Stimulation by glucose of gluconeogenesis in hepatocytes isolated from starved rats

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Control properties of the gluconeogenic pathway in hepatocytes isolated from starved rats were studied in the presence of glucose. The following observations were made. (1) Glucose stimulated the rate of glucose production from 20 mM-glycerol, from a mixture of 20 mM-lactate and 2 mM-pyruvate, or from pyruvate alone; no stimulation was observed with 20 mM-alanine or 20 mM-dihydroxyacetone. Maximal stimulation was obtained between 2 and 5 mM-glucose, depending on the conditions. At concentrations above 6 mM, gluconeogenesis declined again, so that at 10 mM-glucose the glucose production rate became equal to that in its absence. (2) With glycerol, stimulation of gluconeogenesis by glucose was accompanied by oxidation of cytosolic NADH and reduction of mitochondrial NAD⁺ and was insensitive to the transaminase inhibitor amino-oxyacetate; this indicated that glucose accelerated the rate of transport of cytosolic reducing equivalents to the mitochondria via the glycerol 1-phosphate shuttle. (3) With lactate plus pyruvate (10:1) as substrates, stimulation of gluconeogenesis by glucose was almost additive to that obtained with glucagon. From an analysis of the effect of glucose on the curves relating gluconeogenic flux and the steady-state intracellular concentrations of gluconeogenic intermediates under various conditions, in the absence and presence of glucagon, it was concluded that addition of glucose stimulated both phosphoenolpyruvate carboxykinase and pyruvate carboxylase activity.

INTRODUCTION

Control of hepatic gluconeogenesis has been the subject of numerous investigations (see for review Hers & Hue, 1983). More recently Groen (1984) and Groen *et al.* (1986) have quantified the amount of control exerted by each of the steps in the gluconeogenic pathway in rat hepatocytes incubated with mixtures of lactate and pyruvate under steady-state conditions. It was concluded that under these conditions control of gluconeogenic flux was shared by both pyruvate carboxylase and pyruvate kinase, the latter enzyme exerting negative control on glucose formation, which means that inactivation of pyruvate kinase results in increased gluconeogenic flux. Addition of glucagon stimulated gluconeogenesis, because of inactivation of pyruvate kinase by phosphorylation; with the hormone present, control of gluconeogenesis was largely confined to pyruvate carboxylase.

In the course of a study on the control properties of the gluconeogenic pathway under more physiological conditions, i.e. with glucose present, we discovered that glucose at low concentrations stimulates net glucose formation. This unexpected phenomenon, which has also been observed by Soley *et al.* (1985), is analysed in the present paper. A preliminary account of this work has appeared (Rigoulet *et al.*, 1985).

MATERIALS AND METHODS

Hepatocytes from 20–24 h-starved male Wistar rats (200–250 g) were isolated by the method of Berry &

Friend (1969), as modified by Groen *et al.* (1982). The rats used in this study were obtained from T.N.O. (Zeist, The Netherlands) and were of a strain different from those used previously by Groen *et al.* (1983, 1986) and Groen (1984).

Hepatocytes (10–40 mg dry wt.) were incubated in 3 ml of Krebs–Henseleit bicarbonate buffer (pH 7.4) containing the components indicated in the legends to Figures and Tables; the gas atmosphere was O₂/CO₂ (19:1) and the temperature 37 °C. After 10 s and after 10, 20 and 30 min, 0.7 ml samples of the cell suspension were taken and rapidly centrifuged through a layer of silicone oil into HClO₄ (10%, w/v). The supernatants were immediately quenched with HClO₄ (final conc. 3.5%).

Glucose was measured spectrophotometrically with ATP, NADP⁺, glucose-6-phosphate dehydrogenase and hexokinase (Bergmeyer, 1970). Glucose assays on the same sample were always run in triplicate, whereas incubations in closed flasks were run in duplicate. Values for duplicate incubations differed by no more than 3%. Lactate and pyruvate contents in the extracellular fluid were measured spectrophotometrically by standard enzymic procedures (Bergmeyer, 1970). Intracellular glucose 6-phosphate, fructose 6-phosphate, dihydroxyacetone phosphate, glycerol 1-phosphate, phosphoenolpyruvate, pyruvate, 3-hydroxybutyrate and acetoacetate were measured fluorimetrically, as described by Bergmeyer (1970).

Perfusion of hepatocytes was carried out by the method of Van der Meer & Tager (1976) with the modifications described by Groen *et al.* (1982). In each steady state, 0.7 ml samples of the cell suspension were

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drawn and subjected to silicone-oil centrifugation; metabolites were determined as described above. Glucose, lactate, pyruvate, 3-hydroxybutyrate and acetoacetate were measured in the perfusate.

Although most gluconeogenic intermediates are exclusively located in the cytosol, phosphoenolpyruvate is an exception. According to Siess *et al.* (1977), about 45% of the intracellular phosphoenolpyruvate is located in the mitochondrial matrix. We confirmed this distribution and found that the cytosolic/mitochondrial phosphoenolpyruvate concentration ratio remained constant under all experimental conditions used in the present study.

The concentration of cytosolic oxaloacetate was calculated from the cytosolic malate concentration as measured by the digitonin fractionation procedure (Zuurendonk & Tager, 1974) and the [lactate]/[pyruvate] ratio, assuming equilibrium in the lactate dehydrogenase and cytosolic malate dehydrogenase reactions (Williamson *et al.*, 1967). The concentration of mitochondrial oxaloacetate was calculated from the mitochondrial malate concentration and from the equilibrium of the reactions catalysed by 3-hydroxybutyrate dehydrogenase and malate dehydrogenase.

Collagenase (type IV), lactate, pyruvate and glucagon were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); other reagents and enzymes were purchased from Boehringer (Mannheim, Germany).

3-Mercaptopicolinic acid was generously given by Smith, Kline and French Laboratories (Philadelphia, PA, U.S.A.).

RESULTS

The effect of added glucose on gluconeogenesis from various substrates is shown in Table 1. Addition of 3 mM-glucose stimulated glucose production from glycerol (97%), and from lactate plus pyruvate (74 and 32% in the absence and the presence of oleate respectively), whereas 44% stimulation was observed in the presence of

Table 1. Effect of glucose on gluconeogenesis from various substrates

Hepatocytes (10–15 mg dry wt./ml) were incubated with the substrates at the concentrations indicated in the Table, in the absence or presence of 3 mM-glucose. The net rate of glucose production was determined during the first 30 min (see the Materials and methods section). Values are the means $\pm$ S.E.M., with the numbers of different cell preparations in parentheses: n.s., not significant; * $P < 0.005$; ** $P < 0.01$.

Substrates	3 mM-Glucose added	Rate of glucose production ($\mu\text{mol/min per g dry wt.}$)	Stimulation (%)
None	—	0.3 ± 0.1 (3)	
	+	0.6 ± 0.5 (3)	n.s.
Glycerol (20 mM)	—	3.8 ± 0.2 (7)	
	+	7.5 ± 0.3 (7)*	97
Glycerol + 1 mM-amino-oxyacetate	—	3.0 ± 0.2 (2)	
	+	6.4 ± 1.0 (2)**	113
Lactate (20 mM) + pyruvate (2 mM)	—	1.9 ± 0.1 (4)	
	+	3.3 ± 0.2 (4)*	74
Lactate + pyruvate + oleate (1 mM)	—	4.4 ± 0.2 (8)	
	+	5.8 ± 0.2 (8)*	32
Pyruvate (20 mM)	—	3.2 ± 0.1 (4)	
	+	4.6 ± 0.4 (4)*	44
Dihydroxyacetone (20 mM)	—	7.3 ± 0.3 (3)	
	+	7.3 ± 0.2 (3)	n.s.
Alanine (20 mM)	—	2.4 ± 0.1 (3)	
	+	2.2 ± 0.3 (3)	n.s.

pyruvate alone. On the other hand, no stimulation by glucose was observed in the presence of dihydroxyacetone or alanine.

It must be pointed out that, owing to the presence of

Fig. 1. Quantitative relationship between the rate of glucose synthesis and the concentration of glucose

Hepatocytes [10.5 mg dry wt./ml, (a); 13 mg dry wt./ml, (b), lower curve; 14 mg dry wt./ml, (b), upper curve] were incubated with 20 mM-glycerol (a) or 20 mM-lactate, 2 mM-pyruvate and 1 mM-oleate (+1% bovine serum albumin) (b). In (b): ○, control; ●, 1 μM -glucagon present. Each point is the mean value ($\pm$ S.D.) of the glucose production rate obtained with four separate incubations. Rates of glucose formation (J_{glucose}) were determined over the first 30 min of incubation (see the Materials and methods section). The data are from representative experiments.

Fig. 2. Relationship between the rate of glucose production from glycerol and the concentration of gluconeogenic intermediates, and the cytosolic and mitochondrial redox state

Hepatocytes (15 mg dry wt./ml) were incubated with 20 mM-glycerol (●) or glycerol plus different concentrations of glucose in concentrations up to 5 mM (×). The data are from experiments with four different hepatocyte preparations. Rates of gluconeogenesis (J_{glucose}) were determined over the first 30 min of incubation (see the Materials and methods section). The intracellular concentration of metabolites is expressed as $\mu\text{mol/g dry wt.}$ Abbreviation: DHAP, dihydroxyacetone phosphate.

added glucose, the differences between the measured glucose values were sometimes small. This is illustrated by the following example. At a hepatocyte concentration of 10 mg dry wt./ml, the production of glucose in 30 min with lactate, pyruvate and oleate as substrates was $5.8 \times 0.010 \times 30 = 1.74 \mu\text{mol/ml}$ in the presence of 3 mM-glucose, so that the glucose concentration of the medium increased from 3 to 4.74 mM. If glucose had not stimulated gluconeogenesis under these conditions, a value of $3 + (4.4 \times 0.01 \times 30) = 4.32 \text{ mM}$ would have been found (cf. Table 1). Although this difference is small (11%), it was detectable. At higher concentrations of added glucose, the relative differences were smaller and standard deviations increased (cf. Fig. 1).

The dependence of gluconeogenic flux on the external glucose concentration is shown in Fig. 1. With glycerol, the rate of glucose production increased with the concentration of added glucose until at 3.5 mM a plateau was reached (Fig. 1a); above 6 mM the rate of glucose production decreased again, so that at 10 mM-glucose it became almost equal to that in the absence of glucose. In the presence of lactate and pyruvate (plus oleate) similar observations were made, except that maximal stimulation by added glucose was obtained at 3 mM in the absence of glucagon and at 5 mM in the presence of the hormone (Fig. 1b). The inhibition of glucose production at high glucose concentrations is due to activation of phosphofructokinase by fructose 2,6-bisphosphate (Hue *et al.*, 1984).

Gluconeogenesis from glycerol

With glycerol as substrate, 90% of flux through glycerol kinase is accounted for by glucose synthesis, whereas only 10% of the carbon skeleton is converted into lactate and pyruvate (results not shown, but see Berry *et al.*, 1973). The effect of glucose on glucose formation from glycerol was analysed in Fig. 2. In these experiments the concentration of glucose was increased from 0 to 5 mM; at the concentration of hepatocytes used (45 mg dry wt. in 3 ml of incubation medium) the rate of glucose formation was constant during the first 30 min of incubation. As shown in Fig. 2(a), the increase in glucose

production rate by glucose addition was accompanied by an increase in the intracellular concentration of dihydroxyacetone phosphate; likewise, the concentrations of intracellular glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate increased after glucose addition (results not shown). Addition of glucose led to oxidation of cytosolic NADH, as indicated by the decreases in the [glycerol 1-phosphate]/[dihydroxyacetone phosphate] and the [lactate]/[pyruvate] ratios (Figs. 2b and 2c), both parameters being indicators of the cytosolic NAD redox state (Williamson *et al.*, 1967). On the other hand, the [3-hydroxybutyrate]/[acetoacetate] ratio, which reflects the mitochondrial NAD redox state (Williamson *et al.*, 1967) increased with increasing glucose concentration (Fig. 2d). Apparently, addition of glucose accelerated the rate of transfer of cytosolic reducing equivalents to the mitochondria. This process is known to be an important rate-controlling step in gluconeogenesis from glycerol (Williamson *et al.*, 1971; Berry *et al.*, 1973), because for the synthesis of each mol of glucose 2 mol of cytosolic NADH must be oxidized by the mitochondria.

In liver, two major mechanisms are involved in the transfer of reducing equivalents from cytosol to the mitochondria: the malate-aspartate shuttle and the glycerol 1-phosphate shuttle [see Meijer & van Dam (1974) for a review]. Stimulation by glucose addition of gluconeogenesis from glycerol was largely insensitive to the transaminase inhibitor amino-oxyacetate (Table 1). It was concluded that glucose addition accelerated the flux of reducing equivalents mainly via the glycerol 1-phosphate shuttle.

Gluconeogenesis from lactate plus pyruvate

In order to analyse the stimulatory effect of glucose on gluconeogenesis from lactate plus pyruvate (10:1), gluconeogenic flux was manipulated over a wide range (between 1.8 and 10 $\mu\text{mol/min per g dry wt.}$) by addition of oleate, glucagon and different concentrations of glucose. Under all conditions addition of glucose increased the concentrations of glucose 6-phosphate, fructose 6-phosphate and phosphoenolpyruvate without

Fig. 3. Relationship between gluconeogenic flux from lactate plus pyruvate and the concentration of gluconeogenic intermediates

Hepatocytes (15 mg/ml) were incubated with 20 mM-lactate plus 2 mM-pyruvate, with no further additions (Δ) or with the addition of 3 mM-glucose ($\blacktriangle$), 1 mM-oleate ($\circ$), 3 mM-glucose and 1 mM-oleate ($+$), 1 mM-oleate and 0.1 μ M-glucagon ($\bullet$), 1 mM-oleate, 0.1 μ M-glucagon and glucose at different concentrations between 0.5 and 5 mM ($\times$). The data are from experiments with three different hepatocyte preparations. Rates of gluconeogenesis (J_{glucose}) were determined over the first 30 min of incubation (see the Materials and methods section). Concentrations of metabolites are expressed as μ mol/g dry wt. Abbreviations: PEP, phosphoenolpyruvate; Pyruvate_{in}, intracellular pyruvate.

Fig. 4. Effect of glucose on the relationship between the rate of lactate + pyruvate consumption and the pyruvate concentration

Hepatocytes (260 mg dry wt. in 12 ml) were perfused with different concentrations of lactate + pyruvate (10:1) in the presence of 5 mM-glucose ($\circ$), of 0.1 μ M-glucagon ($\bullet$) or of 5 mM-glucose + 0.1 μ M-glucagon ($\times$). Lactate and pyruvate concentrations were determined in the perfusate. The data are mean values ($\pm$ S.E.M.) for three different hepatocyte preparations.

affecting the shape of the curves relating gluconeogenic flux and the concentrations of these intermediates (Figs. 3a–3c). This indicates that the kinetic properties of the gluconeogenic enzymes between phosphoenolpyruvate and glucose were not affected by the addition of glucose. In contrast, the curve relating gluconeogenic flux and the intracellular concentration of pyruvate was shifted upwards, not only by glucose but also by glucagon (Fig. 3d). The effect of glucagon is in agreement with previous observations by Groen *et al.* (1983). The stimulatory effects of glucose and glucagon were additive. Hence, at a given intracellular concentration of pyruvate, gluco-

neogenic flux was stimulated by glucose, in both the absence and the presence of glucagon. These results can be explained by activation of one or more steps between pyruvate and phosphoenolpyruvate, such as pyruvate carboxylase, mitochondrial anion translocators and phosphoenolpyruvate carboxykinase, or by inhibition of pyruvate kinase.

For further analysis of the mechanism of the glucose effect, gluconeogenic flux was manipulated by varying the concentrations of lactate and pyruvate. These experiments were performed with the perfusion system (Van der Meer & Tager, 1976), which allows one to use

Fig. 5. Relationship between the rate of gluconeogenesis from lactate plus pyruvate and the concentration of cytosolic oxaloacetate in the absence and presence of glucose.

Hepatocytes (260 mg dry wt. in 12 ml) were titrated with lactate + pyruvate (10:1) in the presence of 0.1 μ M-glucagon and 0.1 mM-oleate. In each study state, 0.7 ml of the cell suspension was removed from the perfusion chamber for rapid separation of mitochondria and cytosol by the digitonin fractionation procedure (see the Materials and methods section). The cytosolic oxaloacetate concentration was calculated from the cytosolic malate concentration as described in the Materials and methods section. The concentrations of lactate, pyruvate and glucose were measured in the perfusate. The data are from experiments with two different hepatocyte preparations, in the absence (●) or the presence (×) of 5 mM-glucose.

low, constant, substrate concentrations. In this system, correct determination of gluconeogenic flux in the presence of 5 mM-glucose is only possible at lactate concentrations above 2 mM; under these conditions the rate of glucose production in each steady state was exactly half the rate of consumption of lactate and pyruvate (results not shown), similar to the situation in the absence of glucose (Groen *et al.*, 1983).

Fig. 4 shows the relationship between the rate of lactate plus pyruvate consumption and the concentration of pyruvate in the perfusate at a [lactate]/[pyruvate] ratio of 10, in the presence of glucose, glucagon or both. In the presence of glucagon, 5 mM-glucose stimulated lactate and pyruvate consumption when the pyruvate concentration was above 0.15 mM (Fig. 4a): below this concentration a slight inhibition was observed. As previously observed by Groen *et al.* (1983), the complex pathway of gluconeogenesis behaved as a single enzyme with simple Michaelis-Menten kinetics (Fig. 4b). Addition of glucose in the presence of glucagon increased the K_m for pyruvate from $45 \pm 10 \mu$ M to $125 \pm 20 \mu$ M ($P < 0.01$), whereas the V_{max} of lactate and pyruvate consumption increased from 20 ± 2 to $27 \pm 3 \mu$ mol/min per g dry wt. ($P < 0.05$). Addition of glucagon in the presence of glucose increased the V_{max} from 13.6 ± 1.5

to $27 \pm 3 \mu$ mol/min per g dry wt. ($P < 0.01$) without any significant effect on the K_m for pyruvate (Fig. 4b).

Although glucose addition did not affect the shape of the curve relating gluconeogenic flux and the steady-state cytosolic phosphoenolpyruvate concentration (cf. Fig. 3), a clear effect was noted when gluconeogenic flux in the presence of glucagon was plotted against the cytosolic oxaloacetate concentration (Fig. 5). At high oxaloacetate concentrations, gluconeogenic flux in the presence of glucose was greater than that in its absence. In this experiment, glucagon was present to minimize flux through pyruvate kinase (Groen *et al.*, 1983).

There are two possible explanations for these observations. Firstly, glucose either directly or indirectly activates phosphoenolpyruvate carboxykinase. Secondly, pyruvate kinase was not completely blocked by glucagon alone, and glucose addition further inhibited this enzyme. This second possibility was eliminated by an experiment (results not shown) similar to that described by Groen *et al.* (1986) (cf. Fig. 3 of that paper) and which indicated that in the presence of glucagon pyruvate kinase was completely blocked. Thus activation by glucose between cytosolic oxaloacetate and phosphoenolpyruvate (Fig. 5) must have been due to activation of phosphoenolpyruvate carboxykinase. This conclusion has two important implications. Firstly, the flux control coefficient of this enzyme in the pathway from lactate to glucose cannot be zero. Secondly, activation of phosphoenolpyruvate carboxykinase must feed back to pyruvate carboxylase (cf. Sistare & Haynes, 1985), since glucose accelerates steady-state flux through the entire gluconeogenic pathway from lactate to glucose.

The flux control coefficient of phosphoenolpyruvate carboxykinase can be directly measured by titrating the rate of glucose formation with the non-competitive inhibitor of this enzyme, 3-mercaptopicolinic acid (DiTullio *et al.*, 1974; Jomain-Baum *et al.*, 1976), a procedure also followed by Groen and co-workers (Groen, 1984; Groen *et al.*, 1986). Fig. 6(a) gives the results of such an experiment, in which increasing concentrations of the inhibitor were infused in the presence of glucagon and saturating concentrations of lactate plus pyruvate (10:1), either in the absence or in the presence of 5 mM-glucose. Assuming equilibration of 3-mercaptopicolinic acid across the plasma membrane, the flux control coefficient of phosphoenolpyruvate carboxykinase can be calculated from the following equation (Groen *et al.*, 1984):

$$C = -\frac{K_i}{J} \left(\frac{dJ}{dI} \right)_{I \rightarrow 0}$$

in which J denotes gluconeogenic flux, I the concentration of the inhibitor and K_i the inhibition constant, which is 3 μ M (Jomain-Baum *et al.*, 1976). This calculation yields values of 0.22 and 0.24 in the absence and the presence of glucose respectively.

As discussed above, stimulation of phosphoenolpyruvate carboxykinase must be accompanied by a stimulation of pyruvate carboxylase as well. In principle, this could be due to removal of inhibitory oxaloacetate or diminution of the inhibitory effect of this metabolite. In order to investigate this, the extent of product inhibition of pyruvate carboxylase by oxaloacetate was determined by titration with 3-mercaptopicolinic acid, and gluconeogenic flux was plotted against the mitochondrial concentration of oxaloacetate at the various 3-mercaptopicolinic acid concentrations.

Fig. 6. Inhibition of gluconeogenesis from lactate + pyruvate by 3-mercaptopicolinic acid

Hepatocytes (210 mg dry wt. in 12 ml) were perfused with 5 mM-lactate, 0.5 mM-pyruvate, 0.1 mM-oleate, 0.1 μM -glucagon and different concentrations of 3-mercaptopicolinic acid, in either the absence (●) or the presence (×) of 5 mM-glucose. Glucose was measured in the perfusate. In the experiment in (b), which was similar to that in (a) but which was carried out with a different hepatocyte preparation, in each steady state a 0.7 ml sample of the cell suspension was taken and subjected to digitonin fractionation (see the Materials and methods section). The malate concentration was determined in the mitochondrial fraction. The concentrations of 3-hydroxybutyrate and acetoacetate were measured in the perfusate. The mitochondrial concentration of oxaloacetate was calculated from the malate, 3-hydroxybutyrate and acetoacetate concentrations (see the Materials and methods section).

picolinic acid concentrations (Fig. 6b). Two curves were obtained, one in the absence and one in the presence of glucose. This indicates that stimulation of flux through pyruvate carboxylase by glucose was not only due to removal of inhibitory oxaloacetate, because in that case one single curve would have been found. As indicated by the two curves, at equal oxaloacetate concentrations gluconeogenic flux was higher in the presence of glucose than in its absence. Apparently, in the presence of glucose inhibition of pyruvate carboxylase by oxaloacetate was diminished because of a change in kinetic properties of the enzyme.

Effect of glucose on the magnitude of the flux control coefficients of the enzymes of the gluconeogenic pathway during glucose synthesis in the presence of lactate, pyruvate and glucagon

As pointed out by Groen *et al.* (1986), in the calculation of the flux control coefficients of the gluconeogenic enzymes the elasticity coefficient of pyruvate carboxylase to its product oxaloacetate plays a crucial role, because this coefficient primarily determines the magnitude of the flux control coefficient of pyruvate carboxylase; the data of Fig. 6(b) allows us to calculate this parameter directly.

The elasticity coefficient, ϵ , of pyruvate carboxylase towards oxaloacetate can be calculated from the equation:

$$\epsilon = \frac{\delta v/v}{\delta [\text{OAA}]/[\text{OAA}]} \quad (\text{at constant pyruvate concentration})$$

Table 2. Distribution of control during gluconeogenesis from 5 mM-lactate plus 0.5 mM-pyruvate in the presence of glucagon; effect of glucose

Flux control coefficients, in the absence and the presence of 5 mM-glucose, were calculated as described by Groen *et al.* (1986). In these calculations pyruvate transport across the mitochondrial membrane was assumed to be in equilibrium (see the text), so that the flux control coefficient of this step was assumed to be zero. Abbreviations: PGM, phosphoglycerate mutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; TIM, triosephosphate isomerase; F1,6Pase, fructose 1,6-phosphatase; PGI, phosphoglucose isomerase.

Step	Flux control coefficient (%)	
	– glucose	+ glucose
Pyruvate carboxylase/ oxaloacetate transport	52	63
Phosphoenolpyruvate carboxykinase	28	20
Enolase/PGM/GAPDH/PGK	1	2
TIM/aldolase/F1,6Pase	18	14
PGI/glucose 6-phosphatase	1	1

In this equation v is the flux through pyruvate carboxylase, which is equal to the rate of glucose formation in the presence of glucagon (which inhibits back flow via pyruvate kinase). From Fig. 6(b) ϵ can be calculated at zero mercaptopicolinic acid. This yields a value of -0.25 in the absence of glucose and of -0.22 in the presence of 5 mM-glucose.

Following the principles outlined by Groen *et al.* (1986) for the calculation of the flux control coefficients of the gluconeogenic enzymes from the elasticity coefficients of these enzymes towards their substrates and products, we have calculated these flux control coefficients from our data for hepatocytes in the presence of lactate, pyruvate and glucagon, in the absence and the presence of 5 mM-glucose. In this calculation we have combined pyruvate carboxylase and oxaloacetate transport from the mitochondria, and we have assumed that the pyruvate translocator operates at thermodynamic equilibrium, so that its flux control coefficient was assumed to be zero. The results of these calculations are given in Table 2.

It is important to note that the flux control coefficient of phosphoenolpyruvate carboxykinase calculated via the elasticity coefficients yields a value similar to that calculated from the titration of gluconeogenesis with 3-mercaptopicolinic acid and its inhibition constant, as described above (see the Discussion section).

DISCUSSION

In hepatocytes isolated from 24 h-starved rats and incubated in the presence of physiological concentrations of glucose (below 10 mM), there is little or no net glycolysis (e.g. Woods & Krebs, 1971; Seglen, 1974; Katz *et al.*, 1975; Hue *et al.*, 1984) and negligible synthesis of glycogen (Hems *et al.*, 1972; Seglen, 1973; Hue *et al.*, 1975; Katz *et al.*, 1976; Solanki *et al.*, 1980).

In the study described in the present paper, we reported evidence that in the same preparation glucose can stimulate its own formation. Stimulation of gluconeogenesis from lactate was only observed at lactate concentrations above 1.5 mM (Fig. 4). In principle, therefore, this stimulation can occur *in vivo* as well, since the lactate concentration in the portal vein of the rat is above 3 mM (Rémésy *et al.*, 1978; Soley *et al.*, 1985). It is unlikely that this effect of glucose is related to the stimulation by glucose of hepatic glycogen synthesis from C₃ precursors after the starved/refed transition (Newgard *et al.*, 1983; Katz & McGarry, 1984), for two reasons. Firstly, the effect on glycogen synthesis occurs when the glucose concentration in the portal vein rises from 4–5 mM (starvation value) to about 8 mM (Newgard *et al.*, 1983; Soley *et al.*, 1985), whereas in our study stimulation of glucose formation was observed between 0 and 3–6 mM (Fig. 1). Secondly, stimulation of glycogen synthesis by glucose is between glucose 6-phosphate and glycogen, not between C-3 and glucose 6-phosphate (Newgard *et al.*, 1983), as in our study. If stimulation of gluconeogenesis by glucose, which we have observed *in vitro* with isolated hepatocytes, does indeed occur *in vivo* as well (cf. Soley *et al.*, 1985), one can only speculate about its possible physiological meaning. One possibility might be that, in addition to the glucagon/insulin ratio, it would provide a rapid mechanism by which the glucose concentration can be kept rather constant near the value of about 5 mM (Fig. 1), the blood glucose concentration in the rat in starvation.

Surprisingly, the stimulation of gluconeogenesis by glucose has not been described previously, although the effect of glucose on this pathway has been studied in several laboratories (e.g. Exton & Park, 1967; Seglen, 1974; Solanki *et al.*, 1980; Rognstad, 1982). However, the following must be stressed. (i) Some studies have been carried out with labelled substrates. As pointed out by Grunnet & Katz (1978), in view of the complexity of lactate metabolism, the use of labelled lactate can sometimes lead to an incorrect quantitative evaluation of glucose production rates. (ii) Most studies have been performed with concentrations of glucose of 10 mM or higher, which, indeed, do not stimulate net glucose production (cf. Fig. 1). (iii) In one case, a small but significant stimulation (12%) by 5 mM-glucose of gluconeogenesis from unlabelled lactate (in the absence of pyruvate) was observed, but not commented on (Seglen, 1974).

The stimulation by glucose of gluconeogenesis from glycerol that we observed in our experiments is almost entirely due to stimulation of the transfer of cytosolic reducing equivalents to the mitochondria, for the following reasons. Firstly, the observed cytosolic and mitochondrial redox changes (Fig. 2) are consistent with this interpretation. Secondly, gluconeogenesis from dihydroxyacetone, which follows a pathway similar to that of gluconeogenesis from glycerol, except that there is no requirement for the disposal of excess of reducing equivalents, was not affected by glucose (Table 1). Thirdly, the rate of glucose formation in the presence of glycerol plus glucose was equal to that in the presence of dihydroxyacetone alone (Table 1). This situation is analogous to that observed in hepatocytes from hyperthyroid rats, in which the activity of the glycerol 1-phosphate shuttle is increased because of an increased

activity of mitochondrial glycerol 1-phosphate dehydrogenase (Werner & Berry, 1974; Rognstad, 1977). It is noteworthy that in pancreatic β -cells, too, addition of glucose results in oxidation of cytosolic NADH, owing to activation of the glycerol 1-phosphate shuttle (Matschinsky *et al.*, 1986).

With regard to the effect of glucose on gluconeogenesis from lactate plus pyruvate, the mechanism is more complex, in that both phosphoenolpyruvate carboxykinase and pyruvate carboxylase must have been activated. Different mechanisms of action of glucose and glucagon are suggested by the fact that stimulation of glucose production from lactate plus pyruvate by these two agents was almost additive (Fig. 1b) and that glucagon, in contrast with glucose, does not stimulate glycerol metabolism (Veneziale, 1972; Garrison & Haynes, 1973; Pilkis *et al.*, 1976).

The effects of added glucose are strikingly similar to some of the effects of noradrenaline. Addition of this hormone, too, accelerates flux through the glycerol 1-phosphate shuttle with glycerol as the substrate (Kneer *et al.*, 1979). We have also found (results not shown) that glucose, like noradrenaline (Ochs & Lardy, 1981), accelerates ethanol oxidation for the same reason. Furthermore, noradrenaline, like glucose, stimulates glucose formation from 10 mM-pyruvate or 10 mM-lactate plus 1 mM-pyruvate in the absence of an appreciable effect on pyruvate kinase and without a change in the cytosolic oxaloacetate concentration (Ochs & Lardy, 1983); this indicates that noradrenaline, too, must have activated both phosphoenolpyruvate carboxykinase and pyruvate carboxylase. On the other hand, an important difference between the two agents is that 5 mM-glucose, in contrast with noradrenaline, did not stimulate glycogen breakdown in hepatocytes isolated from fed rats (results not shown).

The mechanism by means of which addition of glucose results in simultaneous activation of phosphoenolpyruvate carboxykinase, pyruvate carboxylase and the glycerol 1-phosphate shuttle remains an open question. It is as yet not even clear whether glucose itself or a metabolite derived from it is responsible for these effects. In this context it is noteworthy that, in pancreatic islets, glucose stimulation of insulin release appears to be accompanied by an increase in cytosolic free Ca²⁺ after an increase in inositol 1,4,5-trisphosphate, possibly as the consequence of inhibition of its degradation by certain diphosphorylated glucose metabolites (Rana *et al.*, 1986). Whether glucose also affects Ca²⁺ metabolism in hepatocytes remains to be established. If it does, it would provide a satisfactory explanation for many of the similarities between the effects of glucose and noradrenaline, as discussed above.

In order to quantify the amount of control that the gluconeogenic enzymes exert on glucose synthesis, we have followed the methods used by Groen *et al.* (1986). The results of these calculations show that, under our experimental conditions (lactate, pyruvate and glucagon present), flux control was not confined to one enzyme only, but was shared among several steps (Table 2). In the presence of glucose, control was also shared, major control still being exerted by pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Thus the analysis shows that, even after activation of these two enzymes, they still exert major control on flux.

Some of our results differ slightly from those of Groen

(1984) and Groen *et al.* (1986). In their experiments, in the presence of glucagon and saturating concentrations of lactate plus pyruvate, the flux control coefficient of pyruvate carboxylase was 0.83 and that of phosphoenolpyruvate carboxykinase 0.08, whereas in our experiments these values were 0.52 and 0.28 respectively. A possible reason for this quantitative difference is that in our hepatocyte preparations the $V_{\max}$ of phosphoenolpyruvate carboxykinase relative to that of pyruvate carboxylase was about 20% lower than in the preparations used by Groen *et al.* (1983, 1986) and Groen (1984). It is well known that the activity of phosphoenolpyruvate carboxykinase (Tilghman *et al.*, 1976; Krone *et al.*, 1976), in contrast with that of pyruvate carboxylase (Söling & Kleineke, 1976), increases several-fold during starvation, and even the circadian rhythm of the enzyme shows a 2-fold change (Kida *et al.*, 1980). Thus a small change in $V_{\max}$ of phosphoenolpyruvate carboxykinase relative to that of pyruvate carboxylase is easily accounted for. An additional factor may also be that the ratio of the two enzymes is not always the same in different strains of rats. Since the $V_{\max}$ values of the two enzymes in starved rats are of the same magnitude [cf. Groen *et al.* (1983) and also our Figs. 3(d) and 5], a small change in phosphoenolpyruvate carboxykinase relative to that of pyruvate carboxylase will have large effects on the flux control coefficients of these enzymes at saturating lactate and pyruvate concentrations. This phenomenon possibly explains apparently conflicting results obtained in various laboratories with regard to the role of phosphoenolpyruvate carboxykinase in the control of gluconeogenesis (cf. Sistare & Haynes, 1985; Groen *et al.*, 1986).

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