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Glucose 6-Phosphate Hydrolysis Is Activated by Glucagon in a Low Temperature-sensitive Manner*

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Glucagon affects liver glucose metabolism mainly by activating glycogen breakdown and by inhibiting pyruvate kinase, whereas a possible effect on glucose-6-phosphatase has also been suggested. Although such a target is of physiological importance for liver glucose production it was never proven. By using a model of liver cells, perifused with dihydroxyacetone, we show here that the acute stimulation of gluconeogenesis by glucagon (10^{-7} M) was not related to the significant inhibition of pyruvate kinase but to a dramatic activation of the hydrolysis of glucose 6-phosphate. We failed to find an acute change in glucose-6-phosphatase activity by glucagon, but the increase in glucose 6-phosphate hydrolysis was abolished at 21 °C; conversely the effect on pyruvate kinase was not affected by temperature. The activation of glucose 6-phosphate hydrolysis by glucagon was confirmed *in vivo*, in postabsorptive rats receiving a constant infusion of glucagon, by the combination of a 2-fold increase in hepatic glucose production and a 60% decrease in liver glucose 6-phosphate concentration. Besides the description of a novel effect of glucagon on glucose 6-phosphate hydrolysis by a temperature-sensitive mechanism, this finding could represent an important breakthrough in the understanding of type II diabetes, because glucose 6-phosphate is proposed to be a key molecule in the transcriptional effect of glucose.

The metabolic effects of glucagon on liver glucose metabolism have long been described, mediated by both cAMP- and calcium-dependent signalings (1–3). Besides a powerful and well documented effect on glycogen breakdown, glucagon also increases liver gluconeogenesis mainly by the allosteric inhibition of pyruvate kinase by its phosphorylation (4, 5). In addition, some effects of glucagon on the fructose phosphate cycle have been described, *i.e.* inhibition of 6-phosphofructo-1-kinase and activation of fructose-1,6-bisphosphatase (6, 7). Apart from these indisputable effects, a stimulation of glucose-6-phosphatase, a key enzyme in glucose production, has been proposed but never proven (8–10). In view of a potential key role of glucose 6-phosphate as a cellular intermediate in the transcriptional effects of glucose (11, 12), an effect of glucagon on glucose-6-phosphatase could be of major importance. Indeed, despite a prominent effect on liver glucose metabolism, the role of glucagon in the pathogenesis of type II diabetes is still unclear and a matter of debate (13).

By studying glucose production under steady state conditions in a model of rat hepatocytes perifused with dihydroxyacetone (DHA)[1] as an exogenous source of carbohydrate, we show in the present work that despite a clear inhibitory effect of glucagon on pyruvate kinase, this effect was not responsible for the enhanced glucose production, indicating that this step is actually not controlling this pathway. Actually, the dramatic increase in the glucose production after glucagon addition was related to a substantial activation of glucose 6-phosphate hydrolysis in glucose and inorganic phosphate. This finding *in vitro* in liver cells was confirmed *in vivo* in postabsorptive rats depleted in glycogen, where we showed that glucagon increased hepatic glucose production whereas liver glucose 6-phosphate concentration decreased. Because we failed to find any change in the glucose-6-phosphatase activity in perifused hepatocyte with or without glucagon and only a minor effect *in vivo*, we hypothesized the possibility of an effect linked to a membrane vesicle traffic-based pathway originating from the endoplasmic reticulum, as it was recently proposed (14, 15). This hypothesis was supported by the finding that the effect of glucagon on glucose 6-phosphate hydrolysis was temperature-sensitive, because it was completely abolished at 15 °C, converse to the effect on pyruvate kinase, which was not affected by temperature. Therefore we propose glucagon to be involved in hepatic glucose 6-phosphate concentration by activating its hydrolysis in a temperature-dependent pathway. Hence this hormone could be involved in the glucose sensing by liver cells *via* its phosphorylated metabolite, glucose 6-phosphate, and in turn in the transcriptional effects of glucose (11, 12). Such a finding could lead to reconsider the role of glucagon in the pathogenesis and treatment of type II diabetes.

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[1] The abbreviations used are: DHA, dihydroxyacetone; *J*, metabolic flux (μmol/min/g of dry cells); (L+P), lactate plus pyruvate; DHAP, dihydroxyacetone phosphate; P-enolpyruvate, phosphoenolpyruvate; phosphoglycerate kinase, EC 2.7.2.3; enolase, EC 4.2.1.11; glucose-6-phosphatase, EC 3.1.3.9; glucose-6-phosphate 1-dehydrogenase, EC 1.1.1.49; glyceraldehyde 3-phosphate dehydrogenase, EC 1.2.1.12; glycerokinase, EC 2.7.1.30; glycerol-3-phosphate dehydrogenase, EC 1.1.1.8; L-lactate dehydrogenase, EC 1.1.1.27; phosphoenolpyruvate carboxykinase, EC 4.1.1.32; phosphoglucoisomerase, EC 5.3.1.9; phosphoglycerate mutase, EC 5.4.2.1; pyruvate kinase, EC 2.7.1.40; HPLC, high pressure liquid chromatography; ANOVA, analysis of variance.

MATERIALS AND METHODS

Male Wistar rats (200–250 g), fasted for 24 h, were anesthetized intraperitoneally with sodium thiopental (125 mg/kg). Hepatocytes were isolated by the method of Berry and Friend (16) as modified by Groen *et al.* (17). Liver cells (200 mg of dry cells in 15 ml) were perifused by the method of van der Meer and Tager (18) as modified by Groen *et al.* (17). Hepatocytes were perifused at 37 or 21 °C as indicated at a flow rate of 5 ml·min^{-1} with Krebs bicarbonate buffer (pH 7.4) continuously saturated with O_2/CO_2 (19:1) and containing calcium (1.3 mM) (19, 20). To study the metabolic effect of glucagon, perifused liver cells were titrated with DHA in the presence or absence of glucagon (10^{-7} M). After a first steady state was reached (45 min) in the absence of DHA, seven successive steady states were obtained in the presence of increasing DHA concentrations (0.15, 0.30, 0.60, 1.20, 2.40, 4.80, and 9.60 mM) as indicated. Each steady state was obtained within 20 min after which both perifusate and cell samples were taken for subsequent analysis. Proteins in the perifusate were denatured by heating the samples (80 °C for 10 min) before centrifugation (21). Glucose, lactate, and pyruvate were measured in the perifusate, and DHAP, glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate, and P-enolpyruvate were measured in the cellular fraction as described previously (20, 22, 23). The net fluxes (μmol/min/g dry cells) of gluconeogenesis ($J_{glucose}$), glycolysis ($J_{(L+P)}$), and DHA metabolism (J_{DHA}) were calculated from the total cell content of the perifusion chamber, the perifusate flow rate, and the concentration of glucose, lactate, and pyruvate in the perifusate. All determinations were made by enzymatic procedures (24) with either spectrophotometric or fluorometric determination of NADH.

The effects of glucagon on cytosolic and mitochondrial adenine nucleotide contents were studied in hepatocytes perifused in the presence of a constant DHA concentration (9.6 mM). After an initial period of 45 min, liver cells were exposed to glucagon (10^{-7} M), and samples were taken from the chamber after 5, 10, 15, 20, and 30 min. The samples of cell suspension were quickly removed from the chamber, and cellular content was separated from the extracellular medium by centrifugation through a layer of silicone oil as described previously (25). The mitochondrial fraction was obtained by liver cell fractionation with digitonin as described (26). Cytosolic adenine nucleotides were calculated by subtraction of the mitochondrial from the total intracellular value. ATP, ADP, and AMP were determined by HPLC (25).

Pyruvate kinase activity was assessed in hepatocytes incubated in closed vials containing Krebs bicarbonate buffer saturated with carbogene and 20 mM dihydroxyacetone with or without glucagon (10^{-7} M) and at 15, 21, or 37 °C as indicated. After 30 min, 500 μl of cell samples were taken from the vials, and pyruvate kinase activity was assessed on cell pellets resuspended in 1.5 ml of a buffer containing 20 mmol/liter potassium phosphate (pH 7.4), 0.25 mol/liter sucrose, 1 mmol/liter EDTA, 1 mmol/liter dithiothreitol. After homogenization for 1 min with an Ultraturax, the sample was centrifuged at 30,000 × *g* for 15 min (Beckman J 21). Pyruvate kinase activity in the supernatant was determined in a solution of 2 ml of buffer containing 50 mM TRIS-HCl (pH 7.4), 20 mM KCl, 5 mM $MgCl_2$, and 10 μl of the supernatant. Partially purified enzyme (L form) was obtained from 0.4 ml of the supernatant, washed with 0.3 ml of 100% $(NH_4)_2SO_4$ (final concentration = 40%), and centrifuged at 30,000 × *g* for 15 min. The pellets were suspended in a medium (2 ml) containing 20 mmol/liter potassium phosphate, pH 7.4, 30% glycerol, 1 mmol/liter EDTA, 1 mmol/liter dithiothreitol, 50 mmol/liter NaF, and pyruvate kinase activity was measured in a buffer containing 50 mM TRIS-HCl (pH 7.4), 20 mM KCl, 5 mM $MgCl_2$ (27, 28). Enzyme activity was expressed as the ratio of activity measured in 0.4 mmol/liter P-enolpyruvate to that of 4 mmol/liter P-enolpyruvate (v/V_{max}), because this ratio has been shown to accurately reflect the phosphorylated state of the enzyme (29). Glucose-6-phosphatase was determined in similar experimental conditions of liver cells incubation as described for pyruvate kinase activity at 37 °C. Glucose-6-phosphatase activity was determined after hypo-osmotic shock and cell disruption by sonication and assayed as described previously (30). The contribution of nonspecific phosphatase activity was estimated via the hydrolysis of β-glycerophosphate under the same assay conditions and subtracted from all measurements.

The metabolic effects of glucagon were also studied *in vivo*. Rats were deprived of food for 5 h with free access to water before each experiment. After the rats were anesthetized, a polyethylene catheter was inserted into the right jugular vein for the infusion of saline or glucagon (60 ng/kg/min), and [3-^{3}H]glucose (specific activity 370 GBq/mmol; Isotopchim, Ganagobie, France). A second catheter was placed in the left carotid artery for blood sampling. Blood glucose was monitored every 10 min during infusions using a glucometer II (Bayer Diagnostics, Puteaux, France). Body temperature was maintained at 37.5 °C by a rectal probe-monitored blanket. After 180 min, a laparotomy was performed to allow free access to the liver. A liver lobe was frozen *in situ* with tongs cooled with liquid nitrogen (−196 °C), weighed, and stored at −80 °C. Blood was collected for the determination of plasma glucose and glucagon concentration and the specific activity of glucose. A local ethics committee for animal experimentation approved this protocol. Glucose-6-phosphatase activity was assayed at 30 °C and pH 7.3 in liver homogenates obtained from freeze-clamped samples as described previously (30). Glucokinase was assayed in 12,000-g supernatants of liver homogenates at 30 °C and pH 7.3, as described by Bontemps *et al.* (31). Glucose 6-phosphate concentration was determined as described by Lang and Michal (32) and glycogen content as described by Keppler and Decker (33). Plasma glucose concentration was determined as indicated above. Hepatic glucose production was assessed from [3-^{3}H]glucose dilution. A bolus (88.8 kBq) was infused during the first minute, and [3-^{3}H]glucose was then infused at 8.88 kBq/min. Plasma [3-^{3}H]glucose radioactivity was measured in triplicate in the supernatants after $ZnSO_4$ and $BaOH_2$ treatment and evaporation to eliminate ^3H_2O. A steady state glucose-specific activity was obtained during the final 60 min of the experiment in rats infused with either saline or glucagon. The rate of disappearance of glucose, which equals the rate of glucose appearance in a steady state, was calculated by dividing the [3-^{3}H]glucose infusion rate by the isotopic enrichment of plasma glucose. Hepatic glucose production was obtained from the rate of glucose appearance.

For statistical analysis, results are expressed as the mean ± S.E. The effects of glucagon effect were assessed by ANOVA (Stat View®; Abacus Concepts, Inc., Berkley, CA, 1992) followed by a Student's *t* test for post-hoc analysis when necessary.

RESULTS

Activation of DHA Metabolism by Glucagon—Fig. 1*A* shows that glucagon activates DHA metabolism, as deduced from glucose and lactate plus pyruvate production ($J_{DHA} = J_{(glucose \times 2)} + J_{(L+P)}$), with only 0.30 mM of infused DHA. Above 2 mM of DHA, J_{DHA} exhibited a plateau with or without glucagon, but J_{DHA} was 30% higher in the presence of glucagon. This stimulatory effect on J_{DHA} was accompanied by a decrease in DHAP concentration at every steady state of infusion of DHA (Fig. 1*B*) allowing us to conclude that glucagon activates the pathway downstream of its first step of phosphorylation of DHA. This is confirmed by the shift to the left of the relationship between the cellular concentration of DHAP and J_{DHA} (Fig. 1*C*); for a given cellular concentration of DHAP, J_{DHA} was higher in the presence of glucagon, confirming the downstream activation of DHAP.

Inhibitory Effect of Glucagon on (L+P) Production from DHA—Glucagon inhibited $J_{(L+P)}$ from DHA, and the extent of inhibition was dependent upon DHA concentration; $J_{(L+P)}$ was almost completely inhibited by glucagon at 0.3 mM DHA (2.9 ± 0.5 *versus* 0.3 ± 0.03 μmol/min/g of dry cells for controls or glucagon). At 0.6 and 1.2 mM DHA the inhibitory effect was 76 and 63%, respectively, whereas at 9.6 mM the inhibition was only 32%. Linear double-reciprocal plots were obtained, as would classically be expected of systems following Michaelis-Menten hyperbolic kinetics (Fig. 2*B*). This allows the extrapolation to an apparent maximal velocity of 10 μmol/min/g of dry cells, which is unaffected by glucagon, whereas the apparent affinity clearly differed, 0.5 mM *versus* 4 mM of DHA for controls and glucagon, respectively. Hence, glycolytic rate was only slightly affected by glucagon at high substrate concentrations.

With the model of perifused hepatocytes, it is possible to assess the kinetics of pyruvate kinase directly in intact cells by determining the relationship between phosphoenolpyruvate concentration and the pyruvate flux under steady state conditions. Indeed under this condition of continuous perifusate rinsing, the pyruvate kinase flux (J_{PK}) can be assumed to be close to $J_{(L+P)}$. As shown in Fig. 3*A*, the classical allosteric inhibition of pyruvate kinase by glucagon was found in intact cells. The maximal inhibition by glucagon was observed between 500 and 1000 nmol P-enolpyruvate/g of dry cells,

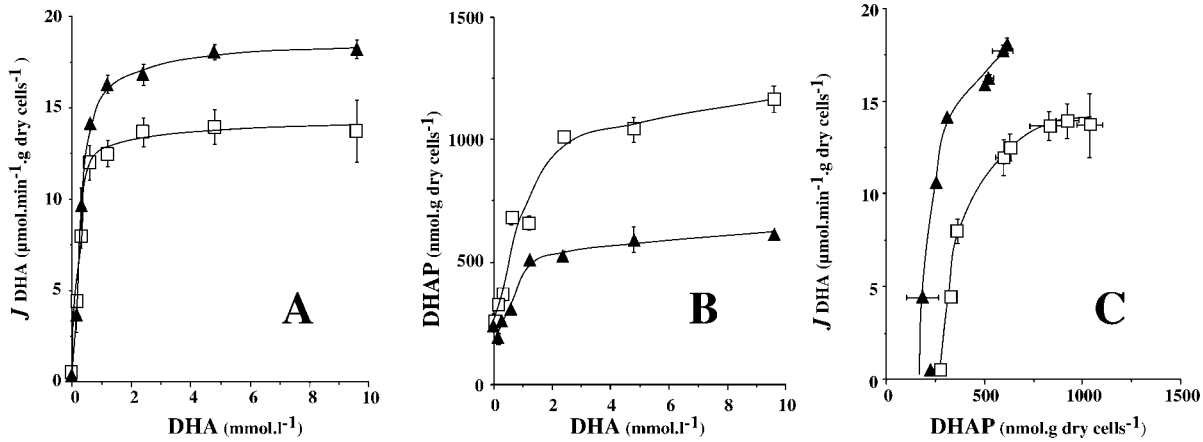
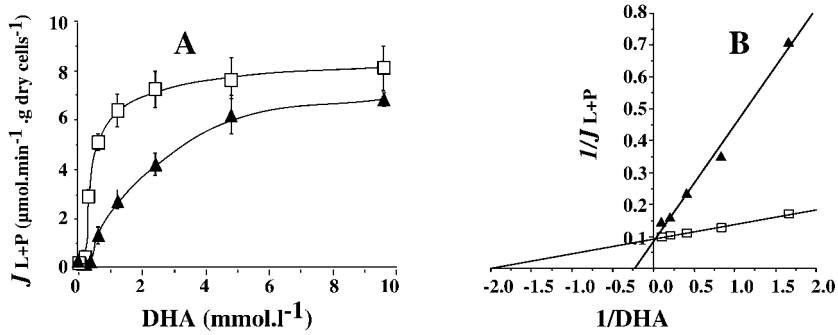


FIG. 1. **Effect of glucagon on DHA metabolism in isolated perifused hepatocytes.** Hepatocytes (200 mg of dry cells in 15 ml), isolated from 24-h-starved Wistar rats, were perifused at 37 °C with 9.6 mM DHA. Flow rate of perifusate was 5 ml·min^{-1} (Krebs-Ringer bicarbonate buffer, pH 7.4) continuously gassed with 95% O_2 − 5% CO_2. Glucagon (10^{-7} M) was continuously infused in one chamber (▲) compared with a control chamber (□). After a first steady state has been reached in absence of exogenous substrate, corresponding to the endogenous rates, DHA was titrated by infusing increasing concentrations (0.15, 0.30, 0.60, 1.20, 2.40, 4.80, and 9.60 mM) as indicated. DHA metabolism (J_{DHA}; *A* and *C*) was calculated from the glucose, lactate, and pyruvate concentrations in the perifusate; J_{DHA} = flow rate × ([glucose] × 2 + [lactate] + [pyruvate]). At each steady state, 0.5-ml samples of cell suspension were removed from the chamber and centrifuged. Intracellular DHAP concentration (*B* and *C*) was measured in the neutralized cell fraction. *Panel A*, effect of glucagon on DHA metabolism; *panel B*, effect of glucagon on steady state concentration of DHAP, first intermediate of the pathway; *panel C*, effect of glucagon on DHA metabolism downstream of DHAP. Results are expressed as the mean ± S.E.; n = 4 per group.

FIG. 2. **Inhibition of glycolysis from DHA by glucagon in hepatocytes perifused.** Hepatocytes (200 mg of dry cells in 15 ml) were perifused as described for Fig. 1 with (▲) or without (□) glucagon (10^{-7} M). *Panel A*, relationship between the rates of glycolysis ($J_{(L+P)}$) calculated from lactate and pyruvate concentrations in the perifusate and steady state concentrations of infused DHA. Results are expressed as the mean ± S.E.; n = 4 per group. *Panel B*, Lineweaver-Burk (double-reciprocal) representation of the relationship between DHA concentration and the flux through the glycolytic pathway.



whereas above this value the fluxes reached almost a similar maximal rate in both groups indicating that the V_{max} was not affected by glucagon. In rat liver cells, phosphoenolpyruvate is present in both the cytosol and the mitochondria, whereas the substrate for pyruvate kinase is in the cytoplasm. The 3-phosphoglycerate (Fig. 3*B*), a purely cytosolic metabolite, is an indicator of cytosolic P-enolpyruvate concentration, because it is in equilibrium with cytosolic P-enolpyruvate. A similar relationship as that reported for P-enolpyruvate confirmed the data presented in Fig. 3*A*. In fact, the linear relationship between 3-phosphoglycerate and P-enolpyruvate in both controls and glucagon-treated cells (Fig. 3*C*) permits us to exclude a significant effect of glucagon on the subcellular distribution of P-enolpyruvate.

When the cellular concentration of DHAP was plotted against $J_{(L+P)}$, to investigate the pathway from DHAP to (L+P) production, it appears that a unique relationship was found with or without glucagon (Fig. 4*A*). This excludes any net effect of the hormone on this part of the pathway, which, however, involves pyruvate kinase. Hence, whereas glucagon inhibited the pathway from P-enolpyruvate to (L+P) production (Fig. 3), this effect was no longer present when the pathway was considered from DHAP (Fig. 4*A*). Fig. 4*B* shows the relationship between the steady state concentrations of DHAP and P-enolpyruvate, which was downshifted in the presence of glucagon; whereas P-enolpyruvate was in a similar range of concentration in both groups, DHAP was lowered by glucagon.

Effect of Glucagon on Redox State and on ATP to ADP Ratio—The steps located between DHAP and P-enolpyruvate are believed to work at near equilibrium under similar experimental conditions (34); therefore the relationship between DHAP and P-enolpyruvate is expected to depend on the cytosolic ATP to ADP ratio (at the phosphoglycerate kinase level; EC 2.7.2.3) and on the redox state (at the glyceraldehyde dehydrogenase level; EC 1.2.1.12). There was no major effect of glucagon on the lactate to pyruvate ratio; therefore a change in cytosolic redox state is unlikely (Fig. 4*C*). Table I shows ATP, ADP, and total nucleotide (ATP + ADP + AMP) concentrations, as well as ATP to ADP ratios in the cytosol and the mitochondrial matrix, with or without glucagon. Although no significant effect was found in the mitochondrial matrix, glucagon significantly lowered cytosolic ADP ($p < 0.01$), leading to a marked increase in the ATP to ADP ratio ($p < 0.01$). Because, converse to the present results (Fig. 4*B*), this effect would increase the ratio of DHAP to P-enolpyruvate, it indicates that this ratio of cytosolic intermediates might not be close to equilibrium with the cytosolic concentrations of ATP and ADP in these conditions. Taken together these results show that the inhibition of pyruvate kinase by glucagon does not explain the inhibition of (L+P) production, because pyruvate kinase is probably not a controlling step of the glycolytic pathway between DHAP and $J_{(L+P)}$.

Effect of Glucagon on Gluconeogenesis from Dihydroxyacetone—Fig. 5*A* shows that glucagon activated the pathway between DHA and glucose production resulting in nearly a 2-fold stimulation in $J_{glucose}$. This effect was because of an activation downstream of DHAP as evidenced by the double

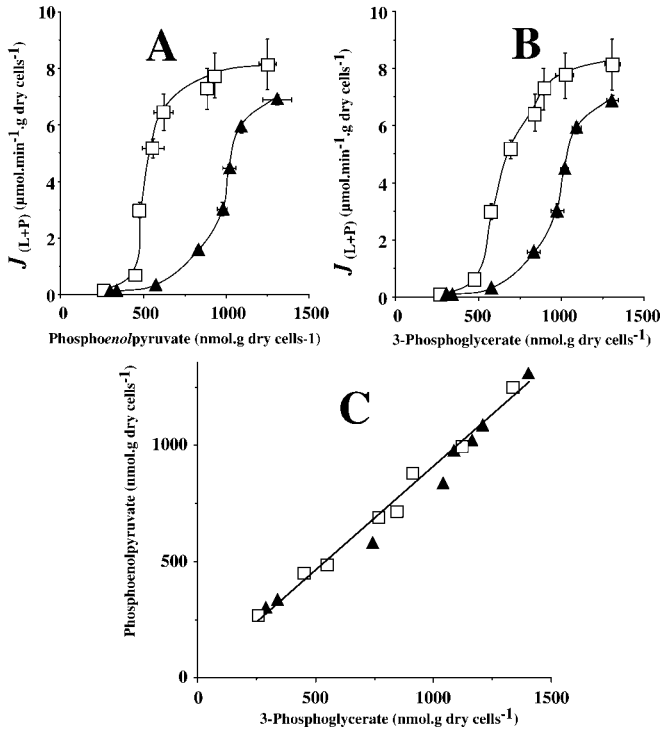


FIG. 3. **Effect of glucagon on pyruvate kinase in intact perifused liver cells.** Hepatocytes (200 mg of dry cells in 15 ml) were perifused as described for Fig. 1 with (▲) or without (□) glucagon (10^{-7} M). *Panel A*, relationship between the rates of glycolysis ($J_{(L+P)}$) and intracellular phosphoenolpyruvate concentrations; *panel B*, relationship between the rates of glycolysis ($J_{(L+P)}$) and 3-phosphoglycerate concentrations; *panel C*, relationship between phosphoenolpyruvate and 3-phosphoglycerate concentrations. Intracellular phosphoenolpyruvate and 3-phosphoglycerate concentrations were measured in the neutralized cell fractions. Results are expressed as the mean ± S.E.; $n = 4$ per group.

relationship between DHAP and $J_{glucose}$ with or without glucagon (Fig. 5*B*). Phosphoglucoisomerase works at near equilibrium under these conditions (Fig. 5*C*), and the dramatic activation of gluconeogenesis by glucagon was because of a stimulatory effect located downstream of glucose 6-phosphate (Fig. 5*D*). Glucose-6-phosphatase activity was determined in our conditions with and without glucagon, and we failed to find any difference according to the presence of the hormone; glucose-6-phosphatase activity was 1654 ± 70 µmol/min/g of dry cell and 1620 ± 74 µmol/min/g of dry cell with or without glucagon, respectively ($n = 6$; not significant). Glucose 6-phosphate hydrolysis is located in the lumen of the endoplasmic reticulum (35, 36), requiring glucose 6-phosphate to be transported from the cytosol into the endoplasmic reticulum and the glucose to be transported back into the cytosol (37). Because glucose-6-phosphatase *per se* is not involved in the effect of glucagon, another step must be involved. In a recent work based on GLUT2-deficient mice, an alternative mechanism for hepatic glucose output other than GLUT2-facilitated diffusion was proposed based on a membrane-traffic mechanism, which was temperature-sensitive (14, 15). We have investigated this possibility, and the data presented in Table II show that the glucagon-related activation of glucose production was inhibited at low temperature in hepatocytes incubated in closed vials. Conversely, the allosteric inhibition of pyruvate kinase by glucagon was not effected by low temperature as assessed by the ratio of v 0.4/v 4 mM P-enolpyruvate (see "Material and Methods"), which was almost identical at 15, 21, and 37 °C. The finding of a low temperature sensitivity of the effect of glucagon on glucose production was confirmed in hepatocytes perifused at 21 °C. (Fig. 6*A*). In this condition the activation of glucose production by glucagon was still present at 21 °C, albeit to a less extent as compared with the effect observed at 37 °C (see Fig. 5). This activation was located downstream of dihydroxyacetone phosphate, as evidenced by the shift to the left of the relationship between DHAP and $J_{glucose}$ in the presence of glucagon (Fig. 6*B*). Interestingly the shape of the relationship between fructose 6-phosphate (Fig. 6*C*) or glucose 6-phosphate (Fig. 6*D*) and $J_{glucose}$ was not affected by glucagon, converse to the results obtained at 37 °C (see Fig. 5*D*). This finding indicates that the effect of glucagon on glucose 6-phosphate hydrolysis and glucose release was temperature-sensitive, because it was present at 37 °C but not at low temperature (21 °C). Conversely, as already shown in Table II, the effect of glucagon on (L+P) production was not affected by the temperature, because it was still present at 21 °C, even at subsaturating DHA concentration (Fig. 6*E*).

Effect of Glucagon on Gluconeogenesis in Vivo—The data from liver cells perifused with dihydroxyacetone are rather far from the actual physiological conditions. Hence, we have undertaken *in vivo* experiments to determine whether glucagon also affects glucose-6-phosphatase under these conditions. Table III shows the results from postabsorptive rats receiving a continuous infusion of glucagon for 180 min. A large and significant increase in blood glucagon concentration was seen, whereas blood glucose did not differ between saline and glucagon groups after 3 h of infusion. Hepatic glucose production was increased 2-fold in the glucagon group, indicating a marked increased in the rate of gluconeogenesis, because liver glycogen was depleted at the time. Although glucokinase was unaffected by glucagon under these conditions, glucose-6-phosphatase activity was significantly increased, the most striking resultant effect being a large decrease in glucose 6-phosphate concentration. Hence the increase in glucose-6-phosphatase flux, as demonstrated by the increased hepatic glucose production, associated with a decreased glucose 6-phosphate concentration, supports the finding of an activation of glucose 6-phosphate hydrolysis by glucagon *in vivo*.

DISCUSSION

In the present work we report that the acute stimulation of liver gluconeogenesis by glucagon was related to a potent activation of glucose 6-phosphate hydrolysis by a low temperature-sensitive pathway, whereas glucose-6-phosphatase activity was activated to a minor extent and only *in vivo* after 3 h of glucagon infusion. Although glucagon affected pyruvate kinase and ATP-to-ADP ratios in our conditions *in vitro*, as expected, the acute activation of glucose 6-phosphate hydrolysis was responsible for both gluconeogenesis activation and glycolysis inhibition at 37 °C. The dramatic activation by glucagon of the gluconeogenic pathway downstream of glucose 6-phosphate was also found *in vivo*, because the activation of gluconeogenesis by this hormone was accompanied by a 2-fold decrease in glucose 6-phosphate concentration.

As it has long been known, glucagon is responsible for an allosteric inhibition of pyruvate kinase by a cAMP-dependent mechanism (4, 5), which was evidenced in the present work in both intact cells and protein extracts, purified or not (see Fig. 3*A* and Table II). It is generally believed that the stimulatory effect of glucagon on glucose production from DHA is mainly the consequence of the inhibition of the glycolytic flux compensating for the increased glucose production in such a way that the total flux (J_{DHA}) is almost unaffected (21, 38, 39). The present finding of a 30% increase of J_{DHA} at 37 °C in the presence of glucagon is not in agreement with this view. From the comparison between Fig. 2*A* and Fig. 4*A* it appears that glucagon was responsible for the inhibition of (L+P) production

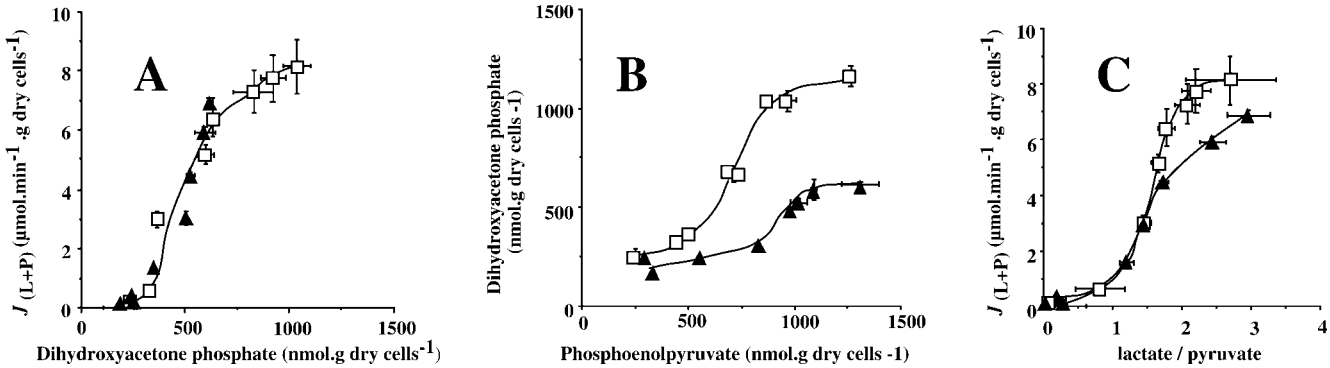


FIG. 4. **Lack of effect of glucagon on glycolytic metabolism of DHAP and on the lactate to pyruvate ratio.** Hepatocytes (200 mg of dry cells in 15 ml) were perifused as described for Fig. 1 with (▲) or without (□) glucagon (10^{-7} M). The rates of glycolysis ($J_{(L+P)}$) and the lactate to pyruvate ratios (lactate/pyruvate) were calculated from lactate and pyruvate concentrations in the perifusate. *Panel A*, relationship between rates glycolysis ($J_{(L+P)}$) and intracellular DHAP concentrations; *panel B*, relationship between intracellular concentrations of P-enolpyruvate and DHAP investigating the effect of glucagon on the pathway between these two intermediates; *panel C*, relationship between lactate to pyruvate ratios (as indicator of cytosolic redox state) and the rates of glycolysis. Intracellular DHAP and P-enolpyruvate concentrations were measured in the neutralized cell fractions. Results are expressed as the mean ± S.E.; $n = 4$ in each group.

TABLE I

Effect of glucagon on adenine nucleotides in isolated perifused hepatocytes

Hepatocytes were perifused for 45 min at 37 °C and then at $t = 0$, 5, 10, 15, 20, and 30 min after the addition of glucagon (or in controls); 0.3-ml samples were removed from the chamber for mitochondria and cytosol separation (see "Materials and Methods"). At the same time, 0.7-ml samples were removed for the separation of extracellular and intracellular compartments by centrifugation through a layer of silicone oil. ATP, ADP, and AMP concentrations were measured in mitochondrial and in total intracellular compartments by HPLC. Cytosolic concentrations were calculated by subtracting mitochondrial values from total intracellular concentrations. Results are expressed as the mean ± S.E. for four different cell preparations. Statistical comparisons were made by ANOVA; the lowering of cytosolic ADP and the increased ATP/ADP ratio by glucagon were significant ($p < 0.01$).

	Time											
	0		5		10		15		20		30	
	Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito
	min											
ATP												
Control	8.5 ± 0.4	2.3 ± 0.4	8.5 ± 0.9	2.3 ± 0.2	8.4 ± 0.6	2.5 ± 0.2	12.1 ± 0.5	2.8 ± 0.2	10.9 ± 1.3	2.3 ± 0.3	9.8 ± 1.3	2.2 ± 0.2
Glucagon	8.3 ± 0.3	2.1 ± 0.3	10.9 ± 0.5	2.4 ± 0.1	8.9 ± 1.5	2.5 ± 0.1	8.9 ± 1.1	2.2 ± 0.2	9.4 ± 1.3	2.5 ± 0.2	9.9 ± 1.3	2.2 ± 0.1
ADP												
Control	1.4 ± 0.2	2.3 ± 0.5	1.3 ± 0.2	2.0 ± 0.1	2.0 ± 0.2	2.2 ± 0.2	2.9 ± 0.5	2.0 ± 0.1	2.2 ± 0.5	1.8 ± 0.1	2.2 ± 0.5	1.6 ± 0.1
Glucagon	1.5 ± 0.2	1.9 ± 0.1	1.0 ± 0.1	2.4 ± 0.2	0.9 ± 0.4	2.1 ± 0.2	0.6 ± 0.2	1.9 ± 0.1	0.8 ± 0.3	1.8 ± 0.1	0.9 ± 0.3	1.6 ± 0.1
ΣAN[a]												
Control	10.5 ± 0.1	3.7 ± 0.5	10.4 ± 1.3	5.7 ± 0.3	9.4 ± 0.9	5.2 ± 0.3	14.2 ± 0.9	5 ± 0.3	12.9 ± 1.7	4.6 ± 0.3	12.1 ± 1.7	4.2 ± 0.3
Glucagon	10.3 ± 0.5	3.3 ± 0.1	12.0 ± 0.7	5.0 ± 0.1	9.4 ± 2.0	5.0 ± 0.1	9.2 ± 1.5	4.4 ± 0.5	10.3 ± 2.1	4.5 ± 0.1	10.8 ± 1.7	4.1 ± 0.1
ATP/ADP												
Control	6.2 ± 1.3	1.0 ± 0.0	6.4 ± 0.3	1.1 ± 0.1	5.3 ± 0.2	1.2 ± 0.1	4.0 ± 0.4	1.42 ± 0.1	5.5 ± 0.5	1.3 ± 0.1	4.5 ± 0.8	1.4 ± 0.1
Glucagon	6 ± 0.6	1.1 ± 0.1	10.8 ± 0.6	1.0 ± 0.1	11.5 ± 3.2	1.2 ± 0.1	14.6 ± 2.2	1.2 ± 0.1	12.2 ± 2.6	1.3 ± 0.1	12.1 ± 2.6	1.4 ± 0.1

[a] ΣAN, ATP + ADP + AMP.

from DHA (Fig. 2*A*) but not from DHAP (Fig. 4*A*). This indicates that the inhibitory effect of glucagon on (L+P) production was because of a lowering of DHAP rather than of its effect on pyruvate kinase. Indeed glucagon was responsible for an activation of the DHA pathway downstream of DHAP *i.e.* between glucose 6-phosphate and glucose (Fig. 5*D*). Hence the effect of glucagon on either glucose or (L+P) fluxes is not because of pyruvate kinase inhibition. The role of pyruvate kinase inhibition by glucagon as a mechanism of explaining the stimulation of gluconeogenesis has been questioned on the basis of a re-evaluation of isotopic tracer studies (40). Using a similar model of perifused hepatocytes, Groen *et al.* (41) have suggested that the stimulating effect of glucagon on gluconeogenesis is almost entirely because of its effect on pyruvate kinase, and this effect is proportional to the flux through pyruvate kinase (41). Conversely, we have found in the present study that the inhibition of pyruvate kinase by glucagon is lessened at high flux and is not responsible for the increased glucose production. The main difference between these two studies relies on the nature of the substrates, lactate/pyruvate in the work by Groen *et al.* (41) and DHA in our study. With lactate/pyruvate as a substrate, the cycling between P-enolpyruvate and pyruvate should likely play a major role in the control of the entire pathway. In contrast, with DHA as a substrate, this cycling is probably very limited because of the low pyruvate concentration achieved by continuous medium rinsing. In the presence of exogenous lactate/pyruvate, cytosolic P-enolpyruvate is provided from cytosolic oxaloacetate by P-enolpyruvate carboxykinase, and its concentration is dependent on a negative feedback mechanism on its own synthesis (*i.e.* its elasticity) (34). This is not likely the case with DHA where P-enolpyruvate is provided from DHAP by a cascade of near equilibrium reactions (34). It is clear that P-enolpyruvate reaches much higher concentrations with DHA (1500 nmol/g of dry cells; see Fig. 3*A*) as compared with the values obtained by Groen *et al.* (41) with lactate/pyruvate (600 nmol/g of dry cells; see Ref. 41). Therefore both views may be reconciled, because (i) the inhibitory effect of glucagon increases whereas P-enolpyruvate concentration rises from 0 to 700 nmol/g of dry cells, and (ii) the lessening of this effect occurs above these values, *i.e.* above the highest concentration reported by Groen *et al.* (41) with lactate/pyruvate (Fig. 3*A*). Nevertheless, if the inhibitory effect of glucagon on pyruvate kinase is not responsible for the decreased (L+P) production then another mechanism must be. Glucagon decreased the

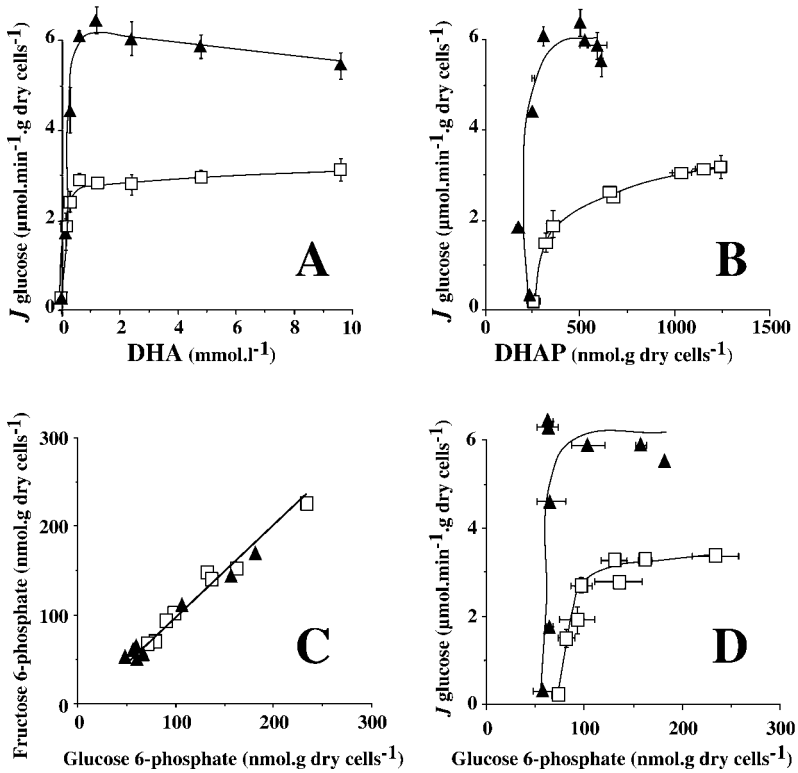


FIG. 5. **Effect of glucagon on gluconeogenesis in isolated hepatocytes perifused with DHA.** Hepatocytes (200 mg of dry cells in 15 ml) were perifused as described for Fig. 1 with (▲) or without (□) glucagon (10^{-7} M). *Panel A*, effect of glucagon on the gluconeogenesis from DHA. The rate of gluconeogenesis ($J_{glucose}$) was calculated from glucose concentration in the perifusate; *panel B*, effect of glucagon on gluconeogenesis downstream of DHAP; *panel C*, linear relationship between fructose 6-phosphate and glucose 6-phosphate; *panel D*, effect of glucagon on glucose 6-phosphate hydrolysis. Intracellular dihydroxyacetone phosphate, fructose 6-phosphate, and glucose 6-phosphate were measured in the neutralized cell fractions. Results are expressed as the means ± S.E.; $n = 4$ per group.

TABLE II

Effect of glucagon on glucose and (L+P) production from DHA and on pyruvate kinase allosteric inhibition at different temperatures

Hepatocytes were incubated in closed vials containing Krebs bicarbonate buffer saturated with carbogene and 20 mM dihydroxyacetone with or without glucagon (10^{-7} M). Temperature of the incubation was 15, 21, or 37 °C as indicated. After 30 min, 500 µl of cell samples were taken from the vials and treated for pyruvate kinase activity as described under "Materials and Methods." At the same time, the rates of glycolysis ($J_{(L+P)}$) and gluconeogenesis ($J_{glucose}$) were calculated from lactate, pyruvate, and glucose accumulations in the cell suspensions. Results are expressed as the mean ± S.E. Statistical comparisons between controls and glucagon were made using a Student's *t* test; *, $p < 0.05$.

	$J_{glucose}$	$J_{(L+P)}$	Pyruvate kinase activity ($V_{0.4}/V_{max}$)	
			Nonpurified	Partially purified
	µmol/min/g dry cell	*µmol/min/g dry cell*		
15 °C				
Control	0.33 ± 0.03	0.90 ± 0.17	0.55 ± 0.02	0.58 ± 0.03
Glucagon	0.37 ± 0.03	0.53 ± 0.03* (−41%)	0.44 ± 0.02* (−20%)	0.47 ± 0.02* (−19%)
	($n = 12$)		($n = 6$)	
21 °C				
Control	3.37 ± 0.10	1.80 ± 0.23	0.60 ± 0.04	0.56 ± 0.07
Glucagon	3.37 ± 0.17	1.20 ± 0.07* (−33%)	0.47 ± 0.02* (−22%)	0.44 ± 0.06* (−21%)
	($n = 6$)		($n = 6$)	
37 °C				
Control	10.17 ± 0.30	7.17 ± 0.57	0.58 ± 0.05	0.64 ± 0.05
Glucagon	13.47 ± 0.60* (+32%)	1.60 ± 0.30* (−78%)	0.31 ± 0.01* (−47%)	0.46 ± 0.01* (−28%)
	($n = 15$)		($n = 6$)	

steady state concentration of DHAP (Fig. 1*C*), such a decrease being responsible for the inhibition of (L+P) production, as it is evidenced by the unique relationship between DHAP and $J_{(L+P)}$ with or without glucagon (Fig. 4*A*). This indicates that, under our conditions, the hormone had no net effect on this part of the pathway.

Glucagon affects the fructose phosphate cycle by inhibiting 6-phosphofructo-1-kinase and activating fructose-1,6-bisphosphatase (6, 7). From the data at 37 °C it is not possible to exclude an acute role of glucagon on this cycle, the presumed effect being more likely on fructose-1,6-bisphosphatase than on 6-phosphofructo-1-kinase, because the latter is probably inhibited in fasting conditions (42). The marked effect of glucagon on glucose 6-phosphate concentration at 37 °C (Fig. 5*D*) could hide an effect at the fructose phosphate cycling. Indeed, it is quite interesting to note that at 21 °C, a condition where the activation downstream of glucose 6-phosphate is abolished, the activation by glucagon of the gluconeogenic pathway downstream of DHAP remains, evidencing the effect of the hormone at the level of the frucose phosphate cycling (Fig. 6).

The main effect of glucagon is a dramatic activation of glucose release from glucose 6-phosphate. Glucose production is a good reflection of the rate of gluconeogenesis, because glycogen synthesis is almost negligible in comparison to the flux of glucose reported here (43). The lack of effect of glucagon on glucose production in the absence of DHA (Fig. 5*A*) confirms

the classical depletion of liver glycogen after 24 h of fasting. The flux through glucokinase is most likely very low under these experimental conditions because of the continuous washout of glucose; the highest glucose concentration in the perifusate was 0.25 mM, whereas the K_m of glucokinase for glucose is 10 mM (44). Hence, the main effect of glucagon on DHA metabolism appears to be because of an activation of glucose 6-phosphate hydrolysis. This result has been confirmed *in vivo* in postabsorptive state after 3 h of glucagon infusion, because the increased hepatic glucose production was accompanied by a dramatic decrease in the glucose 6-phosphate. The last step in glucose production, hydrolysis of glucose 6-phosphate to glucose and phosphate, is catalyzed by the glucose-6-phosphatase in the lumen of the endoplasmic reticulum (35, 36). Considering the modest activation (15%) of glucose-6-phosphatase activity *in vivo* after 3 h of glucagon infusion and the lack of change *in vitro*, it is not likely that glucagon has any acute effect on glucose-6-phosphatase activity. The topological organization of glucose-6-phosphatase in the lumen of the endoplasmic reticulum requires glucose 6-phosphate, produced in the cytosol, to be transported into the endoplasmic reticulum for hydrolysis. It is then assumed that glucose is transported back into the cytosol for its release out of the liver cells by facilitated diffusion through the plasma membrane glucose transporter GLUT2 (37). On GLUT2-deficient mice Guillam *et al.* (14, 15) have shown that hepatic glucose production does not need the presence of GLUT2 in the plasma membrane nor of any other facilitated diffusion mechanism for glucose. This finding led them to propose an alternative mechanism for hepatic glucose output based on a membrane-traffic mechanism. Indeed this group has proposed the existence of two pathways for glucose release, one temperature sensitive and one relying on diffusion of glucose through the plasma membrane transporter GLUT2. On the basis of the effects of glucagon presented in the present work, substantial activation of gluconeogenesis downstream of glucose 6-phosphate, lack of effect on glucose-6-phosphatase, and suppression of the activation at low temperature (contrasting with the lack of effect of temperature on pyruvate kinase inhibition), we would like to propose that glucagon activates the temperature-sensitive pathway. As proposed by Guillam *et al.* (15) this pathway could be a direct membrane-traffic mechanism from the endoplasmic reticulum to the plasma membrane. It is of interest to note that the GLUT2-deficient mice are hyperglycemic with markedly elevated plasma glucagon levels (15), a hormonal change that could be viewed as an adaptive mechanism permitting to compensate for the impaired glucose release because of the lack of GLUT2. Furthermore in Fanconi-Bickel disease, where GLUT2 is not functional because of mutations in both alleles of this gene (45), the patients increase both glycemia (46) and glycosuria (47) in response to glucagon, indicating that despite the lack of functional GLUT2, hepatic glucose production can be stimulated by this hormone. It could be noted that our data do not exclude other hypothetical mechanisms. For instance a subtle and fragile activational effect by glucagon on the catalytic site unit of the glucose-6-phosphatase (48) may occur. But it must be noted that such a hypothetical activation seems not to be present *in vitro*, because we failed to find any activation even in permeabilized cells (data not shown), and as judged from our *in vivo*

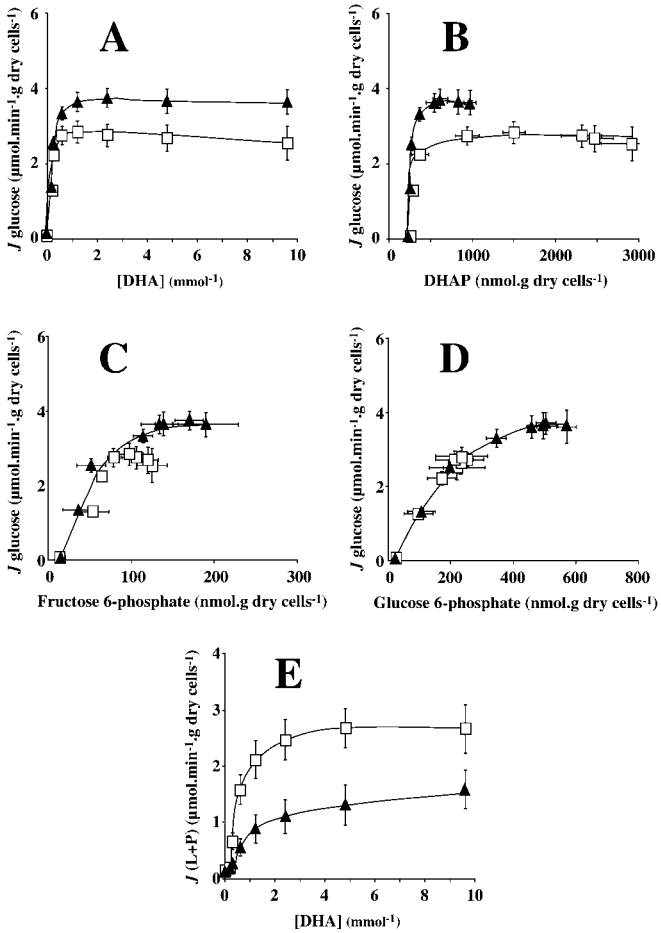


FIG. 6. **Effect of glucagon on gluconeogenesis at low temperature.** Hepatocytes (200 mg of dry cells in 15 ml) were perifused as described for Fig. 1 with (▲) or without (□) glucagon (10^{-7} M) except for the temperature of the perifusate, which was 21 °C. *Panel A*, effect of glucagon on the gluconeogenesis from DHA; *panel B*, effect of glucagon on gluconeogenesis downstream of DHAP; *panel C*, effect of glucagon on the relationship between fructose 6-phosphate and glucose production; *panel D*, effect of glucagon on the relationship between glucose 6-phosphate and glucose production; *panel E*, effect of glucagon on the rate of glycolysis ($J_{(L+P)}$). Intracellular dihydroxyacetone phosphate, fructose 6-phosphate, and glucose 6-phosphate were measured in the neutralized cell fractions. Results are expressed as the means ± S.E.; $n = 3$ per group.

TABLE III
In vivo effects of glucagon on rat liver glucose metabolism

Postabsorptive rats were anesthetized and equipped with arterial and venous catheters, and a laparotomy was performed (see "Materials and Methods") to allow free access to the liver. After 180 min of glucagon infusion, blood samples were taken for glucose and glucagon determination. Hepatic glucose production (HGP) was assessed from [3-^{3}H]glucose dilution. A liver lobe was frozen *in situ* with tongs cooled with liquid nitrogen weighed and stored at −80 °C. Glycogen, glucose 6-phosphate (G6P), glucose-6-phosphatase (G6Pase), and glukokinase were assayed in 12,000-g supernatants of centrifuged liver homogenates. The results are expressed as enzymatic units; 1 unit is the amount of enzyme that converts 1 µmol of substrate per min under the conditions of the assay. Statistical analysis was performed by analysis of variance, and two-tailed unpaired Student's *t* test was used for *post-hoc* analysis. NS, not significant.

Infusion	Glucose	Glucagon	HGP	G6Pase	Glucokinase	G6P	Glycogen
	mmol/l	*µmol/l*	*µmol/kg/min*	*µmol/min/g*	*µmol/min/g*	*nmol/g*	*mg/g*
Saline	8.6 ± 0.2	219 ± 30	77 ± 9	8.1 ± 0.1	1.65 ± 0.03	175 ± 9	13.4 ± 5.1
Glucagon	8.6 ± 0.3	877 ± 19	137 ± 7	9.2 ± 0.4	1.56 ± 0.07	63 ± 2	<0.1
	NS	$P < 0.01$	$P < 0.001$	$P < 0.05$	NS	$P < 0.01$	$P < 0.001$

data, its extent seems limited. Moreover this effect must also be temperature-dependent. Glucagon may also affect the auxiliary component of the glucose 6-phosphate system (auxiliary protein or T1 or glucose 6-phosphate transporter; see Refs. 35, 36, 49, and 50), although the effect of glucagon on glucose production was already observed after a few minutes (data not shown).

It is important to consider that besides a clear effect on flux (*i.e.* gluconeogenesis and glycolysis), glucagon markedly affects the cellular concentration of some metabolites. Hence, except at very low and very high $J_{(L+P)}$, *i.e.* where glucagon has almost no effect on pyruvate kinase, phosphoenolpyruvate concentration is 2-fold increased by glucagon for a similar $J_{(L+P)}$ (Fig. 3*A*). In addition, glucagon was responsible for a 60% decrease in glucose 6-phosphate concentration *in vivo* whereas hepatic glucose production was increased 2-fold, and glucose concentration was identical. Therefore, we may consider that one of the major roles of glucagon could be related to its effect on intracellular signaling molecules such as glucose 6-phosphate (51), as it was proposed for glucose-6-phosphatase (52). Indeed this metabolite could play a key role as a signaling molecule to transmit the effect of glucose on gene regulation (12, 53). We have also suggested recently that glucose 6-phosphate could be a crucial signaling metabolite in the short term inhibition of hepatic glucose production under the action of insulin and hyperglycemia (54). In this view, glucagon may act as a modulator of the liver glucose-sensing mechanism. It has been shown that liver glucose-6-phosphatase overexpression is responsible for several features classically associated with diabetes, glucose intolerance, hyperinsulinemia, and decreased liver glycogen (55). Glucagon is also known to be significantly increased in diabetes (56, 57). Hence the results herein connecting glucagon and glucose 6-phosphate hydrolysis could represent an important clue in the understanding and the treatment of the illness.

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Glucose 6-Phosphate Hydrolysis Is Activated by Glucagon in a Low Temperature-sensitive Manner

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