were those at which a glucagon effect on the production of glucose and lactate could be observed. 3. Glycerol had no effect on fructose 2,6-bisphosphate content or on production of lactate, and glucagon did not stimulate the production of glucose from this precursor. 4. With alanine or lactate/pyruvate as substrates, glucagon stimulated glucose production whether the concentration of fructose 2,6-bisphosphate was increased or not. 5. The extent of inactivation of pyruvate kinase by glucagon was not affected by the presence of the various gluconeogenic precursors. 6. The role of fructose 2,6-bisphosphate in the effect of glucagon on gluconeogenesis from precursors entering the pathway at the level of triose phosphates or pyruvate is discussed.

Fructose 2,6-bisphosphate is a very powerful stimulator of liver phosphofructokinase and, under aerobic conditions, changes in its concentration in the liver have been related to changes in the glycolytic flux. It disappears after the addition of glucagon to hepatocytes. This is explained by the inactivation of phosphofructokinase-2 and activation of fructose 2,6-bisphosphatase resulting from the phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. It is by this action that glucagon controls glycolysis and gluconeogenesis at the level of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle (for a review see Hers & Hue, 1983).

In a previous study (Hue *et al.*, 1982) it was shown that, when hepatocytes from fed rats were incubated with high concentrations (20 mM) of various gluconeogenic precursors, the concentration of fructose 2,6-bisphosphate was decreased,

thus favouring the gluconeogenic pathway. On the other hand, it has been reported (Katz *et al.*, 1975; Clark *et al.*, 1979) that either 2.5 mM-fructose or 10 mM-dihydroxyacetone caused an increase in $^3\text{H}_2\text{O}$ production from [$3\text{-}^3\text{H}$]glucose, indicating that the phosphorylation of fructose 6-phosphate was increased. The present work was undertaken to see whether a small concentration of gluconeogenic precursors could affect fructose 2,6-bisphosphate content. The study was performed with hepatocytes from starved rats, which contain very low concentrations of fructose 2,6-bisphosphate in the absence of substrate and in which an increase in fructose 2,6-bisphosphate is easily detectable.

In addition, there are several reports in the literature (Ross *et al.*, 1967; Exton & Park, 1969; Venezia, 1972; Garrison & Haynes, 1973; Pilkis *et al.*, 1976) indicating that glucagon has no effect on gluconeogenesis in liver preparations incubated with high concentrations (above 5 mM) of fructose or, at least in the starved state, with glycerol. Our purpose was to study the role of fructose 2,6-bisphosphate in the control of gluconeogenesis by glucagon and, more particularly, to see whether changes in fructose 2,6-bisphosphate content are related to the stimulation of glucose production by glucagon.

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Materials and methods

Preparation and incubation of hepatocytes

Hepatocytes from overnight-starved rats were prepared as described previously (Hue *et al.*, 1978). After 20 min of incubation at 37°C in the presence of O₂/CO₂ (19:1), the gluconeogenic precursors were added to the cell suspension (2 ml, containing about 100 mg of cells). The incubation was continued for the indicated periods of time in the presence or absence of 0.1 μM-glucagon. For the measurement of glucose and lactate, hepatocytes were incubated for 15 or 30 min and the reaction was stopped by the addition of 0.5 ml of ice-cold 2 M-HClO₄ to the cell suspension. For the measurement of fructose 2,6-bisphosphate, samples of the cell suspension were taken after 15 min of incubation, frozen in acetone/solid CO₂ and processed further as described below.

Measurement of fructose 2,6-bisphosphate

The method currently used for measuring fructose 2,6-bisphosphate is based on its ability to stimulate phosphofructokinase activity (Van Schaftingen *et al.*, 1980; Richards & Uyeda, 1980; Pilkis *et al.*, 1981). Since tissue extracts contain metabolites, active on phosphofructokinase, the concentration of which can vary under the conditions studied, this method may give erroneous results. We have developed a more specific method which is based on the enzymic measurement of fructose 2,6-bisphosphate after its purification and hydrolysis to fructose 6-phosphate; this method avoids the interference by other metabolites (Hue *et al.*, 1982). During the course of the present study, another very sensitive method of measuring fructose 2,6-bisphosphate was discovered. It is based on the stimulation by fructose 2,6-bisphosphate of pyrophosphate:fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90) from potato tubers (Van Schaftingen *et al.*, 1982). Because of its great sensitivity, it allows the measurement of fructose 2,6-bisphosphate in the pmol range, i.e. when the concentrations of other metabolites are very diluted.

The first method was used in experiments reported in Figs. 1, 2 and 3. The method described by Van Schaftingen *et al.* (1982) was applied to the experiments reported in Figs. 4 and 5. For the first method, extracts were prepared from frozen samples as described by Hue *et al.* (1982). For the second method, 0.1 ml samples of frozen cell suspension were thawed in 0.1 ml of 50 mM-NaOH and kept at 80°C for 10 min. After cooling and addition of 0.2 ml of water, the extracts were neutralized by adding 0.05 ml of 25 mM-acetic acid and 0.05 ml of 0.1 M-Tris, pH 7.5.

It has been checked that the two methods of measurement of fructose 2,6-bisphosphate gave

very similar results by comparing the contents of fructose 2,6-bisphosphate measured in cells incubated with various concentrations of fructose.

Measurement of metabolites

The concentrations of lactate (Hohorst, 1963) and glucose (Huggett & Nixon, 1957) were measured enzymically in neutralized HClO₄ extracts as indicated. Biochemicals used for these measurements were from Boehringer. The production of glucose and lactate in the presence of gluconeogenic precursors was corrected for the rates observed in the absence of substrates.

Measurement of pyruvate kinase activity

Samples of the cell suspension (0.1 ml, corresponding to about 5 mg of liver cells) were taken after 15 min of incubation and immediately frozen in acetone/solid CO₂. They were homogenized in 100 mM-KF/15 mM-EGTA/5 mM-potassium phosphate/50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4. The activity was measured spectrophotometrically at 340 nm in the presence of a low (0.15 mM) and a saturating (5 mM) concentration of phosphoenolpyruvate as described by Felú *et al.* (1976).

Results

Incubation of hepatocytes from overnight-starved rats, in the presence of various concentrations of fructose, caused a dose-dependent increase in glucose and lactate production (Fig. 1). Maximal rates of glucose (1.8 μmol/min per g) and lactate (1.2 μmol/min per g) production were reached at 10 mM-fructose, whereas the rates reached a plateau at higher concentrations. Half-maximal effects were obtained with 2–4 mM-fructose. Small concentrations (2–5 mM) of fructose produced a large increase in fructose 2,6-bisphosphate concentration, which became similar to that obtained after addition of 10 mM-glucose. Concentrations of fructose higher than 10 mM did not cause an increase in fructose 2,6-bisphosphate. In the presence of glucagon, the rate of glucose formation was significantly increased at concentrations of fructose less than 10 mM. In addition, the concentration of fructose 2,6-bisphosphate was significantly decreased, as was the production of lactate. No effect of glucagon on glucose production and fructose 2,6-bisphosphate was observed at 10–20 mM-fructose (Fig. 1).

With dihydroxyacetone as a substrate (Fig. 2), the production of glucose increased with the concentration of the precursor to reach a maximal value (1.6 μmol/min per g) at concentrations of 10 mM or higher; half-maximal effect was observed at 2 mM-dihydroxyacetone. In contrast, the same relationship did not hold for lactate production,

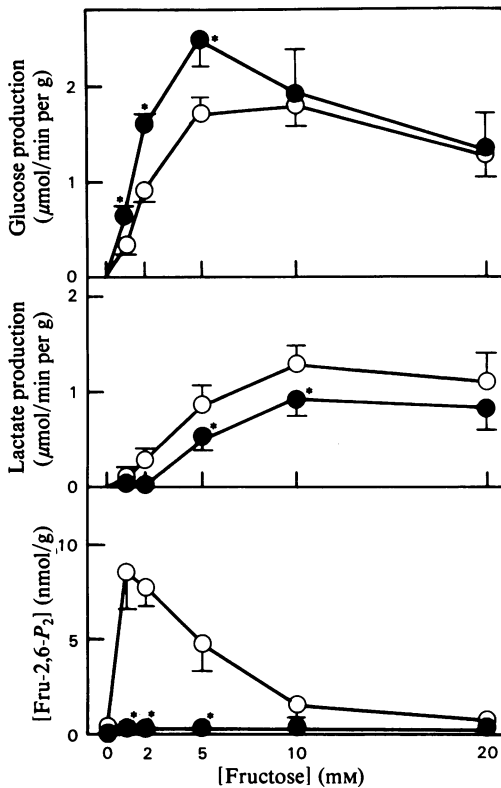


Fig. 1. Influence of $0.1 \mu\text{M}$ -glucagon (●) on the production of glucose and lactate, and on the concentration of fructose 2,6-bisphosphate (Fru-2,6-P₂), in hepatocytes from starved rats incubated with various concentrations of fructose. Hepatocytes were incubated for 15 min in the presence of fructose. Values shown are means \pm S.E.M. for four separate experiments; * indicates a statistically significant difference (*t* test for paired data: $P < 0.05$).

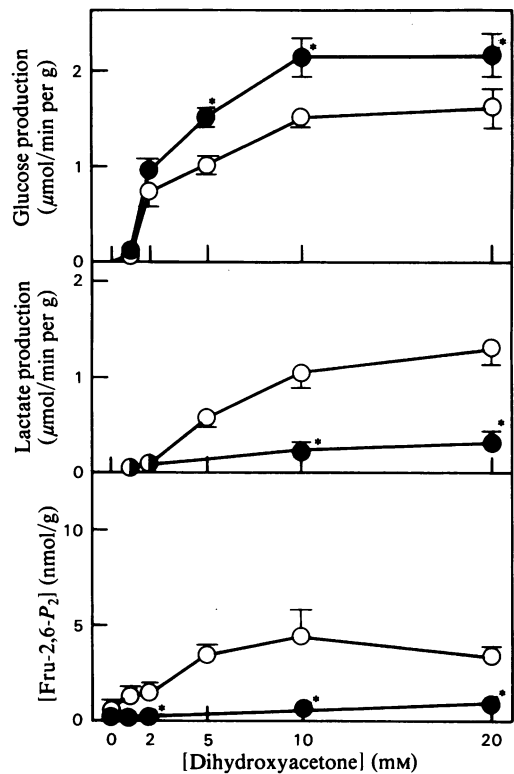


Fig. 2. Influence of $0.1 \mu\text{M}$ -glucagon (●) on the production of glucose and lactate, and on the concentration of fructose 2,6-bisphosphate (Fru-2,6-P₂), in hepatocytes from starved rats incubated with various concentrations of dihydroxyacetone.

Hepatocytes were incubated for 15 min in the presence of dihydroxyacetone. Values shown are means \pm S.E.M. for four separate experiments; * indicates a statistically significant difference (*t* test for paired data: $P < 0.05$).

which was very small at concentrations of dihydroxyacetone below 2 mM and increased progressively with higher concentrations. Dihydroxyacetone caused an increase in fructose 2,6-bisphosphate concentration, reaching a maximum at 10 mM-dihydroxyacetone. In the presence of glucagon and concentrations of dihydroxyacetone higher than 2 mM, the production of glucose was stimulated, whereas the production of lactate and the concentration of fructose 2,6-bisphosphate were decreased.

When glycerol was the precursor (Fig. 3), glucose production increased in a dose-dependent fashion (maximal rate $0.7 \mu\text{mol}/\text{min per g}$), with a half-maximal effect being observed at a concentration of 0.8 mM. The glucose production from glycerol was not affected by glucagon treatment. There was little or no influence of glycerol, with or without glucagon, on the production of lactate and

on the concentration of fructose 2,6-bisphosphate.

With precursors such as alanine (Fig. 4), and lactate together with pyruvate in the molar ratio 10:1 (Fig. 5), there was also a dose-dependent increase in glucose production, reaching a maximum at substrate concentration of approx. 5 mM. A half-maximal effect was obtained with concentrations of precursors close to 1 mM. These substrates caused a small, but significant, increase in fructose 2,6-bisphosphate content at concentrations of less than 5 mM. Glucagon stimulated glucose production at all precursor concentrations tested; however, when 1 mM-lactate was the substrate, no increase was observed.

The activity of pyruvate kinase is another point of hormonal control of gluconeogenesis, and we wished to know whether the well-known effect of glucagon to inactivate pyruvate kinase (for a review, see Engström, 1978) would be influenced

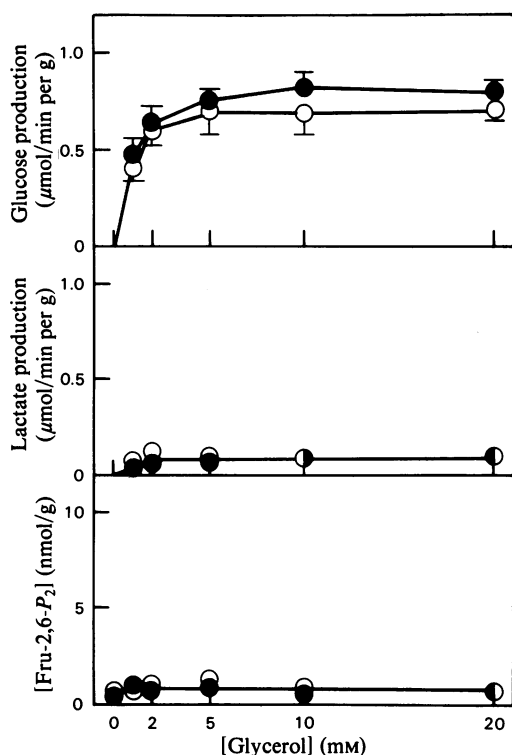


Fig. 3. Influence of $0.1 \mu\text{M}$ -glucagon (●) on the production of glucose and lactate, and on the concentration of fructose 2,6-bisphosphate (Fru-2,6-P₂), in hepatocytes from starved rats incubated with various concentrations of glycerol. Hepatocytes were incubated for 30 min in the presence of glycerol. Values shown are means \pm S.E.M. for four separate experiments.

by the presence of the various gluconeogenic precursors. It appeared that the basal activity of pyruvate kinase, as well as the inactivation brought about by glucagon, was not influenced by the gluconeogenic precursors present in a 1–20 mM concentration range (results not shown).

A series of experiments were performed to see whether ethanol, which is known to cause a fall in fructose 2,6-bisphosphate concentration (Claus *et al.*, 1982), could cause an increase in gluconeogenesis from 2 mM-fructose or 10 mM-dihydroxyacetone. The results, however, showed that 10 mM-ethanol did not stimulate the formation of glucose from the two precursors (results not shown).

Discussion

Effect of gluconeogenic precursors on fructose 2,6-bisphosphate concentration

Some gluconeogenic precursors such as dihydroxyacetone, in the concentration range 2–20 mM, and fructose, at concentrations less than

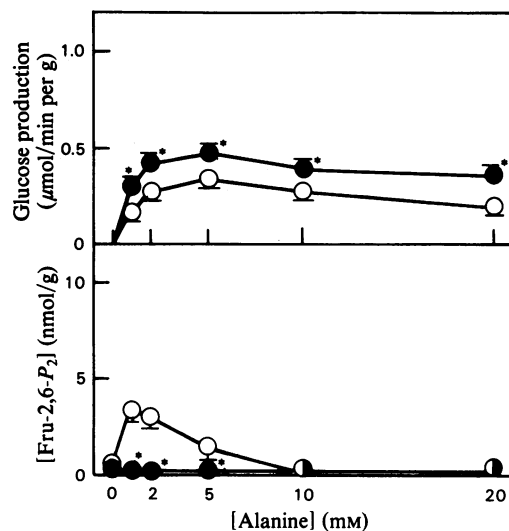


Fig. 4. Influence of $0.1 \mu\text{M}$ -glucagon (●) on the production of glucose, and on the concentration of fructose 2,6-bisphosphate (Fru-2,6-P₂), in hepatocytes from starved rats incubated with various concentrations of alanine.

Hepatocytes were incubated for 30 min in the presence of alanine. Values shown are means \pm S.E.M. for three separate experiments; * indicates a statistically significant difference (*t* test for paired data; $P < 0.05$).

5 mM, caused an increase in fructose 2,6-bisphosphate concentration in hepatocytes from starved rats. The increase after alanine or lactate (1–2 mM) was much smaller than after 1 mM-fructose, and glycerol had no effect. The fact that glycerol and 10–20 mM-fructose did not increase fructose 2,6-bisphosphate in cells from starved rats, but decreased it in cells from fed rats (Hue *et al.*, 1982), may be related to the depletion of adenine nucleotides and P_i that these agents are known to cause in the liver (Van den Berghe, 1978); such a toxic effect has not been found with dihydroxyacetone. Since fructose 2,6-bisphosphate is a powerful stimulator of phosphofructokinase, an increase in fructose 2,6-bisphosphate should result in the stimulation of the flux through phosphofructokinase. Such an increased flux has been observed. Clark *et al.* (1979) have indeed shown that 2.5 mM-fructose, but not 7.5 mM- or 25 mM-fructose, caused a 4-fold increase in the apparent oxidation of [U -¹⁴C]glucose and a 3-fold increase in the production of ³H₂O from [3 -³H]glucose, which allows the measurement of phosphofructokinase flux (Katz & Rognstad, 1976; Hue, 1981). It has also been reported that dihydroxyacetone stimulated the flux through phosphofructokinase (Katz *et al.*, 1975; Rognstad & Katz, 1976; Rognstad, 1981). Previous experiments indicating that phosphofructokinase

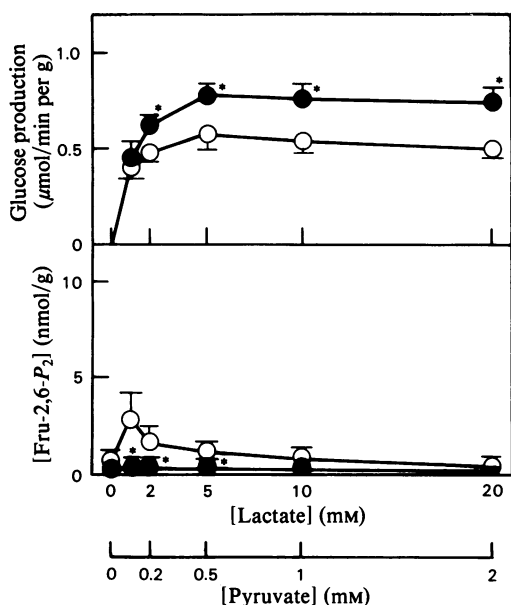


Fig. 5. Influence of $0.1 \mu\text{M}$ -glucagon (●) on the production of glucose, and on the concentration of fructose 2,6-bisphosphate (Fru-2,6- P_2), in hepatocytes from starved rats incubated with various concentrations of lactate and pyruvate

Hepatocytes were incubated for 30 min in the presence of lactate and pyruvate in a molar ratio of 10:1. Values shown are means \pm S.E.M. for three separate experiments; * indicates a statistically significant difference (t test for paired data: $P < 0.05$).

flux was stimulated by fructose and dihydroxyacetone (Clark *et al.*, 1974) are difficult to interpret because the isotope technique used is subject to criticism (Hue & Hers, 1974).

Since the flux through phosphofructokinase is stimulated while there is a net transformation of fructose and dihydroxyacetone into glucose, it follows that futile cycling should occur under these conditions. This conclusion is sustained by the observations by Jarrett *et al.* (1979) and Clark *et al.* (1979), who showed that, in isolated rat hepatocytes, either 2.5 mM-fructose or 10 mM-dihydroxyacetone stimulated heat production more than O_2 consumption. This indicated a less efficient metabolism, which was attributed, at least in part, to an increased cycling at the level of fructose 6-phosphate/fructose 1,6-bisphosphate (Clark *et al.*, 1979). With 7.5 mM- or 20 mM-fructose no such increase in cycling has been observed. Our data suggest that the changes in cycling caused by dihydroxyacetone and small doses of fructose may result from an increase in fructose 2,6-bisphosphate concentrations observed under these conditions.

It is also remarkable that, with 1 mM-fructose, very little lactate is produced despite the presence of relatively high concentrations of fructose 2,6-bisphosphate. This indicates that stimulation of the flux through phosphofructokinase is not sufficient to yield net lactate production; inhibition of fructose 1,6-bisphosphatase is required to diminish futile cycling. Therefore it follows that fructose 1,6-bisphosphatase is less sensitive to fructose 2,6-bisphosphate than is phosphofructokinase, as previously suggested from kinetic and binding studies (Kitajima & Uyeda, 1983).

Effect of glucagon on gluconeogenesis

The rates of glucose formation that we observed in the absence of glucagon are comparable with the values reported in the literature (Ross *et al.*, 1967; Exton & Park, 1969; Garrison & Haynes, 1973; Claus *et al.*, 1975; Söling & Kleineke, 1976). Also in agreement with the data from the literature (Ross *et al.*, 1967; Exton & Park, 1969; Veneziale, 1971, 1972; Blair *et al.*, 1973; Garrison & Haynes, 1973; Clark *et al.*, 1974; Pilkis *et al.*, 1976) is the stimulation by glucagon of the formation of glucose from alanine and lactate, and from dihydroxyacetone and low doses of fructose, but not from glycerol or high doses of fructose. We were, however, unable to obtain a stimulation of glucose formation from 1 mM-dihydroxyacetone or -lactate.

The most striking observation is that, with precursors entering the pathway at the level of triose phosphates, the concentrations of fructose and dihydroxyacetone which gave rise to an increase in fructose 2,6-bisphosphate are those at which an effect of glucagon on the production of glucose was observed. In addition, glucagon did not stimulate the formation of glucose from glycerol, which did not increase the concentration of fructose 2,6-bisphosphate. In all these cases, the inactivation of pyruvate kinase by glucagon was the same, and thus cannot explain the different effect of glucagon. Therefore it is very tempting to suggest that an increase in fructose 2,6-bisphosphate concentration is a prerequisite for the glucagon action on gluconeogenesis from substrates entering at the level of triose phosphates. The evidence is, however, merely circumstantial, and the importance of pyruvate kinase inactivation cannot be ruled out. After glucagon treatment, lactate production from dihydroxyacetone was indeed greatly diminished, whereas with fructose the production of lactate was slightly decreased at concentrations less than 10 mM-fructose, but not for higher concentrations. In the latter condition, fructose 1-phosphate accumulates (Van den Bergh, 1978) and stimulates pyruvate kinase (Eggleston & Woods, 1970). In addition, ethanol, which is known to decrease fructose 2,6-bisphos-

phate, was unable to stimulate glucose production from 2mM-fructose or 10mM-dihydroxyacetone. This lack of effect could, however, result from a shift in the redox state of the cell.

With alanine and lactate entering the pathway at the level of pyruvate, glucagon stimulated glucose production whether fructose 2,6-bisphosphate was present or not. It should also be added that the increase in fructose 2,6-bisphosphate, in the case of lactate, was very small. In this case we may suggest that the most important control is at the level of pyruvate kinase, which becomes inactive and thus re-directs the metabolic flux towards glucose and prevents recycling.

In conclusion, it appears that the physiological importance of two effects of glucagon, of inactivating pyruvate kinase and decreasing fructose 2,6-bisphosphate, depends on the gluconeogenic substrates. With substrates entering at the level of pyruvate, the inactivation of pyruvate kinase seems to be essential, whereas, with substrate entering at the level of triose phosphates, a decrease in fructose 2,6-bisphosphate appears to be more important.

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