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The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos

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Summary

Preimplantation mouse embryos utilize pyruvate preferentially during the early cleavage stages before switching to glucose at around the time of compaction. This switch in substrate preference has been studied using a non-invasive ultramicrofluorometric analytical technique on single mouse embryos.

On the basis of transport kinetic studies and inhibition by phloretin, cytochalasin B and sugar analogues, a component of glucose uptake by mouse blastocysts was found to be mediated by facilitated diffusion. The J_{max} and K_t of this facilitated component were 3·53 pmol embryo^{-1} h^{-1} and 0·14 mM, respectively. At physiological concentrations of glucose, the facilitated component accounts for around 75 % of glucose uptake. Glucose uptake by blastocysts was found to be insensitive to insulin, added at a range of concentrations. There was no evidence for glucose active transport. The carrier-mediated component of glucose entry was detectable from the 2-cell stage onwards.

Pyruvate uptake was also mediated by a carrier throughout development. In the absence of glucose in the incubation medium, the characteristic decline in pyruvate uptake does not occur. The data are consistent with a role for embryonic cell transport in regulating glucose utilization prior to compaction, but do not exclude the involvement of metabolic factors, such as the allosteric regulation of the enzymes hexokinase and phosphofructokinase.

Key words: preimplantation embryo, glucose transport, pyruvate transport, metabolism, mouse.

Introduction

During the development of mammalian embryo culture techniques, it became apparent that mouse zygotes had an absolute requirement for pyruvate to support the first cleavage division (Biggers *et al.* 1967). Lactate supports the second cleavage division and acts synergistically with pyruvate. Glucose as sole energy source is unable to support development until the late 4-cell/early 8-cell stage (Brinster & Thomson, 1966).

Nutrient uptake studies carried out on single embryos showed that preimplantation mouse embryos switch from a pyruvate to a glucose-based metabolism at around the time of compaction (Leese & Barton, 1984; Gardner & Leese, 1986). The only work to have examined the biochemical nature of this switch in any detail is that of Barbehenn *et al.* (1974, 1978), who proposed that the block to glucose utilization resided in glycolysis at the level of phosphofructokinase. However, a role for hexokinase and other factors controlling the intracellular glucose-6-phosphate concentration was not ruled out.

The presence of a specific transport system for pyruvate has been demonstrated in unfertilized mouse oocytes by Leese & Barton (1984). Glucose transport has been less-well characterized, though there is indirect evidence of a facilitated glucose entry system (Wales & Brinster, 1968). The aim of this work was to determine the extent to which the switch in nutrient preference was due to the appearance or disappearance of specific transport systems for pyruvate and glucose across the plasma membrane of the embryo.

Materials and methods

Animals and analytical technique

Embryos were obtained from F_1 (CBA/Ca×C57BL/6) hybrid females. 6-week-old virgin females were superovulated with 5 i.u. pregnant mares serum (PMS), (Fol-

ligon, Intervet), administered at noon, followed 48 h later by 5 i.u. human chorionic gonadotrophin (hCG), (Chorulon, Intervet). Females were placed with F_1 males immediately after the second injection. The presence of a vaginal plug the following morning indicated that mating had taken place. Zygotes, 2-cell and 8-cell embryos and blastocysts were collected at 21, 45, 69 and 93 h post hCG, respectively. Zygotes were teased from the oviduct and the cumulus removed with hyaluronidase (Bovine testes, Sigma), at a concentration of $1\,mg\,ml^{-1}$ in substrate-free M2 (Quinn *et al.* 1982). Later stages were flushed from the reproductive tract, washed three times in the substrate-free medium and then twice in M2 containing either 1 mM-glucose or 0·5 mM-pyruvate as the sole energy source. The osmolarity of the medium was kept at 285–287 mOsmol by altering the sodium chloride concentration.

Embryos were then transferred individually to 25 nl microdrops of M2 containing either 1 mM-glucose or 0·5 mM-pyruvate, and incubated for up to 3 h at 37 °C. Serial 1 nl samples of medium were removed every 20 to 30 min and assayed for nutrient content using the ultramicrofluorometric technique of Leese *et al.* (1984). This is a miniaturization of the conventional methods of enzymic analysis, in which the reduced pyridine nucleotides NADH and NADPH are generated or consumed in coupled reactions (Lowry & Passonneau, 1972; Bergmeyer & Gawehn, 1974). The assay reactions were performed in 10 nl microdrops of reaction mixture on siliconized microscope slides, under a layer of mineral oil, which had previously been saturated overnight with 0·9 % w/v NaCl. The products of the assay were quantified using a Leitz Diavert fluorescent microscope, with photomultiplier and photometer attachments (Gardner & Leese, 1987). Micropipettes, capable of delivering volumes in the nano- and picolitre range, were used to deliver the microdrops. They were constructed on a microforge and calibrated with tritiated water.

Analyses of results

The results were analysed using Analysis of Variance and Student's *t*-test.

Results

Initial experiments were designed to discover whether a component of glucose uptake by the early mouse embryo was carrier-mediated. These studies were conducted on the blastocyst, since glucose uptake is well established at this stage.

Fig. 1 shows the uptake of glucose by single mouse blastocysts at increasing concentrations of glucose. The rates of uptake remained linear at each concentration. The pattern obtained did not conform to that expected of either simple or facilitated diffusion, but was best explained by a combination of the two processes.

Phloretin is known to inhibit glucose-facilitated diffusion in erythrocytes and other systems (LeFevre & Marshall, 1959). When the effect of increasing concentrations of phloretin (0·1–1·0 mM) on the up-

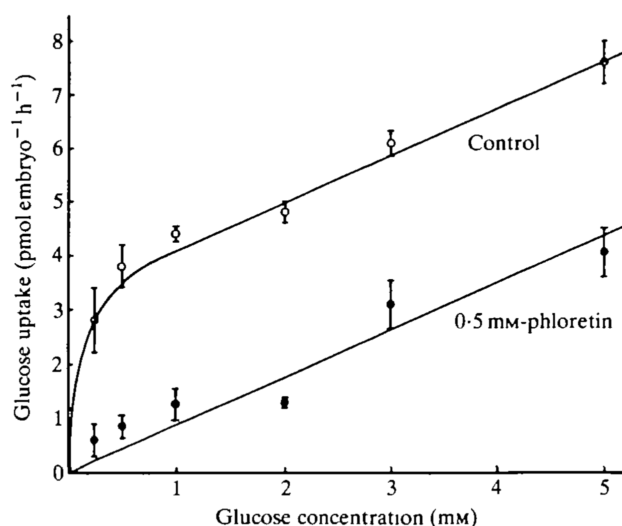

Fig. 1. The effect of increasing concentrations of glucose on glucose uptake by single mouse blastocysts in the presence and absence of 0·5 mM-phloretin. Values are mean (±S.E.) of at least six embryos.

take of 1 mM-glucose by single mouse blastocysts was examined, maximum inhibition occurred with 0·5 mM-phloretin. The effect of 0·5 mM-phloretin on the uptake of glucose at a range of concentrations by single mouse blastocysts is shown in Fig. 1. Uptakes in the presence of 0·5 mM-phloretin conform to the pattern expected for simple diffusion, with uptake increasing linearly with substrate concentration. The facilitated component of glucose transport can be determined by subtracting the values for glucose uptake in the presence of phloretin from the control values in Fig. 1. The J_{max} and K_t for the glucose transport system can then be calculated from a double-reciprocal plot (the slope of the line being K_t/J_{max}, and the intercept on the y-axis being $1/J_{max}$). From such a plot, the J_{max} and K_t for glucose uptake were found to be 3·53+0·33 pmol embryo^{-1} h^{-1} and 0·14+0·01 mM, respectively.

The effect of a second inhibitor of glucose facilitated diffusion, cytochalasin B, was investigated. At a concentration of 4 μm, the concentration at which Dick *et al.* (1984) obtained maximum binding to the glucose carrier in cerebral microvessels, uptake of 1 mM-glucose by single blastocysts was reduced by just over 60 %.

Phlorizin inhibits glucose active transport in the small intestine and kidney (Alvarado & Crane, 1962). The effect of increasing concentrations of phlorizin on 1 mM-glucose uptake by single blastocysts was therefore examined. Over the range of concentrations employed (0–0·5 mM), phlorizin had no effect on glucose transport.

The effect of four sugar analogues on the uptake of 1·0 mM-glucose by single blastocysts is shown in

Table 1. *The effect of sugar analogues on 1 mM-glucose uptake by single mouse blastocysts*

Treatment	Glucose uptake (pmol embryo^{-1} h^{-1} ±S.E.)	% Inhibition	Number of embryos
Control	4·27 ± 0·11	–	27
5 mM-glucosamine	2·78 ± 0·20**	35	4
50 mM-glucosamine	1·46 ± 0·21**	66	5
5 mM-galactose	3·15 ± 0·13**	26	4
50 mM-galactose	2·00 ± 0·11**	47	4
5 mM-3-*O*-methyl-glucose	3·75 ± 0·22	12	7
50 mM-3-*O*-methyl-glucose	2·54 ± 0·23**	40	4
50 mM-xylose	3·52 ± 0·35	17	5

An analysis of variance was performed on the controls for each experiment. There was no significant difference between the controls which were therefore grouped together.
** Significantly different from control values ($P < 0{\cdot}01$).

Table 2. *The effect of sodium ions and temperature on 1 mM-glucose uptake by single mouse blastocysts*

Treatment	Glucose uptake (pmol embryo^{-1} h^{-1} ± S.E.)	Number of embryos
Control	3·99 ± 0·15	9
No sodium	3·79 ± 0·26	9
4°C	0·52 ± 0·05**	6

** Significantly different from control values ($P < 0{\cdot}01$).

Table 1. 1 mM-glucose uptake was significantly reduced in the presence of 5 and 50 mM-glucosamine, 5 and 50 mM-galactose, and 50 mM-3-*O*-methylglucose. 5 mM-3-*O*-methylglucose and 50 mM-xylose had no significant effect. The greatest inhibition of glucose uptake occurred in the presence of 50 mM-glucosamine.

Table 2 shows the effect of omitting sodium ions and of reduced temperature on the uptake of glucose by single blastocysts. There was no significant difference in rates of glucose uptake in the presence or absence of sodium ions. At 4°C glucose uptake was reduced by nearly 90 %.

The effect of insulin (Bovine Pancreas, Sigma) on the uptake of 1 mM-glucose by single mouse blastocysts was studied. Over the range of concentrations employed (0–5000 μunits ml^{-1}), there was no significant increase.

Glucose may be metabolised after entering the embryo. Conversion to other metabolites will lower the intracellular glucose concentration and act, indirectly, as a driving force for further glucose entry. The conversion of glucose to one such metabolite, lactate, was therefore measured at a range of glucose concentrations (Fig. 2). Lactate production increased as the glucose concentration was increased from 0·25 to 2·0 mM, after which there were no significant increases.

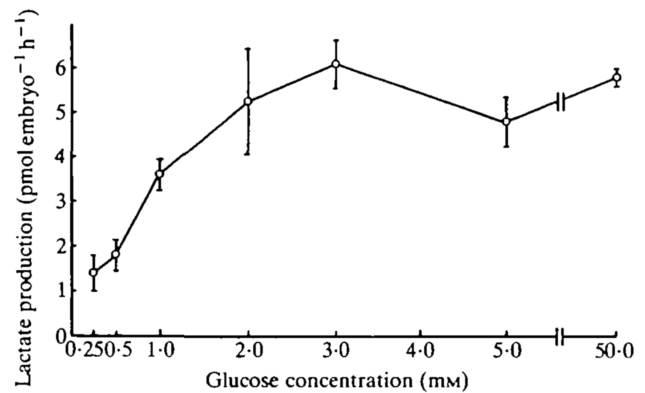

Fig. 2. The effect of increasing concentrations of glucose on lactate production by single mouse blastocysts. Values are mean (±S.E.) of at least six embryos.

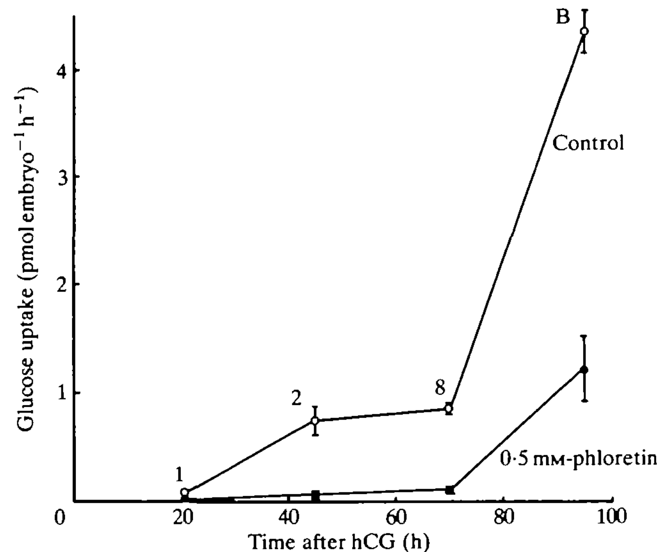

Fig. 3. The uptake of 1 mM-glucose by single mouse embryos in the presence and absence of 0·5 mM-phloretin. 1-cell (1), 2-cell (2), 8-cell (8), blastocyst (B). Values are mean (±S.E.) of six embryos.

The above data indicate that a component of glucose transport into the blastocyst is, in all probability, mediated by a carrier. Experiments were therefore carried out in the presence and absence of phloretin to determine whether carrier-mediated glucose transport was present in the earlier stages of embryo development. In the absence of phloretin, glucose uptake increased from 0·05 pmol embryo^{-1} h^{-1} at the 1-cell stage to 0·73 pmol embryo^{-1} h^{-1} at the 2-cell stage (Fig. 3). Between the 2-cell and the 8-cell stages, the ability to take up

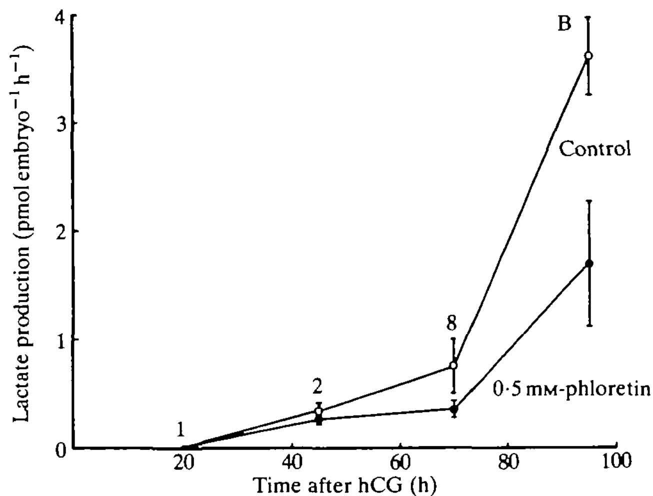

Fig. 4. The production of lactate by single mouse embryos, incubated with 1 mM-glucose, in the presence and absence of 0·5 mM-phloretin. 1-cell (1), 2-cell (2), 8-cell (8), blastocyst (B). Values are mean (±S.E.) of six embryos.

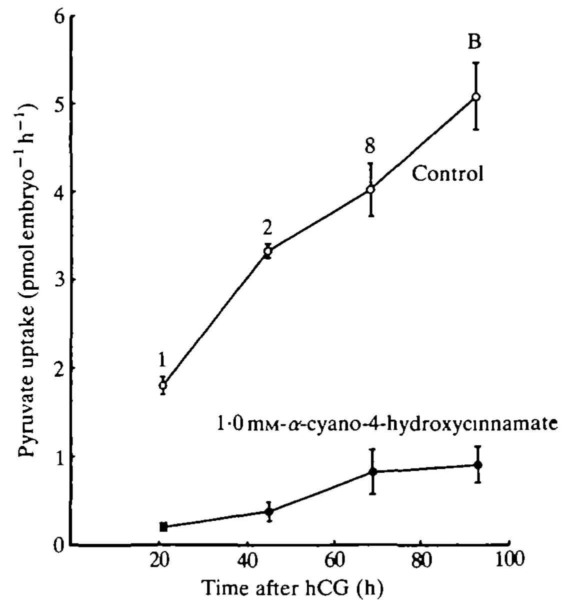

Fig. 5. The uptake of 0·5 mM-pyruvate by single mouse embryos in the presence and absence of 1 mM-α-cyano-4-hydroxycinnamate. 1-cell (1), 2-cell (2), 8-cell (8), blastocyst (B). Values are mean (±S.E.) of six embryos.

glucose remained relatively constant, and then increased from 0·82 to 4·38 pmol embryo^{-1} h^{-1} between the 8-cell stage and the blastocyst. In the presence of phloretin, glucose uptake remained close to zero until the blastocyst stage when its uptake rose to 1·22 pmol embryo^{-1} h^{-1}.

Parallel measurements of lactate formation were also carried out. At the 1-cell stage, there was no detectable lactate production in the presence or absence of phloretin (Fig. 4). In the absence of phloretin, lactate production increased at each developmental stage, reaching 3·6 pmol embryo^{-1} h^{-1} at the blastocyst stage. When phloretin was present, lactate production was detectable at the 2-cell stage and remained low until after the 8-cell stage. By the blastocyst stage, lactate production had increased to 1·7 pmol embryo^{-1} h^{-1}.

An analogous series of experiments was conducted to determine the extent to which carrier-mediated entry of pyruvate persisted throughout development (Leese & Barton, 1984). Fig. 5 shows the uptake of 0·5 mM-pyruvate at each developmental stage in the presence and absence of 1·0 mM-α-cyano-4-hydroxycinnamate, a specific inhibitor of pyruvate transport in erythrocytes and rat liver mitochondria (Halestrap & Denton, 1974).

In the absence of the pyruvate transport inhibitor, uptake steadily increased throughout development. In the presence of α-cyano-4-hydroxycinnamate, the uptake of pyruvate was greatly reduced at each developmental stage.

Discussion

The main finding of this study is that early mouse embryos possess a facilitated entry mechanism for glucose. This conclusion is based on the kinetics of glucose uptake at increasing glucose concentrations (Fig. 1), the inhibition of glucose uptake by phloretin (Fig. 1) and cytochalasin B, its inhibition by sugar analogues (Table 1), its lack of dependence on sodium ions and its sensitivity to temperature (Table 2).

Dabich & Acey (1982) examined glucosamine transport by mouse blastocysts, which was also found to be mediated by a phloretin-sensitive carrier mechanism. The J_{max} and K_t of the glucosamine transport system were 6·6 pmol embryo^{-1} h^{-1} and 0·52 mM, respectively. Our corresponding values for glucose transport were 3·53 pmol embryo^{-1} h^{-1} and 0·14 mM. However, Dabich & Acey (1982) did not attempt to separate the passive and facilitated components of glucosamine transport, making direct comparison of the two systems difficult. If the analysis of glucose transport is repeated using unammended data (Fig. 1), the values for J_{max} and K_t become 6·5 pmol embryo^{-1} h^{-1} and 0·34 mM, respectively, suggesting that glucose and glucosamine share the same carrier. As the K_t is lower for glucose, the carrier has a higher affinity for glucose than for glucosamine.

At 4 °C the uptake of glucose was reduced by nearly 90 %. Although it is reassuring that this finding agrees with those of others (Wales & Brinster, 1968; Dabich & Acey, 1982), the significance of nutrient uptake values at a reduced temperature is open to question. Cooling will affect many intracellular functions such

as rates of metabolism and ion transport, and one cannot be sure of the cells' integrity.

Effect of insulin

Glucose uptake was examined in the presence of increasing concentrations of insulin added to the incubation medium. Insulin is present in mammalian blood at a concentration of about 50 μunits ml^{-1} (Wallis *et al.* 1985). At this concentration, glucose uptake by single mouse blastocysts was slightly elevated (17 %), but the difference was not significant. At concentrations of insulin up to 5000 μunits ml^{-1}, there was no significant elevation of glucose uptake. Gardner & Kaye (1984) found that uptake of the nonmetabolizable analogue, 3-*O*-methylglucose, by mouse blastocysts was increased by 60 % at high concentrations of insulin (about 25 000 μunits ml^{-1}), but not in the presence of physiological concentrations of insulin.

A more recent study, by Rosenblum *et al.* (1986), demonstrated stage-specific binding of physiological concentrations of insulin to the preimplantation mouse embryo. Binding was strongest to the blastocyst. There appeared to be limited binding to the morula, but prior to this stage, no insulin binding was observed. Rosenblum *et al.* (1986) concluded that insulin was required for glucose transport into the embryo. Our evidence is not consistent with this conclusion. Glucose can be transported by mouse embryos in the absence of insulin and the presence of insulin-binding sites does not necessarily mean that the hormone increases glucose transport. For example, isolated placental cells possess intact insulin receptors and yet do not show an enhancement of 3-*O*-methylglucose transport in the presence of the hormone (Bissonette *et al.* 1985).

Pritchard *et al.* (1987) showed that there was an increased incorporation of [^{3}H]uridine into RNA when mouse morulae were incubated in the presence of physiological concentrations of insulin. These results are in agreement with the work of Wales *et al.* (1985), who found that although insulin did not stimulate glucose incorporation into glycogen, glucose incorporation into acid-insoluble macromolecules was increased by 0·05 pmol embryo^{-1} h^{-1}.

It appears that insulin may have an anabolic effect on the formation of RNA and disposal of glucose within the developing embryo, but that this is not reflected as an increase in glucose uptake.

Physiological significance of facilitated glucose transport

Although glucose uptake at the zygote stage was only just greater than zero, it was inhibited by phloretin. The uptake of glucose by 2- and 8-cell embryos was also greatly reduced in the presence of phloretin. These results indicate that a facilitated glucose entry mechanism is present from the first cleavage division onwards. 2- and 8-cell embryos took up similar amounts of glucose, in agreement with the results of Wales & Brinster (1968), who used U-[^{14}C]glucose to study substrate carbon accumulation by the early mouse embryo.

Glucose uptake in the presence of phloretin presumably represents that component of transport due to passive diffusion. This passive component is negligible in the early developmental stages, but increases sharply at the blastocyst stage. This might be the result of the need for increased substrate utilization at blastocoele formation.

The concentration of glucose in the uterine lumen has been reported to be approximately 1 mM (Edirisinghe & Wales, 1985). At this concentration, up to 75 % of the glucose uptake into the early mouse embryo is likely to be carrier-mediated. In other words, facilitated glucose transport is likely to be essential to preimplantation embryo survival. It is not necessary to postulate the active transport of glucose into the early embryo, since it is likely that there will be a substantial gradient in glucose concentration between the extracellular and intracellular compartments. The absence of glucose active transport was confirmed by experiments using phlorizin and sodium-free media. This is supported by the work of Benos (1981), who found that glucose transport by rabbit blastocysts was sodium-independent.

Glucose metabolism

Lactate production was not detectable at the zygote stage, but then increased at each developmental stage, reflecting the embryo's ability to utilize glucose as an energy source as development proceeds. 8-cell embryos convert significantly more glucose (40 %), ($P < 0·05$) to lactate than at the 2-cell stage (25 %), although they both have similar glucose uptakes. This is further evidence of a block to glycolysis at these early developmental stages. Although glucose uptake had increased greatly by the blastocyst stage, the proportion of uptake accounted for by lactate formation was similar.

This pattern of lactate production follows that observed by Wales (1969, 1986), who found that production gradually increased up to the 8-cell stage and then rose sharply at the blastocyst.

Pyruvate transport

In the presence of α-cyano-4-hydroxycinnamate, pyruvate uptake by the embryo was substantially inhibited at each developmental stage. It is known that α-cyano-4-hydroxycinnamate can inhibit pyruvate transport in erythrocytes and across the inner mitochondrial membrane of isolated liver mitochon-

dria (Halestrap & Denton, 1974). Although it is possible that pyruvate transport across the inner mitochondrial membrane of the mouse embryo was inhibited, a recent study (Edlund & Halestrap, 1988) demonstrated that α-cyano-4-hydroxycinnamate, in the mM range, can specifically inhibit the transport of 0·5 mM-pyruvate across the plasma membrane of hepatocytes. At such concentrations, the inhibitor has little, if any, effect on the enzymes of pyruvate metabolism (Halestrap & Denton, 1975). The evidence suggests that α-cyano-4-hydroxycinnamate acts as a transport inhibitor, indicating that a pyruvate carrier is present throughout mouse embryo development.

Fig. 5 shows that in the absence of glucose in the incubation medium the characteristic decline in pyruvate uptake after compaction does not occur (Leese & Barton, 1984; Gardner & Leese, 1986). In other words, the embryo is able to compensate for the absence of glucose by maintaining its uptake of pyruvate. This indicates that the switch to a glucose-based metabolism after compaction is not due to the loss of a pyruvate transport system and that the preimplantation mouse embryo can adapt to its environment in culture by altering its substrate preference. This ability to utilize different substrates may be of importance to the embryo *in vivo* and allow it to respond to differing concentrations of nutrients in the upper reproductive tract.

Biochemical basis of the block to glucose utilization

Glucose uptake by the early embryo could be regulated at two levels; transport across the plasma membrane and/or intracellular utilization. Phosphofructokinase (PFK) is generally recognized as the principal regulatory enzyme of glycolysis. However, Brinster (1971) found that the activity of PFK remained constant during the preimplantation period (around 30 pmol embryo^{-1} h^{-1}). The activity of hexokinase shows a different pattern to that of PFK. Up to the 2-cell stage, the activity is 1·7 pmol embryo^{-1} h^{-1}. This increases slightly at the 8-cell stage to 2·2 pmol embryo^{-1} h^{-1}, and by the blastocyst it has risen to 7·9 pmol embryo^{-1} h^{-1} (Brinster, 1968). In other words, the activity of both these enzymes exceeds the maximum rate of glucose uptake at all stages measured, from the zygote to the blastocyst (Fig. 3). It is therefore possible that the block to glucose utilization resides at the level of glucose transport across the plasma membrane. However, hexokinase and PFK measurements represent the maximum catalytic activity *in vitro* and it is likely that both enzymes are inhibited allosterically *in vivo*, such that they contribute to the limited glucose utilization. This proposition is supported by the observation that the 2- and 8-cell embryos take up similar amounts of glucose and yet differ in their ability to synthesize lactate.

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