Glucose and pyruvate metabolism in preimplantation blastocysts from normal and diabetic rats

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Glucose metabolism was analysed in day-5 rat blastocysts incubated in the presence of [$5\text{-}^3\text{H}$]-, [$6\text{-}^{14}\text{C}$]- or [$\text{U-}^{14}\text{C}$]glucose. Glycolysis, quantified by $^3\text{H}_2\text{O}$ recovery rate, was the main pathway of glucose utilization by fresh (11.5 ± 0.36 pmol per embryo h^{-1}) or cultured (24 h) blastocysts (20.4 ± 0.6 pmol per embryo h^{-1}). Glucose consumption rate was almost saturated at a medium glucose concentration of 0.28 mmol l^{-1} (K_m : 0.17 mmol l^{-1} ; $V_{\max}$: 23 pmol per embryo h^{-1}). A further 10% increase in glucose utilization was obtained with a tenfold higher glucose concentration (3 mmol l^{-1}). Phloretin completely abolished the rapid component of glucose utilization kinetics, suggesting the existence of a Na^+ -independent glucose transport system. Less than 1% of [$6\text{-}^{14}\text{C}$]glucose consumed by cultured blastocysts was oxidized through the Krebs cycle. [$1\text{-}^{14}\text{C}$]pyruvate, however, was oxidized at a rate of 2 pmol per embryo h^{-1} by fresh blastocysts. The pentose-phosphate pathway accounted for about 2% of glucose utilization. One to two per cent of the total glucose metabolized in 24 h was retained in macromolecules. Insulin had no effect on glucose uptake, utilization, incorporation and turnover, or on pyruvate oxidation. Blastocysts from diabetic mothers utilized glucose at a rate similar to that of normal blastocysts. These results show that glucose is actively taken up by rat blastocysts and utilized mainly through the Embden–Meyerhof pathway, which is rapidly saturated at low glucose concentrations. Retention of glucose-derived products in macromolecules, although relatively small, may modulate the effect of high glucose concentrations on embryo growth. The lack of effect of insulin on the main metabolic pathways of glucose metabolism does not rule out a more discrete insulin effect at some molecular levels.

Introduction

Experimental diabetes induced in rodents by streptozotocin or alloxan administration before pregnancy provokes an increased incidence of congenital malformations in the offspring (Eriksson, 1984). Besides the deleterious effect of maternal diabetes on early fetal organogenesis, recent observations by ourselves in rats (De Hertogh *et al.*, 1989, 1992; Pampfer *et al.*, 1990; Vercheval *et al.*, 1990), and by others in mice (Diamond *et al.*, 1989; Beebe and Kaye, 1991) have shown that embryo growth is already impaired at the preimplantation stage, mainly at the expense of the inner cell mass of the blastocysts (Pampfer *et al.*, 1990). Moreover, *in vitro* experiments demonstrated that high glucose concentrations and high insulin concentrations in the presence of glucose can impair rat blastocyst development, whereas low insulin concentrations stimulate growth of the inner cell mass (De Hertogh *et al.*, 1991). Insulin stimulates protein and nucleic acid synthesis (Harvey and Kaye, 1988; Heyner

et al., 1989) and growth of the inner cell mass (Harvey and Kaye, 1990) in mouse blastocysts, in which insulin receptors have been reported (Rosenblum *et al.*, 1986).

Preimplantation mouse embryos shift from predominantly using pyruvate to glucose at the late morula and early blastocyst stages (Leese and Barton, 1984; Brown and Whittingham, 1991). This shift has recently been confirmed in rat preimplantation embryos, by measuring substrate consumption and lactate production with a noninvasive method (Brison and Leese, 1991). However, glucose metabolic pathways have not yet been described in the rat blastocyst. It has been suggested (Gardner and Kaye, 1984) but not confirmed (Gardner and Leese, 1988) that, in mice, insulin acts on glucose uptake or incorporation into preimplantation blastocysts.

In view of the recent observations made on preimplantation embryos from diabetic rats, and on the interplay of glucose and insulin on blastocyst growth in *in vitro* cultures, we decided to analyse the different pathways of glucose metabolism and the effect of insulin on glucose consumption, incorporation and retention in fresh and cultured rat blastocysts.

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Materials and Methods

Animals, blastocyst retrieval and culture

Ten- to twelve-week-old female Wistar rats from the University breeding centre were mated overnight with males. The presence of a vaginal plug on the following morning was considered as day 1 of pregnancy. On the morning of day 5, pregnant rats were submitted to light ether anaesthesia, the uterine horns removed and the embryos retrieved by gentle flushing with 0.5 ml prewarmed glucose-free Ham's F-10 medium (074-00088, Gibco, Grand-Island, NY) containing 1 g BSA l⁻¹ (A 9647, Sigma, St Louis, MO). Blastocysts, characterized by the presence of a clear blastocoel, were either put in metabolic chambers (fresh embryos), or transferred to incubating cells (Multidish, Nunc, Intermed, Roskilde, Denmark), and cultured for 24 h in Ham's F-10 medium, containing 6 mmol glucose l⁻¹, 100 iu penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ with or without added purified bovine insulin (Gibco) at a concentration of 600 pmol l⁻¹ as previously described (De Hertogh *et al.*, 1991). At the end of the incubation period, embryos were recovered and transferred to metabolic chambers (cultured embryos).

Diabetic rats were obtained by injecting streptozotocin (50 mg kg⁻¹) to fasted rats as described by De Hertogh *et al.* (1982) and Vercheval *et al.*, (1990). Diabetes was confirmed after 24 h by the presence of high glycosuria and a blood glucose concentration exceeding 14 mmol l⁻¹. Diabetic animals were mated one to three weeks after the onset of diabetes. On day 5 of pregnancy, the diabetic state of the mother was confirmed and embryos were recovered as described above. Fully formed blastocysts only were used for metabolic studies.

Metabolic studies

The metabolic chamber was similar to that described by O'Fallon and Wright (1986). In brief, it consisted of a 1.5 ml tube containing 1 ml 0.1 mol NaOH l⁻¹. The embryos were suspended in a 5 µl microdrop of glucose-free Ham's F-10-BSA medium, enriched with Hepes (6 g l⁻¹) and the appropriate metabolic substrate (see below), from the inside surface of the cap, which was then gently fixed on the top of the tube. The tubes were incubated at 37°C for 4 h. At the end of the incubation time, the NaOH was quantitatively transferred to a counting vial containing 15 ml of scintillation mixture (Instagel, Packard Instrument, Groningen) and radioactivity was measured (Tri-Carb 4000, Packard). A correction was made for radioactivity measured in a blank metabolic chamber containing the same metabolic substrate but no embryo in the suspended microdrop.

Experiments allowed the measurement of glucose utilization by the main metabolic pathways (O'Fallon and Wright, 1986, 1987). Hence, [5-³H]-, [U-¹⁴C]- or [6-¹⁴C]glucose, or [1-¹⁴C]pyruvate were included in the Ham's F10-Hepes medium at different concentrations, in the presence or absence of insulin. In all experiments, labelled glucose or pyruvate concentrations are expressed in mmol l⁻¹ of total substrate, taking into account the concentration of unlabelled compound present or added to the incubation medium.

From preliminary experiments measuring the kinetic equilibrium between 5 µl microdrops of ³H₂O suspended in metabolic

chambers and NaOH layers, it was concluded that 82% ³H₂O released by embryos during a 4 h period was quantitatively recovered in the NaOH layers. Similar experiments performed with 5 µl microdrops of Ham's F-10 medium containing a tracer amount of [¹⁴C] bicarbonate indicated that about 76% of ¹⁴CO₂ produced by embryos during 4 h would be recovered. A correction for recovery has therefore been included in the results. A linear relationship between ¹⁴CO₂ recovery and time of incubation or number of embryos in the metabolic chambers was observed. The results of the metabolic studies were therefore expressed in terms of pmol of total substrate (labelled and unlabelled) consumed per embryo h⁻¹.

Glucose incorporation and retention

Glucose incorporated into blastocysts by the end of the incubation in the presence of [³H]- or [¹⁴C]glucose was measured. Embryos were recovered from the metabolic milieu and quickly washed six times in Earle's medium (EBSS, Gibco), supplemented with 5 mmol sodium pyruvate l⁻¹ and 1 mmol L-glutamine l⁻¹. Embryos were then digested in 500 µl solune (Packard) for 24 h, and radioactivity measured in 10 ml ionic fluor (Packard). The retention of the incorporated label was studied by pulse-chase experiments in which the embryos, after washing, were further incubated for several hours in the original medium, in which unlabelled glucose was substituted for [³H]- or [¹⁴C]labelled compound. At the end of the incubation period, the embryos were recovered, digested in solune and counted. Concentrations of incorporated and retained substances are expressed as pmol per embryo, taking into account the specific activity of glucose in the culture medium.

Chemicals

Phloretin (Sigma) was used to block Na⁺-independent glucose transport. Phenazine ethosulfate (Sigma), which oxidizes NADPH to NADP⁺, was used to stimulate the pentose-phosphate shunt. Dinitrophenol (Sigma), uncouples oxidative phosphorylation, and was used to stimulate Krebs cycle (Rieger and Guay, 1988). [6-¹⁴C]glucose (50 to 60 mCi mmol⁻¹; Amersham, UK) was used to measure glucose oxidation through the Krebs cycle, on the basis of a one to one molar ratio between ¹⁴CO₂ recovered and [6-¹⁴C]glucose oxidized. [U-¹⁴C]glucose (> 230 mCi mmol⁻¹; Amersham, UK) was used to estimate the Krebs cycle and pentose-phosphate shunt. In the latter pathway, one mole of glucose was considered to yield three moles of ¹⁴CO₂ after complete recycling.

[1-¹⁴C]pyruvate (32 mCi mmol⁻¹; Amersham, UK) was used to measure the activity of the pyruvate dehydrogenase complex and of the Krebs cycle, on the basis of a one to one molar ratio between the ¹⁴CO₂ produced and the pyruvate oxidized. Finally, [5-³H]glucose (10 to 20 mCi mmol⁻¹; Amersham, UK) allowed the measurement of the glycolytic pathway, assuming a one to one molar ratio between ³H₂O produced and [5-³H]glucose metabolized.

Statistical analysis

Two-tailed unpaired *t* tests were used to determine the significance of differences between experimental groups.

Table 1. Glucose utilization by fresh and cultured day-5 rat blastocysts in the presence and absence of insulin

Insulin concentration (pmol l ⁻¹)	[5- ³ H]glucose ^a (pmol per embryo h ⁻¹)		[U- ¹⁴ C]glucose ^{a,e} (pmol per embryo h ⁻¹)	
	Fresh embryos	Cultured embryos	Fresh embryos	Cultured embryos
0	11.5 ± 0.36 (7) ^d	20.4 ± 0.6* (48)	0.29 ± 0.01 (14)	0.46 ± 0.02* (52)
600 ^b	12.0 ± 0.36 (6)	19.2 ± 0.84* (52)	0.29 ± 0.01 (16)	0.42 ± 0.02* (58)
600 ^c	12.0 ± 0.36 (6)	19.2 ± 0.5* (47)	0.29 ± 0.01 (16)	0.42 ± 0.02* (50)

Values given as means ± SEM.

^aGlucose concentration was 0.28 mmol l⁻¹ throughout the 4 h incubation.

^bInsulin was present throughout the culture (24 h) and incubation (4 h).

^cInsulin was present during the incubation only (4 h).

^dNumbers in parentheses are the number of metabolic chambers of three embryos each.

^eResults calculated on the assumption that all ¹⁴CO₂ was produced via the pentose-phosphate pathway and that complete recycling had occurred (see text).

*P < 0.001 versus fresh embryos.

Fig. 1. Glucose utilization by day-5 rat blastocysts, cultured for 24 h and further incubated in the presence of [5-³H]glucose at increasing concentrations for 4 h, with (○) or without (●) insulin (600 pmol l⁻¹) or phloretin (■) (0.5 mmol l⁻¹). Each point is the mean ± SEM of indicated numbers of cells incubated from three embryos.

Results

[5-³H]glucose utilization

At a concentration of 0.28 mmol l⁻¹, overall glucose utilization through glycolysis amounted to 11.5 pmol per embryo h⁻¹ in fresh, day 5 blastocysts, and to 20.4 pmol per embryo h⁻¹ in blastocysts cultured for 24 h (Table 1). Insulin had no effect on these utilization rates.

Kinetic studies showed a curvilinear relationship between glucose utilization by cultured embryos and glucose concentration, with a sharp increase up to 0.28 mmol l⁻¹, and a slow linear increase at higher concentrations (Fig. 1). Insulin had no

Fig. 2. Double reciprocal plot of the rapid component of glucose utilization (from Fig. 1) versus glucose concentration. The intercept on the y axis is 1/V_{max}. The slope of the curve is K_t/V_{max}.

effect on the kinetic profile. Phloretin (0.5 mmol l⁻¹) abolished the rapid utilization phase at low glucose concentration (Fig. 1). By subtracting the slow glucose utilization phase from the total utilization rate, the rapid component could be isolated and analysed on a double reciprocal plot (Fig. 2). From such a plot, the mean maximal velocity for glucose utilization (V_{max} = 1/intercept on the y axis), and the mean apparent K_t/V_{max} being the slope of the line) were found to be 23 pmol per embryo h⁻¹ and 0.17 mmol l⁻¹, respectively.

[U-¹⁴C]- and [6-¹⁴C]glucose utilization

¹⁴CO₂ produced from [6-¹⁴C]glucose was about 150 times lower than from [U-¹⁴C]glucose (Table 2). On the assumption

Table 2. $^{14}\text{CO}_2$ production from [U- ^{14}C]- or [6- ^{14}C]glucose by day-5 cultured rat blastocysts in the presence or absence of phenazin ethosulfate (PES) or excess of pyruvate

PES ^a (mmol l ⁻¹)	[6- ^{14}C]glucose ^b (pmol per embryo h ⁻¹)	[U- ^{14}C]glucose ^b (pmol per embryo h ⁻¹)	[U- ^{14}C]glucose ^b plus 5 mmol pyruvate l ⁻¹ (pmol per embryo h ⁻¹)
0	0.010 ± 0.001 (7) ^c	1.62 ± 0.10 (14)	0.81 ± 0.04*** (14)
0.01	0.016 ± 0.002* (6)	15.3 ± 0.63** (14)	16.5 ± 1.07** (14)

Values are given as means ± SEM.

^aPES was added at the end of the culture period (24 h) and was present throughout the incubation (4 h).

^bGlucose concentration was 0.28 mmol l⁻¹ throughout the 4 h incubation.

^cNumbers in parentheses are the number of metabolic chambers of three embryos each.

*P < 0.05 versus 0 mmol PES l⁻¹.

**P < 0.001 versus 0 mmol PES l⁻¹.

***P < 0.001 versus [U- ^{14}C]glucose without excess of pyruvate.

Table 3. Effect of dinitrophenol (DNP) and insulin on [1- ^{14}C]pyruvate and [5- ^3H]glucose utilization by fresh, day-5 rat blastocysts

DNP ^a	Insulin ^a	[1- ^{14}C]pyruvate ^a (pmol per embryo h ⁻¹)	[5- ^3H]glucose ^a (pmol per embryo h ⁻¹)
—	—	2.00 ± 0.18 (19) ^b	13.0 ± 0.36 (7)
+	—	7.74 ± 0.5* (20)	22.0 ± 0.50* (7)
—	+	1.40 ± 0.3 (7)	12.9 ± 0.84 (7)
+	+	6.30 ± 0.5* (12)	25.2 ± 0.13* (6)

Values given as means ± SEM.

^aDNP: 50 micromol l⁻¹; insulin: 600 pmol l⁻¹; [1- ^{14}C]pyruvate: 1 mmol l⁻¹ and [5- ^3H]glucose: 0.28 mmol l⁻¹ were present throughout the incubation (4 h).

^bNumbers in parentheses are the number of metabolic chambers of three embryos each.

*P < 0.001 versus respective control without DNP.

that (1) [U- ^{14}C]glucose yields six times more $^{14}\text{CO}_2$ per mole than [6- ^{14}C]glucose via complete oxidation in the Krebs cycle and (2) [6- ^{14}C]glucose was oxidized through the Krebs cycle only, then only 4% of the U- ^{14}C glucose was oxidized through the cycle. Hence, 96% of [U- ^{14}C]glucose was oxidized via other pathways.

The first is the formation of acetyl-CoA from pyruvate, through the action of the pyruvate dehydrogenase complex. The second is the pentose-phosphate pathway.

Phenazin ethosulfate (PES) increased $^{14}\text{CO}_2$ production from [U- ^{14}C]glucose about tenfold and about 1.5 fold, from [6- ^{14}C]glucose (Table 2), indicating that the pentose-phosphate pathway was active and responsive to stimulation. However, $^{14}\text{CO}_2$ production from [U- ^{14}C]glucose was decreased in the presence of a sixfold increase in the concentration of unlabelled pyruvate in the incubation medium (Table 2), suggesting that the pyruvate dehydrogenase complex was responsible for part of the [U- ^{14}C]glucose oxidation, at least in the basal condition.

In the presence of PES, the stimulated $^{14}\text{CO}_2$ production was not modified by excess pyruvate. Ignoring the possible participation of pyruvate dehydrogenase, and assuming that all the [U- ^{14}C]glucose was oxidized through the pentose-phosphate pathway, the maximal glucose oxidation rate through that pathway amounted to about 2% of the total glucose utilization rate (Table 1). PES increased glucose utilization rate through the pentose-phosphate pathway in a dose-dependent manner; up to at least ten times, at a concentration of 0.01 mmol l⁻¹ (from 0.53 ± 0.04 ($n = 12$) to 5.1 ± 0.20 ($n = 14$) pmol per embryo h⁻¹) ($P < 0.001$). However, [5- ^3H]glucose utilization (that is glycolysis) was depressed by about 50% (from 22.1 ± 1.5 ($n = 12$) to 9.7 ± 0.36 ($n = 14$) pmol per embryo h⁻¹) ($P < 0.001$).

As Ham's F10 medium contains pyruvate (1 mmol l⁻¹), the oxidation of [6- ^{14}C]glucose through the Krebs cycle might be underestimated by dilution of the endogenously produced [^{14}C]pyruvate. The maximum dilution factor can be derived on the assumption that glucose metabolized through the glycolytic

Fig. 3. Incorporation of [^{14}C]labelled compounds (in terms of glucose) into day-5 rat blastocysts cultured for 24 h and further incubated for 1 to 8 h in the presence of 0.28 mmol [^{14}C]glucose l^{-1} , with (○) or without (●) insulin at a concentration of 600 pmol l^{-1} . Each point is the mean $\pm$ SEM of the indicated numbers of cells incubated from three embryos.

Fig. 4. Retention of [^{14}C]labelled compounds (in terms of glucose) by day-5 rat blastocysts incubated in the presence of 0.28 mmol [^{14}C]glucose l^{-1} for 24 h, with (○) or without (●) insulin (600 pmol l^{-1}), and further incubated for 1 to 6 h in the presence of 0.28 mmol unlabelled glucose l^{-1} , with (○) or without (●) 600 pmol insulin l^{-1} . Each point is the mean $\pm$ SEM of indicated numbers of cells incubated from three embryos each.

pathway yields 1 mole of labelled pyruvate per mole of glucose (20.5 pmol per embryo $\text{h}^{-1} \times 4 \text{ h} \times 3 \text{ embryos} = 246 \text{ pmol}$), and from the amount of pyruvate present in 5 μl of Ham's F10 (1 mmol $\text{l}^{-1} \times 5 \times 10^{-6} \text{ l} = 5000 \text{ pmol}$).

This dilution factor does not exceed 20. Taking this maximum dilution factor and the rate of $^{14}\text{CO}_2$ produced from [$6\text{-}^{14}\text{C}$]glucose (Table 2), it can be calculated that glucose utilization through the Krebs cycle could account for no more than 0.2 pmol per embryo h^{-1} , i.e. about 1% of the total glucose utilization in cultured blastocysts.

Fig. 5. Incorporation of [^3H]labelled compounds (in terms of glucose) into day-5 rat blastocysts cultured for 24 h and further incubated with [$5\text{-}^3\text{H}$]glucose at increasing concentrations, with (○) or without (●) 600 pmol insulin l^{-1} . Each point is the mean $\pm$ SEM of the indicated numbers of cells incubated from three embryos each.

[1- ^{14}C]pyruvate utilization

Fresh embryos were incubated in the presence of 1 mmol [$1\text{-}^{14}\text{C}$]pyruvate l^{-1} and [$5\text{-}^3\text{H}$]glucose (0.28 mmol l^{-1}), with or without dinitrophenol (DNP) at a dose of 50 $\mu\text{mol l}^{-1}$, and insulin (600 pmol l^{-1}). Pyruvate was utilized at a rate of 2.0 pmol per embryo h^{-1} (Table 3), i.e. about 8% of the amount of pyruvate formed through the glycolytic pathway ($13 \times 2 = 26 \text{ pmol per embryo h}^{-1}$) (Table 3). This value is at least eight times higher than the amount expected from the maximum calculated rate (1%) of [$6\text{-}^{14}\text{C}$]glucose utilization by the Krebs cycle (see above). DNP induced a fourfold activation of pyruvate oxidation, whereas glycolysis was stimulated only twofold. Insulin was without effect on pyruvate utilization in the presence, or absence, of DNP.

Incorporation and retention of [$5\text{-}^3\text{H}$]- or [^{14}C]glucose

Incorporation of glucose-derived [^3H]- or [^{14}C]labelled compounds into cultured day-5 embryos was studied in relation to incubation time in the presence of 0.28 mmol labelled glucose l^{-1} . There was a non-linear increase in ^{14}C incorporation, which levels off with increased incubation time (Fig. 3).

^3H incorporation rate was half that of ^{14}C incorporation at 4 h (1.0 ± 0.2 ($n = 10$) versus 2.1 ± 0.2 ($n = 15$) pmol glucose equivalent per embryo) ($P < 0.001$).

Embryos, cultured for 24 h in the presence of 0.28 mmol [$5\text{-}^3\text{H}$]- and [^{14}C]glucose l^{-1} incorporated slightly less ^3H than ^{14}C compounds (3.6 ± 0.3 ($n = 9$) versus 5.1 ± 0.3 ($n = 9$) pmol per embryo) ($P < 0.01$).

Replacement of labelled with unlabelled glucose at the same concentration was followed by a slow and only partial release of incorporated ^{14}C (Fig. 4) and ^3H (not shown). Insulin had no effect on the incorporation kinetics, or on the retention of glucose-derived labelled compounds (Figs 3 and 4).

Table 4. Glucose utilization by day-5 blastocysts from diabetic rats

	[5- ³ H]glucose ^a (pmol per embryo h ⁻¹)		[U- ¹⁴ C]glucose ^a (pmol per embryo h ⁻¹)	
	Fresh embryos	Cultured embryos	Fresh embryos	Cultured embryos
Control rats	12.5 ± 0.5 (17) ^b	20.4 ± 0.5*	0.24 ± 0.02 (17)	0.60 ± 0.04* (8)
Diabetic rats	11.5 ± 0.36 (17)	20.8 ± 0.5* (8)	0.26 ± 0.02 (16)	0.49 ± 0.03* (8)

Values given as means ± SEM.

^aGlucose concentration was 0.28 mmol l⁻¹ throughout the 4 h incubation.

^bNumbers in parentheses are the number of metabolic chambers of three embryos each.

*P < 0.001 versus fresh embryo.

The incorporation of [5-³H]glucose was also studied as a function of glucose concentration in the medium. A biphasic curve was obtained (Fig. 5) that was similar to that obtained for glucose utilization (Fig. 1), with a break point at 0.28 mmol l⁻¹. Insulin had no effect at low or high glucose concentrations.

Glucose utilization by fresh and cultured blastocysts from diabetic rats

Day 5 blastocysts from diabetic rats showed a normal glucose utilization rate before (fresh) and after (cultured) a 24 h culture period (Table 4).

Discussion

Using a non-invasive technique for measuring substrate consumption, Brison and Leese (1991) showed that rat blastocysts used glucose as their main source of energy, and that glycolysis is the main metabolic pathway, accounting for almost all of the glucose consumed. Lactate production was quantitatively equivalent to the amount of glucose taken up by the embryo on a 2 to 1 molar basis. In the present work, we determined the rate of glycolysis by measuring the production of ³H₂O from [5-³H]glucose, and obtained values similar to those reported by Brison and Leese. Fresh embryos used about 11.5 pmol glucose per embryo h⁻¹; a figure similar to that reported by Brison and Leese (1991) in rats, but about three times higher than measurements made in mice (Gardner and Leese, 1988), and less than half the glucose consumption rate reported for human blastocysts (Hardy *et al.*, 1989). After culture for 24 h when the cell population had approximately doubled (De Hertogh *et al.*, 1991), the glucose consumption by rat blastocysts through glycolysis amounted to 20.4 pmol per embryo h⁻¹, showing a close relationship between cell population and glucose consumption.

A curvilinear relationship was observed between glucose consumption via glycolysis and glucose concentration in the medium. A sharp increase was observed up to about 0.28 mmol glucose l⁻¹, followed by a slow, linear increase up to 10 mmol l⁻¹. The slope of this curve was similar to that

described for glucose uptake by mouse blastocysts (Gardner and Leese, 1988). The fast part corresponds to a rapidly saturable metabolic process, with an apparent K_t as low as 0.17 mmol l⁻¹ and a V_{max} of about 23 pmol per embryo h⁻¹ in cultured blastocysts. The K_t value is similar to that described for mouse blastocysts and is in the range of K_m values for some of the glycolytic enzymes (Lehninger, 1982; Rawn, 1989).

Hence, glucose utilization via glycolysis is rapidly saturated at low extra-embryonic glucose concentrations, such that higher glucose concentrations (up to 10 mmol l⁻¹) would increase glucose consumption by only about 40%. Blocking the Na⁺-independent glucose-transport mechanism with phloretin completely abolished the rapid component of glucose utilization, as also shown in mice (Gardner and Leese, 1988), indicating the presence of facilitated glucose transport in rat blastocysts. In agreement with observations made in other species (Gardner and Leese, 1988; Robinson *et al.*, 1990), this transport system is not dependent on insulin, as shown by the lack of activation by this hormone of either the first or second component of the glucose consumption kinetics.

Insulin-dependent glucose transporter (Glut-1 and Glut-2) gene expressions have recently been described in mouse blastocysts. The Glut-4 isoform, which is insulin dependent, is not expressed (Hogan *et al.*, 1991). The present study suggests that there is a similar insulin-independent glucose transport in rat blastocysts. However, formal characterization of the transporter isoforms has not yet been described in rats.

Glucose oxidation was also studied by ¹⁴CO₂ production from labelled precursors. A minimal release of ¹⁴CO₂ was observed from [6-¹⁴C]glucose indicating a very low oxidation of glucose via the Krebs cycle. Taking into account the maximum dilution of endogenously produced [¹⁴C]pyruvate by unlabelled pyruvate present in the medium, glucose oxidation via the Krebs cycle did not exceed 1% of total glucose consumption in cultured blastocysts. These results agree with those of Brison and Leese (1991), who accounted for almost 100% of glucose consumption as lactate formation.

However, when [¹⁴C]pyruvate was used in the incubation medium, a much higher ¹⁴CO₂ production was observed with fresh embryos, amounting to about 2.0 pmol per embryo h⁻¹, that is, about 8% of the amount of glucose metabolized through

glycolysis, and thus likely to produce pyruvate. This discrepancy between $^{14}\text{CO}_2$ production from $[6\text{-}^{14}\text{C}]\text{glucose}$ and from $[^{14}\text{C}]\text{pyruvate}$ could be explained if the products of glycolysis were not able to enter the tricarboxylic cycle. Such a situation has been reported in cattle blastocysts by Rieger and Guay (1988), who suggest a block at the pyruvate kinase level, i.e. the last glycolytic step before pyruvate formation. This hypothesis however diverges from the observations of Brison and Leese (1991), in which lactate accumulated in rat blastocysts incubated in the presence of glucose. Another explanation could be that endogenously produced pyruvate is unable to equilibrate with extracellular pyruvate and fails to gain access to the oxidative pathways. A third possibility is that oxidation of $[1\text{-}^{14}\text{C}]\text{pyruvate}$ is restricted to the activity of the pyruvate dehydrogenase complex leading to the formation of acetyl CoA and $^{14}\text{CO}_2$. This hypothesis is supported by our observations of $[\text{U-}^{14}\text{C}]\text{glucose}$ metabolism, showing that $^{14}\text{CO}_2$ production slightly decreased when pyruvate concentration in the incubation medium was increased, probably as a result of dilution of the $[1\text{-}^{14}\text{C}]\text{pyruvate}$ produced via the glycolytic pathway. The activity of the Krebs cycle, although limited in basal conditions, could be stimulated, as shown by increased $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{pyruvate}$ in the presence of DNP, as previously observed in cattle blastocysts by Rieger and Guay (1988). Insulin, however, did not influence pyruvate utilization in the presence or absence of DNP.

A limited amount of glucose may be utilized via the pentose-phosphate shunt as shown in mouse (O'Fallon and Wright, 1987) and pig (Flood and Wiebold, 1988) blastocysts. We obtained a similar result for rat blastocysts using $[\text{U-}^{14}\text{C}]\text{glucose}$ as a precursor; a maximum of 2% of the total glucose used was oxidized via the pentose-phosphate pathway. Phenazin ethosulfate (PES) stimulated this cycle at least tenfold, despite an overall 50% decrease in glycolysis. The latter effect has also been described in other species (O'Fallon and Wright, 1987; Flood and Wiebold, 1988) and may be due to the nonspecific toxic effect of PES on the embryos. The morphological appearance of the embryos had deteriorated after 4 h incubation in the presence of 0.01 mmol l^{-1} PES. It has been shown in mouse embryos, that PES, at a tenfold higher concentration (0.1 mmol l^{-1}), induces non-enzymatic decarboxylation of pyruvate, thereby giving an apparent rise in the pentose-phosphate shunt activity and decreasing glycolysis by removing NAD^+ (O'Fallon and Wright, 1991). Whether such a mechanism plays a role in rat embryos at the much lower PES concentration used here is unknown. However, increasing the concentration of pyruvate in the medium decreased $^{14}\text{CO}_2$ production in the basal, but not in the PES-stimulated, state, strongly suggesting that PES was able to induce glucose oxidation through a mechanism not dependent on exogenous pyruvate, i.e. the pentose-phosphate shunt. Our experiments thus show that the pentose-phosphate pathway was operating at a submaximal rate and could be stimulated though not by insulin.

Previous experiments (De Hertogh *et al.*, 1991) showed that rat blastocysts can develop *in vitro* in glucose-free Ham's F-10 medium, although to a slightly lesser extent than in Ham's F-10 containing $5.5\text{ mmol glucose l}^{-1}$. Hence, alternative sources of energy can be used by blastocysts. One of them, might be glutamine. However, all our experiments were performed in the

same concentration of glutamine, thus allowing a valid comparison between groups. Another could be external pyruvate, as shown in the present study. Pyruvate, if fully oxidized in the tricarboxylic cycle, would yield almost eight times more ATP per mmol than glucose metabolized via the glycolytic pathway. Considering that fresh embryos utilize about six times more glucose than pyruvate (13 versus $2\text{ pmol per embryo h}^{-1}$), energy production could still be 1.5 times higher from pyruvate than from glucose. However, as discussed earlier, the present work does not allow a clearcut estimate of the amount of pyruvate oxidized in the Krebs cycle, beyond the pyruvate dehydrogenase step and acetyl CoA formation. This first metabolic step *per se* would yield only 20% of the extra energy, if not followed by the high energy producing oxidative steps of the tricarboxylic cycle (Rawn, 1989).

Besides its role as an energy source, glucose or glucose-derived carbon atoms might be incorporated into macromolecules such as glycogen, lipids, nucleic acids or proteins (Murdoch and Wales, 1973; Flynn and Hillman, 1978; Pike and Wales, 1982; Eridisinghe *et al.*, 1984). In rabbit embryos, glucose may be used more as an anabolic compound than as an energy source (Robinson and Benos, 1991). Insulin could stimulate such incorporation, and eventually play an important role in anabolic processes (Wales *et al.*, 1985).

We investigated this problem, by studying the total incorporation of glucose-derived $[^3\text{H}]$ - or $[^{14}\text{C}]$ -labelled substrate into rat blastocysts. We showed that accumulation of radioactivity increased slowly with incubation time with a plateau of about $5\text{ pmol per embryo after } 24\text{ h in culture}$. This amount is very small compared with the total glucose utilization during that time. Hence, considering that fresh blastocysts use about $11.5\text{ pmol per embryo h}^{-1}$ and consumption is about $20.4\text{ pmol per embryo h}^{-1}$ after 24 h of culture, we may conclude that glucose utilization during the whole incubation time is between $276 (11.5 \times 24)$ and $490 (20.4 \times 24)\text{ pmol per embryo}$. Glucose-derived labelled products incorporated into the embryo therefore amounted to less than 1 to 2% of this consumption. The nature of the accumulated products was not determined. However, ^{14}C was incorporated to a slightly greater extent than ^3H , suggesting that most of the glucose retained in the embryo did not follow the glycolytic pathway.

It has been shown in other species that glycogen may constitute most of the retained glucose (Kaye, 1986), and this observation fits in with our results. A small amount of carbon, derived from glucose oxidation products, is also accumulated, but accounts for much less than 1% of the total glucose consumption.

Retention kinetics showed that $[^3\text{H}]\text{glucose}$ was incorporated as a function of glucose concentration in a curvilinear relationship, similar to that of glucose utilization. Hence, a common mechanism may control utilization and accumulation of glucose above a concentration of 0.28 mmol l^{-1} in the medium. The slower part of the retention kinetics was however depressed less than that of the consumption kinetics, suggesting that another step, beyond glucose-6-phosphate, might be involved in limiting glucose utilization but allowing a relatively higher retention rate. Accumulation of glucose-derived molecules may proceed to a relatively higher amount at high glucose concentration, even though glucose consumption remains very much the same. Another aspect of the glucose-derived products, is their slow turnover as shown by the pulse-chase experiments in

which unlabelled glucose was substituted for labelled glucose in the medium. In 6 h, while the overall glucose utilization amounted to about 122 pmol per embryo (6×20.4 pmol per embryo h^{-1}), less than 1 pmol of labelled product (in terms of labelled glucose) was released. This ruled out the possibility that retained products were either labelled glucose itself or breakdown products, and suggests that the glucose-derived label had been incorporated into slow turnover pools of macromolecules, most of which retain the intact glucose structure. A slow turnover of glycogen has been described in other species (Kaye, 1986). Insulin was without effect, either on the incorporation kinetics, retention concentrations or the turnover kinetics of [3H] or [^{14}C]labelled products. Hence, we were unable to show an effect of insulin on the gross incorporation of glucose-derived metabolites into the embryo, although more discrete insulin effects on proteins or nucleic acids cannot be ruled out (Wales *et al.*, 1985).

Blastocysts from diabetic mothers were shown to use glucose at a similar rate to blastocysts from normal mothers. A similar observation was reported recently by Brison and Leese (1990). However, we discarded blastocysts showing signs of degeneration to compare structures of similar appearance. We may thus conclude that apparently healthy blastocysts, from diabetic mothers, put into a similar medium to normal blastocysts, can utilize glucose at a normal rate.

In conclusion, the present work shows that glucose is taken up by a carrier-mediated mechanism in rat blastocysts, and metabolised predominantly through glycolysis. The Krebs cycle and pentose-phosphate pathways, although active, account for less than 1 and 2%, respectively, of the total glucose consumption, which is rapidly saturated at glucose concentrations as low as 0.28 mmol l^{-1} . At higher concentrations, glucose consumption changes only minimally, although incorporation into macromolecules is increased, and could be involved in the toxic effect that high glucose concentrations have on blastocyst growth (De Hertogh *et al.*, 1991).

The observed growth anomalies described in blastocysts from diabetic mothers (Vercheval *et al.*, 1990) are unlikely to be mediated by significant changes in glucose metabolism, unless abnormal accumulation of glucose-derived products plays a role, as yet to be defined.

The significant effect of insulin on blastocyst growth observed by De Hertogh *et al.* (1991) does not result from any gross modification of glucose uptake, metabolism or incorporation.

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