Antagonistic regulation of the glucose/glucose 6-phosphate cycle by insulin and glucagon in cultured hepatocytes*

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Flux through the glucose/glucose 6-phosphate cycle in cultured hepatocytes was measured with radiochemical techniques. Utilization of [2-³H]glucose was taken as a measure of glucokinase flux. Liberation of [¹⁴C]glucose from [U-¹⁴C]glycogen and from [U-¹⁴C]lactate, as well as the difference between the utilization of [2-³H]glucose and of [U-¹⁴C]glucose, were taken as measures of glucose-6-phosphatase flux. (1) At constant 5 mM-glucose and 2 mM-lactate concentrations insulin increased glucokinase flux by 35%; it decreased glucose-6-phosphatase flux from glycogen by 50%, from lactate by 15% and reverse flux from external glucose by 65%, i.e. overall by 40%. (2) Glucagon had essentially no effect on glucokinase flux; it enhanced glucose-6-phosphatase flux from glycogen by 700%, from lactate by 45% and reverse flux from external glucose by 20%, i.e. overall by 110%. (3) At constant glucose concentrations cellular glucose 6-phosphate concentrations were essentially not altered by insulin, but were increased by glucagon by 230%. In conclusion, under basic conditions without added hormones the glucose/glucose 6-phosphate cycle showed only a minor net glucose uptake, of 0.03 $\mu\text{mol/min per g}$ of hepatocytes; this flux was increased by insulin to a net glucose uptake of 0.21 $\mu\text{mol/min per g}$ and reversed by glucagon to a net glucose release of 0.22 $\mu\text{mol/min per g}$. Since the glucose 6-phosphate concentrations after hormone treatment did not correlate with the glucose-6-phosphatase flux, it is suggested that the hormones influenced the enzyme activity directly.

INTRODUCTION

The liver is the glucostat of the organism. It removes glucose when offered in excess as after a normal meal, and it liberates glucose when needed, as in between meals, by glucose-dependent organs such as the brain and the erythrocytes. Whether glucose, as in the classical model (Hue & van de Werve, 1981; Hers & Hue, 1983), or C₃ substrates, as in a more recent model (Hems *et al.*, 1972; Sugden *et al.*, 1983; Newgard *et al.*, 1983, 1984b; Katz & McGarry, 1984), are the main precursors for glycogen synthesis, the decisive parameter for net uptake or release of glucose by the liver is the net flux at the glucose/glucose 6-phosphate 'futile' cycle (Katz & Rognstad, 1976; Hue, 1981, 1982; Hers & Hue, 1983). It is generally believed that the rates of the cycle reactions are determined by the availability of the substrates, i.e. by the concentrations of glucose and glucose 6-phosphate, rather than by a hormone-dependent on/off mechanism (Hue, 1981, 1982; Hers & Hue, 1983). Regulation of the cycle by substrate availability may explain substrate flow into glycogen in the classical and in the zonation model only if a 'pull' (Stalmans, 1976; Hers, 1981) rather than a 'push' (El-Refai & Bergman, 1976; van de Werve & Jeanrenaud, 1984) mechanism is operative. This matter is still highly controversial: the 'pull' mechanism requires a decrease in glucose 6-phosphate and UDP-glucose concentrations triggered by an activation of glycogen synthase; the 'push' mechanism is associated with an increase in glucose 6-phosphate and UDP-glucose and requires inhibition of glucose-6-phosphatase.

The cycle has been studied in the liver *in vivo* and in hepatocyte suspensions from fed and starved animals

(for reviews see Katz & Rognstad, 1976; Hue, 1981, 1982); the direct influence of hormones, however, has so far not been investigated. It was therefore the object of the present investigation to study the regulation of the cycle by insulin and glucagon. Primary cultures of hepatocytes were chosen as the experimental model, because in this system glycolysis is sensitive not only to glucagon but also to insulin (Probst *et al.*, 1982; Probst & Jungermann, 1983). It was found that insulin shifted the cycle to a net uptake, and glucagon shifted it to a net release, of glucose by respectively decreasing or increasing mainly the glucose-6-phosphatase flux. The glucose 6-phosphate concentrations did not correlate with the flux; they were either too high or too low with respect to the measured flux through the enzyme. These findings support the view that the glucose/glucose 6-phosphate cycle is regulated more directly by the pancreatic hormones than by substrate availability. Parts of the present study have been communicated previously in preliminary form (Christ *et al.*, 1984).

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade and obtained from commercial sources. Insulin (bovine), penicillin, bovine serum albumin and chromatography materials were from Serva (Heidelberg, Germany). Glucagon, dexamethasone and streptomycin were supplied by Sigma (Taufkirchen, Germany). Collagenase was purchased from Worthington/Biochrom (Berlin, Germany), and medium 199 and fetal-calf serum were from Boehringer

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(Mannheim, Germany). Radiochemicals were from Amersham-Buchler (Braunschweig, Germany), Quickszint 2000 was from Zinsser (Frankfurt, Germany).

Cell culture

Hepatocytes were isolated from male fed Wistar rats (200 g) by the collagenase perfusion technique (Katz & Jungermann, 1976). Cells were suspended in medium 199, which contained 5 mM-glucose and was supplemented with 4% (v/v) fetal-calf serum, 0.5 nM-insulin and 10 nM-dexamethasone for the first 4 h of culture. The cells were then plated on 60 mm-diam. Falcon culture dishes (35 µg of DNA/2.5 ml per dish). From 4 to 48 h cells were maintained in basal medium 199 containing 5 mM-glucose, 10 nM-insulin and 100 nM-dexamethasone. The gas atmosphere was O₂/CO₂/N₂ (16:5:79) mimicking to a first approximation arterial oxygen partial pressures (Nauck *et al.*, 1981; Wölflé *et al.*, 1983). The medium was changed at 24 h, 32 h and 48 h. DNA was determined as described by Oliver *et al.* (1978).

Radiochemical determination of flux rates

After 48 h cell culture, the dishes were washed twice with insulin-free medium (containing 5 mM-glucose, 2 mM-lactate and 100 nM-dexamethasone) and pre-incubated for 20 min with radiochemicals and hormones as indicated before the zero-time sample was taken. Incubations were continued for 1–4 h. Utilization of radioactive substrates and formation of products were linear with time.

In the first series of experiments, glucose was labelled uniformly with ¹⁴C (1.2 µCi/culture dish) and on C-2 with ³H (2 µCi/culture dish). Flux through glucokinase was determined as the utilization of [2-³H]glucose. The yield of ³H₂O should equal the utilization of [2-³H]glucose, if glucose 6-phosphate totally equilibrated with fructose 6-phosphate. 'Reverse' flux through glucose-6-phosphatase was estimated as the difference between [2-³H]glucose and [U-¹⁴C]glucose utilization (Katz & Rognstad, 1976; Hue, 1981, 1982).

In the second series of experiments, glycogen was prelabelled by supplying the cell cultures from 24 h to 48 h with [U-¹⁴C]glucose (3 µCi/culture dish) (Wölflé *et al.*, 1983). After 48 h cells were washed twice as described above and supplied with fresh medium containing hormones as indicated and [2-³H]glucose (2 µCi/culture dish). Flux through glucokinase was determined as indicated above; flux through glucose-6-phosphatase was measured by the generation of [¹⁴C]glucose from prelabelled glycogen.

In the third series of experiments, [2-³H]glucose (2 µCi/culture dish) and [U-¹⁴C]lactate (0.3 µCi/culture dish) were added. Flux through glucokinase was measured as outlined above; flux through glucose-6-phosphatase was determined from the formation of [¹⁴C]glucose and [U-¹⁴C]lactate.

Radioactive glucose, water and lactate were separated by ion-exchange chromatography. Incubation medium (100 µl) was applied to a double glass column (0.5 cm diameter), the upper column containing both 1 cm of Dowex 1 X 8 (formate form; 200–400 mesh) at the top and 1 cm of Amberlite AG (type CG-120 I; Na⁺ form; 100–200 mesh) at the bottom, the lower column containing 2 cm of Dowex 1 X 8 (borate form; 200–400 mesh). The columns were washed with 2 ml of distilled water; the corresponding effluent contained the ³H₂O.

Glucose was eluted from the borate column with another 2 ml of 1 M-acetic acid. Lactate was separated by applying 100 µl of incubation medium to a separate column (2 cm × 0.5 cm diam.) of Dowex 1 X 8 (formate form; 200–400 mesh). Radioactive glucose and water were eluted with 1.5 ml of water; this fraction was discarded. Elution was continued with 0.4 M-sodium formate. Lactate was quantitatively recovered with the next 1.5 ml of eluent. The identity of [¹⁴C]glucose and [¹⁴C]lactate formed had been verified previously by autoradiography after t.l.c. (Probst *et al.*, 1982). The effluent fractions were collected directly in counting vials, mixed with 12 ml of Quickszint 2000 and counted for radioactivity. The amounts of products formed or substrates utilized were calculated by dividing the total radioactivity (d.p.m.) in a product or substrate fraction by the specific radioactivity (d.p.m./µmol) of the labelled substrate. This was permissible, since there was only a very small dilution of the labelled substrates in the medium during the experimental period (Probst *et al.*, 1982; Wölflé *et al.*, 1983). If prelabelled glycogen was the substrate, the specific radioactivity of [U-¹⁴C]glucose used for the prelabelling was taken into account. This was allowable for the following reasons. (i) It has been shown that those glucose residues added last during synthesis are released first during degradation (Devos & Hers, 1979). (ii) Besides glucose, lactate (pyruvate) may be a major precursor of glycogen. Prelabelling was started at 24 h in a lactate/pyruvate-free medium; it was ended at 48 h when the medium contained 0.5 mM-lactate with essentially half the specific radioactivity of [U-¹⁴C]glucose. Thus glucose residues in glycogen formed from lactate (pyruvate) had the same specific radioactivity as those derived from glucose (results not shown). (iii) If the prelabelling of glycogen was started in a medium containing 2 mM-[U-¹⁴C]lactate in addition to the routinely added 5 mM-glucose, only 20% of the newly formed glycogen was derived from lactate (results not shown), in keeping with a report on glycogen synthesis in cell cultures (Parniak & Kalant, 1985). Thus, even if high concentrations of C₃ substrates were added, dilution of the glucose label in glycogen was only minor; it was negligible in the presence of low concentrations of lactate [see (ii)].

If [U-¹⁴C]lactate was the substrate, a dilution of its specific radioactivity on its pathway to glucose by one-third was taken into account. This procedure was arrived at empirically in previous studies with hepatocyte suspensions (Katz & Jungermann, 1976); it is theoretically permissible if it is assumed that, in the conversion of two lactate molecules into glucose, one-sixth of the carbon atoms are lost owing to equilibration of oxaloacetate with the symmetrical fumarate, and that another one-sixth might be 'lost' owing to dilution with non-radioactive citrate-cycle intermediates.

Flux rates were originally determined as µmol/h per mg of DNA; the data were converted into µmol/min per g wet wt. by using the ratio 2.25 mg of DNA/g wet wt. (Leslie, 1955) in order to facilitate comparison with metabolic studies *in vivo*, in the perfused liver and in cell suspensions.

Assays of enzymes and substrates

Glucokinase (EC 2.7.1.2) and glucose-6-phosphatase (EC 3.1.3.9) were determined in homogenates of the cultured cells, which had been washed with 0.9% NaCl

Table 1. Effect of insulin and glucagon on the flux through the glucose/glucose 6-phosphate cycle in rat hepatocyte cultures

Flux was measured with [2-³H]glucose, [U-¹⁴C]glucose, [U-¹⁴C]glycogen and [U-¹⁴C]lactate as substrates. Cells were preincubated with radiochemicals and insulin (0.1 μ M) or glucagon (0.1 μ M) for 20 min; then the zero-time sample (100 μ l) was taken. Incubation was continued for 1–4 h. Metabolites were separated by ion-exchange chromatography as indicated in the Materials and methods section. Values represent means $\pm$ S.E.M. for the numbers of separate cell preparations given in parentheses, each preparation with three to five culture dishes. Significance of difference from control (paired values; Student's *t* test): ^a*P* < 0.01, ^b*P* < 0.025, ^c*P* < 0.05.

Metabolic parameters	Rate (nmol of C ₆ unit/min per g wet wt.)		
	Insulin	Control	Glucagon
Series 1: labelled substrates, [2- ³ H,U- ¹⁴ C]glucose			
[2- ³ H]Glucose utilization	296 $\pm$ 31 (11) ^a	180 $\pm$ 16 (10)	150 $\pm$ 11 (8)
HO ³ H formation	221 $\pm$ 23 (12) ^a	169 $\pm$ 20 (10)	131 $\pm$ 15 (8)
[U- ¹⁴ C]Glucose utilization	255 $\pm$ 30 (11) ^a	89 $\pm$ 19 (9)	26 $\pm$ 7 (6) ^b
[¹⁴ C]Lactate formation	53 $\pm$ 4 (8) ^b	23 $\pm$ 11 (7)	15 $\pm$ 7 (7)
Series 2: labelled substrates, [2- ³ H]glucose, [U- ¹⁴ C]glycogen			
[2- ³ H]Glucose utilization	426 $\pm$ 37 (6) ^b	312 $\pm$ 36 (6)	289 $\pm$ 40 (8)
HO ³ H formation	261 $\pm$ 19 (7) ^a	211 $\pm$ 19 (8)	188 $\pm$ 15 (8)
[¹⁴ C]Glucose formation	10 $\pm$ 4 (7) ^c	25 $\pm$ 5 (8)	188 $\pm$ 23 (8) ^a
[¹⁴ C]Lactate formation	7 $\pm$ 3 (3)	11 $\pm$ 3 (4)	3 $\pm$ 2 (4) ^c
Series 3: labelled substrates, [2- ³ H]glucose, [U- ¹⁴ C]lactate			
[2- ³ H]Glucose utilization	355 $\pm$ 70 (3) ^c	250 $\pm$ 70 (3)	233 $\pm$ 56 (3)
HO ³ H formation	274 $\pm$ 15 (2)	278 $\pm$ 4 (2)	214 $\pm$ 11 (2)
[¹⁴ C]Glucose formation	83 $\pm$ 15 (8)	94 $\pm$ 15 (8)	135 $\pm$ 26 (8) ^a

as described previously (Probst *et al.*, 1982; Brinkmann *et al.*, 1978). For determination of glucose 6-phosphate, the medium was quickly removed from the culture dishes by suction, and the cells were immediately frozen in liquid N₂. Subsequently 300 μ l of ice-cold 0.02 M-HCl was added per dish on to the frozen cells, which were then scraped off. The resulting homogenate was centrifuged for 15 min in a bench centrifuge (about 8000 *g*) and the supernatant was treated with 2 mg of charcoal. After another 15 min centrifugation in the ultracentrifuge at 100000 *g* the supernatant was neutralized by addition of 10 μ l of 1.5 M-Tris solution and again centrifuged for 15 min at 100000 *g*. The supernatant was used for the determination of glucose 6-phosphate with the glucose-6-phosphate dehydrogenase (EC 1.1.1.49) reaction. The NADPH formed was quantified either by its alkali-increased fluorescence (Katz *et al.*, 1977) or by its *A*₃₄₀ in a double-beam Perkin-Elmer Lambda 3 spectrophotometer. Standards were subjected to the whole procedure. Both methods gave essentially the same results.

In untreated control cultures, the glucose 6-phosphate content was about 50 nmol/g wet wt., which corresponds to a cytosolic concentration of 100 μ M (see Table 2), which should be compared with the values of 135 and 59 nmol/g wet wt. (corresponding to 270 and 118 μ M) found for hepatocyte suspensions from fed and starved animals respectively, and with the values of 131 and 81 nmol/g wet wt. (corresponding to 262 and 162 μ M) reported for freeze-clamped livers from fed and starved rats respectively (Jungermann *et al.*, 1982). The contents in cell cultures were lower than those in fresh hepatocyte suspensions or freeze-clamped livers from fed animals and similar to those in preparations from starved animals, which may be linked to the lower glycogen content in cultured cells (Wölfe *et al.*, 1983) and in livers in the starved state (Jungermann *et al.*, 1982). The comparison is taken to indicate that, during the 1–2 s

required to suck the medium from the culture dish before the freeze-stop in liquid N₂, an artifactual increase in glucose 6-phosphate owing to hypoxia-stimulated glycogenolysis cannot have occurred.

RESULTS

Net flux through the glucose/glucose 6-phosphate cycle is the difference between the total flux through glucokinase (hexokinase) and the glucose-6-phosphatase reaction. Flux through glucokinase has one major source, the extracellular glucose, and one minor source, the glucose formed in the glucose-6-phosphatase reaction. The minor source can be neglected, because normally the glucose pool is large (about 5 mM). Flux through glucose-6-phosphatase has three sources, the glucose 6-phosphate formed via glycogenolysis from glycogen, via gluconeogenesis, e.g. from lactate, and via the glucokinase reaction from glucose. The last flux (reverse flux) cannot be neglected, because the glucose 6-phosphate pool is small (about 0.1 mM). On the basis of these considerations, flux at the cycle was measured in primary cultures of rat hepatocytes under identical substrate conditions with different radiochemical labels: series 1, [2-³H]glucose and [U-¹⁴C]glucose; series 2, [2-³H]glucose and [U-¹⁴C]glycogen; and series 3, [2-³H]glucose and [U-¹⁴C]lactate.

Flux through glucokinase

The disappearance of [2-³H]glucose is a measure of total glucokinase flux, if the [2-³H]glucose 6-phosphate formed equilibrates completely with fructose 6-phosphate, so that the ³H label is quantitatively lost as HO³H and [2-³H]glucose-6-phosphate cannot be recycled to yield [2-³H]glucose. The yield of HO³H averaged 75–80% of the [2-³H]glucose disappearance (Table 1, series 1–3); therefore [2-³H]glucose utilization was a measure of

Fig. 1. Control of the glucose/glucose 6-phosphate cycle in rat hepatocyte cultures by insulin and glucagon

(a) Flux through glucokinase was calculated from the utilization of $[2\text{-}^3\text{H}]\text{glucose}$. (b) Flux through glucose-6-phosphatase starting with $[\text{U-}^{14}\text{C}]\text{glucose}$ was calculated as the difference between $[2\text{-}^3\text{H}]\text{-}$ and $[\text{U-}^{14}\text{C}]\text{-glucose}$ utilization. (c) Flux through glucose-6-phosphatase starting with $[\text{U-}^{14}\text{C}]\text{glycogen}$ was calculated from the formation of $[\text{U-}^{14}\text{C}]\text{glucose}$. (d) Flux through glucose-6-phosphatase starting with $[\text{U-}^{14}\text{C}]\text{lactate}$ was calculated from the formation of $[\text{U-}^{14}\text{C}]\text{glucose}$. Values (means $\pm$ S.E.M.) are calculated (a, b) or taken (c, d) from Table 1; in (a) they represent means $\pm$ S.E.M. for the three series of experiments. The total numbers of experiments are indicated within the bars. Significance of difference from control (paired values in parentheses; Student's *t* test): (a) insulin $P < 0.01$ (17); (b) insulin $P < 0.01$ (8), glucagon $P < 0.01$ (5); (c) insulin $P < 0.05$ (7), glucagon $P < 0.01$ (8); (d) glucagon $P < 0.01$ (8).

glucokinase flux only in a first approximation, leading to a slight underestimation.

Glucokinase flux amounted to an average of $0.24 \mu\text{mol/min per g wet wt.}$ under control conditions without hormones; it was increased by about 35%, to $0.34 \mu\text{mol/min per g wet wt.}$, by insulin, and stayed essentially constant, at $0.22 \mu\text{mol/min per g wet wt.}$, under the influence of glucagon (Fig. 1a).

'Reverse' flux through glucose-6-phosphatase from glucose

The disappearance of $[\text{U-}^{14}\text{C}]\text{glucose}$ is the difference between $[\text{U-}^{14}\text{C}]\text{glucose}$ phosphorylation to $[\text{U-}^{14}\text{C}]\text{glucose 6-phosphate}$ and its hydrolysis back to $[\text{U-}^{14}\text{C}]\text{glucose}$.

glucose. Therefore the difference between $[2\text{-}^3\text{H}]\text{glucose}$ utilization, which is a measure of glucose phosphorylation, and $[\text{U-}^{14}\text{C}]\text{glucose}$ utilization indicates the reverse flux of $[\text{U-}^{14}\text{C}]\text{glucose}$ via $[\text{U-}^{14}\text{C}]\text{glucose 6-phosphate}$ through glucose-6-phosphatase (Table 1, series 1).

Glucose-6-phosphatase 'reverse' flux from glucose was found to be $0.10 \mu\text{mol/min per g wet wt.}$ in the absence of hormones. It was decreased by insulin by 65%, to $0.04 \mu\text{mol/min per g wet wt.}$, but it was slightly increased, to $0.12 \mu\text{mol/min per g wet wt.}$, by glucagon (Fig. 1b).

Insulin acted mainly by causing a slight increase in the utilization of $[2\text{-}^3\text{H}]\text{glucose}$ and a strong enhancement in that of $[\text{U-}^{14}\text{C}]\text{glucose}$; it also clearly stimulated $[\text{U-}^{14}\text{C}]\text{lactate}$ formation (Table 1, series 1). Conversely, glucagon decreased the utilization of $[\text{U-}^{14}\text{C}]\text{glucose}$ more effectively than that of $[2\text{-}^3\text{H}]\text{glucose}$; it also diminished $[\text{U-}^{14}\text{C}]\text{lactate}$ formation (Table 1, series 1).

Flux through glucose-6-phosphatase from glycogen

The formation of $[\text{U-}^{14}\text{C}]\text{glucose}$ from $[\text{U-}^{14}\text{C}]\text{glycogen}$ is a measure of the flux through glucose-6-phosphatase starting from glycogen (Table 1, series 2). Some $[\text{U-}^{14}\text{C}]\text{glucose}$ will be formed directly, circumventing the glucose-6-phosphatase reaction, by hydrolysis of 1,6-linked glucose in the polysaccharide; its contribution will be small and can be neglected.

Glucose-6-phosphatase flux from glycogen was determined to be $0.025 \mu\text{mol/min per g wet wt.}$ under control conditions. It was decreased by insulin by 50%, to $0.01 \mu\text{mol/min per g wet wt.}$, and was increased drastically by glucagon by 700%, to $0.19 \mu\text{mol/min per g wet wt.}$ (Fig. 1c).

Flux through glucose-6-phosphatase from lactate

The formation of $[\text{U-}^{14}\text{C}]\text{glucose}$ from $[\text{U-}^{14}\text{C}]\text{lactate}$ indicates the flux through glucose-6-phosphatase owing to gluconeogenesis (Table 1, series 3). It may be a slight underestimation, since, in addition to gluconeogenesis from exogenous lactate, some glucose might have been synthesized from endogenous substrates such as amino acids and glycerol.

Glucose-6-phosphatase flux from lactate was $0.09 \mu\text{mol/min per g wet wt.}$ in the absence of the glucoregulatory pancreatic hormones. Insulin did not alter the rate significantly, but glucagon increased it by 45% (Fig. 1d).

Fig. 2. Total and net flux through the glucose/glucose 6-phosphate cycle in cultured rat hepatocytes under the influence of insulin and glucagon

The flux rates are given in $\mu\text{mol/min per g wet wt.}$; the values were taken from Fig. 1. Abbreviation: G6P, glucose 6-phosphate.

Table 2. Comparison of measured glucose 6-phosphate concentrations and radiochemically determined glucose-6-phosphatase flux rates in cultured hepatocytes

Glucose 6-phosphate contents were determined after 2 h of incubation and originally related to mg of DNA. They were then converted into cytosolic concentrations by using the following factors: 2.25 mg of DNA/g wet wt. (Leslie, 1955) and 0.5 ml of cytosol/g wet wt. (calculated from Table 1 of Soboll *et al.* 1980). Calculation of the cytosolic concentration was permissible, since it was shown that glucose 6-phosphate is essentially absent from mitochondria (Jungermann *et al.* 1982). Values are means $\pm$ S.E.M., with the numbers of different cell preparations given in parentheses; there were four or five separate dishes per cell preparation. Significance of differences from controls (Student's *t* test): **P* < 0.01; ^{ns}not significant. The flux rates were taken from Fig. 2.

	Glucose 6-phosphate concn.		Glucose-6-phosphatase flux	
	($\mu\text{mol/l}$ of cytosol)	(%)	($\mu\text{mol/min}$ per g wet wt.)	(%)
Insulin	107.6 $\pm$ 6.2 ^{ns} (4)	93	0.13	62
Control	115.6 $\pm$ 7.8 (4)	100	0.21	100
Glucagon	388.5 $\pm$ 41.3 ^a (4)	336	0.44	210

Net flux at the glucokinase/glucose-6-phosphatase cycle as determined by radiochemical techniques

Under control conditions a glucokinase flux of 0.24 $\mu\text{mol/min}$ per g wet wt. was balanced by a total glucose-6-phosphatase flux of 0.21 $\mu\text{mol/min}$ per g wet wt., resulting in a slight net uptake of glucose of 0.03 $\mu\text{mol/min}$ after g wet wt. (Fig. 2). Insulin increased the glucokinase flux to 0.34 $\mu\text{mol/min}$ per g wet wt. and decreased total glucose-6-phosphatase flux to 0.13 $\mu\text{mol/min}$ per g wet wt., and thus drastically enhanced net uptake of glucose, to 0.21 $\mu\text{mol/min}$ per g wet wt. In contrast, glucagon had no influence on the glucokinase flux, but clearly enlarged total glucose-6-phosphatase flux to 0.44 $\mu\text{mol/min}$ per g wet wt., and thus led to a net release of glucose of 0.22 $\mu\text{mol/min}$ per g wet wt. (Fig. 2).

Net flux at the glucokinase/glucose-6-phosphatase cycle as calculated from measured enzyme activities and substrate concentrations

If the flux through glucokinase and glucose-6-phosphatase is controlled only by substrate availability, as is widely believed (Hue, 1981, 1982; Hers & Hue, 1983), the rates should be essentially proportional to the concentrations of the substrates glucose and glucose 6-phosphate respectively. This should be the case as long as the substrate concentrations are lower than the half-saturating concentrations (K_m values) and as long as both enzymes are saturated with their second substrate, ATP and H_2O , respectively. The K_m value reported for glucokinase is 9 mM (Bontemps *et al.*, 1976) and that for glucose-6-phosphatase is 1 mM at pH 7 (Nordlie & Lygre, 1966), which can be regarded as the cytosolic pH of liver cells (Soboll *et al.*, 1980). Saturation with the second substrate can be assumed for both enzymes.

In all experiments the concentration of glucose was held constant at 5 mM in the extracellular space and thus also in the intracellular space (Cahill *et al.*, 1958). Yet the glucokinase flux determined with the radiochemical techniques was the same only in the controls and with glucagon, but not with insulin (Fig. 2). The concentrations of glucose 6-phosphate in the cytosol were determined to be 108 and 116 μM with insulin and in the controls respectively, i.e. essentially the same under these two conditions, but they were increased to 389 μM by

glucagon (Table 2). Yet the total glucose-6-phosphatase flux was 0.13, 0.21 and 0.44 $\mu\text{mol/min}$ per g wet wt. respectively with insulin, in the controls and with glucagon (Fig. 2).

The validity of the approach to compare substrate concentrations with measured flux rates may be indicated by the observation that the flux rates calculated by using the Michaelis-Menten equation $v = (V \times s)/(K_m + s)$ were in the range of the measured rates. The values of V of glucokinase and of glucose-6-phosphatase were determined in the cell cultures and found to be 0.72 and 1.78 $\mu\text{mol/min}$ per g wet wt. With the K_m values and substrate concentrations outlined above, the glucokinase flux under control conditions was calculated to be 0.24 $\mu\text{mol/min}$ per g wet wt., which agrees with the flux determined with the radiochemical technique (Fig. 2); the glucose-6-phosphatase flux in the controls was calculated to be 0.19 $\mu\text{mol/min}$ per g wet wt., which is close to the radiochemical flux of 0.21 $\mu\text{mol/min}$ per g wet wt. (Fig. 2).

Clearly, the measured rates were not proportional to the substrate concentrations (Table 2). For glucose-6-phosphatase the discrepancy is even more pronounced if the substrate concentrations are compared not with the total but with the partial glucose-6-phosphatase flux from glucose, glycogen and lactate (Figs. 1b, 1c and 1d). These observations can be taken to indicate that other mechanisms besides substrate availability could be involved in the regulation of the glucokinase and glucose-6-phosphatase flux.

DISCUSSION

In the present investigation it was shown in rat hepatocyte cultures that the cyclic flux between glucose and glucose 6-phosphate is shifted by insulin to net glucose uptake and by glucagon to net glucose release (Fig. 2).

Net flux and 'reverse' flux at the glucose/glucose 6-phosphate cycle

Cycling between glucose and glucose 6-phosphate has been shown to occur in rat liver *in vivo* (Katz *et al.*, 1976), in the isolated perfused organ (Clark *et al.*, 1975) and in hepatocyte suspensions (Clark *et al.*, 1973, 1975;

Bontemps *et al.*, 1976; Katz *et al.*, 1978); the problem has not been studied in cell cultures previously. The previous studies investigated the fed and starved states, in which the cycle operated in the direction of net glucose release; the eating state, in which the cycle should operate in the direction of net glucose uptake, was not studied. The previous investigations used only the labelled substrate pair [2-³H]- and [U-¹⁴C]-glucose, and determined the net release of glucose. This approach allowed quantification of the glucokinase flux and the total glucose-6-phosphatase flux (net release of glucose plus apparent glucokinase flux; Katz *et al.*, 1978; Clark *et al.*, 1973) as well as the 'reverse' glucose-6-phosphatase flux (recycling of glucose 6-phosphate just formed by glucokinase from external glucose back to glucose); it did not allow, as did the approach in the present study, discrimination between the glycogenolysis- and the gluconeogenesis-dependent glucose-6-phosphatase flux.

The previous results obtained in perfused livers and isolated hepatocytes and the present findings using cell cultures can be best compared if one assumes that livers and hepatocytes from fed animals correspond, to a first approximation, to the control cultures and that preparations from starved animals correspond to glucagon-stimulated cultures. The absolute metabolic rates at the glucose/glucose 6-phosphate cycle were quite variable, but were higher in the perfused liver and in cell suspensions than in cell cultures, which in general exhibit lower specific enzyme activities; the relative rates, however, were quite comparable.

Thus the 'reverse' glucose-6-phosphatase flux (defined two paragraphs above) compared with the glucokinase flux amounted to about 70% in livers from fed and starved rats perfused, without hormones, with 9 mM- and 7 mM-glucose respectively (Clark *et al.*, 1975). It was 65% and 80% respectively in hepatocyte suspensions from fed and starved animals, when in the absence of hormones 10–15 mM- and 5–10 mM-glucose, respectively, were offered (Katz *et al.*, 1978; Clark *et al.*, 1973). In the hormone-free controls and the glucagon-stimulated cell cultures (cf. Fig. 1b with Fig. 1a) recycling was lower, of the order of 40% and 55% respectively; it was remarkably decreased to 10% in the insulin-stimulated cultures.

The 'reverse' glucose-6-phosphatase flux (defined three paragraphs above) compared with the total glucose-6-phosphatase flux was about 35% and 50% in the perfused livers (Clark *et al.*, 1975) and 45% and 60% in hepatocyte suspensions (Katz *et al.*, 1978; Clark *et al.*, 1973) from fed and starved animals respectively. In the control cell cultures the contribution of the 'reverse' to the total glucose-6-phosphatase flux (that in Fig. 1b compared with sum of those in Figs. 1b, 1c and 1d) was similar (of the order of 45%), but lower (around 30%) in the glucagon- and insulin-stimulated cultures. The difference between the preparations from starved animals and the glucagon-treated cultures was to be expected, since in the cell-culture experiments glucagon acutely and strongly stimulated glycogenolysis and gluconeogenesis, thus decreasing the relative contribution of the 'reverse' to the total glucose-6-phosphatase flux.

In conclusion, the present cell-culture studies corroborate previous investigations showing that there is substantial 'futile' cycling, of the order of 30–70%, between glucose and glucose 6-phosphate under most conditions with respect to both the glucokinase and the

total glucose-6-phosphatase flux. They extend them by demonstrating that the 'futile' cycling can be controlled by hormones, the most remarkable effect being the decrease of the 'reverse' flux to 10% by insulin, which contributes decisively to the insulin-triggered shift to net glucose uptake (Fig. 2).

Direct or indirect control of the glucose/glucose 6-phosphate cycle

The measured substrate concentrations were not correlated with the radiochemically determined flux (cf. Table 2). This discrepancy indicates either that the substrate and enzyme pools are heterogeneous, which would preclude calculation of rates from the overall substrate concentrations and enzyme activities, or that the cycle is not regulated by substrate availability alone. Evidence for the heterogeneity of the glucose 6-phosphate pools has been presented (Threlfall & Heath, 1968), but there is also some indication that both glucokinase and glucose-6-phosphatase might be under direct allosteric or hormonal control respectively (Tippett & Neet, 1982; Speth & Schulze, 1981; Suzuki *et al.*, 1984). Long-chain acyl-CoA was found to be an allosteric inhibitor of glucokinase (Tippett & Neet, 1982). Glucagon was shown to activate, and insulin to inhibit, glucose-6-phosphatase in liver microsomal preparations by respectively decreasing or enhancing the enzyme's latency in the membranes (Speth & Schulze, 1981). A putative second messenger of insulin action extracted from liver plasma membranes was observed to decrease glucose-6-phosphatase activity in liver microsomal fractions (Suzuki *et al.*, 1984). The hormone action might be mediated by phosphorylation/dephosphorylation (Begley & Craft, 1981); this possibility was later contested (Burchell *et al.*, 1982). Evidence for a direct suppression of glucose-6-phosphatase flux at unchanged glucose 6-phosphate concentrations by carbohydrate feeding has also been obtained in studies *in vivo* (Newgard *et al.*, 1984a).

The finding that insulin inhibited the 'reverse' glucose-6-phosphatase flux by 70%, but the flux through the enzyme from glycogen by 50% and that from gluconeogenesis only by 15% (see Figs. 1b, 1c and 1d) may be disturbing at first glance. However, the different percentages of inhibition might reflect that glucose-6-phosphatase under the experimental conditions chosen exerted a different control strength (Groen *et al.*, 1982) on the different pathways: the control was strongest on the shortest pathway, i.e. the direct reverse flux of glucose 6-phosphate to glucose; it was intermediate on the medium-length pathway, from glycogen to glucose, and weakest on the longest pathway, from lactate to glucose.

In conclusion, the present study shows that the control of the glucose/glucose 6-phosphate cycle can hardly be determined by substrate availability alone, but that direct hormone-dependent on/off mechanisms must also be operative. Hormone-dependent activation/inactivation would be a prerequisite for the 'push' mechanism of glycogen synthesis (El-Refai & Bergman, 1976; van de Werve & Jeanrenaud, 1984); it would also fit into and complement the 'pull' mechanism (Hers, 1981; Stalmans, 1976). The on/off mechanism would be in line with the classical mechanism of glycogen synthesis from glucose (Hue & van de Werve, 1981; Hers & Hue, 1983), with the more recent model of glycogen formation from pyruvate (Hems *et al.*, 1972; Sugden *et al.*, 1983; Newgard *et al.*,

1983, 1984b; Katz & McGarry, 1984), and also with the model of metabolic zonation (Jungermann & Sasse, 1978; Jungermann & Katz, 1982; Pilkis *et al.*, 1985; Jungermann, 1986), according to which glycogen in the periportal zone should be formed predominantly via gluconeogenesis from pyruvate, but glycogen in the perivenous area mainly from glucose.

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