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Metabolism in preimplantation mouse embryos

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Abstract. The ability of preimplantation mouse embryos to utilize glucose oxidatively is controlled, in part at least, at the level of glycolysis. Various experimental observations are reviewed that indicate the regulatory mechanism in delayed implanting blastocysts involves the classic negative allosteric feedback of high levels of ATP on phosphofructokinase while the situation in 2-cell embryos appears to be more complicated. That is, in addition to the usual negative effect of ATP and citrate on phosphofructokinase, there appears to be a modification of hexokinase that prevents phosphorylation of adequate amounts of glucose and results in low levels of fructose-6-phosphate at the 2-cell stage and consequently there is a failure to release the inhibition of phosphofructokinase even if ATP and citrate levels decrease. Although both types of embryos have limited glycolytic activity, they do have adequate capacity for citric acid cycle activity and oxidative phosphorylation, and are able to maintain a high ATP : ADP. It is argued, therefore, that the reduced levels of macromolecular synthesis characteristic of 2-cell and delayed implanting blastocysts are not due to restricted energy substrates or regulatory controls on glycolysis and a subsequent low energy state. On the contrary, it seems that the reduction in oxidative utilization of glucose in these situations is a result of diminished energy demand because of the low level of synthetic activity. The potential significance of this relationship between energy production and utilization in terms of potential regulatory mechanisms in preimplantation embryos is discussed.

Keywords. Preimplantation; mouse; embryos; carbohydrate metabolism; delayed implantation.

Introduction

Interest in the capacity of mammalian preimplantation embryos to utilize various energy substrates was initiated by Whitten's (1956, 1957) observations that mouse embryos at the 8-cell stage would develop in a Krebs-Ringer bicarbonate solution containing albumin and glucose while 2-cell embryos required the addition of lactate. This demonstration of a difference in substrate requirements led to the realization that there are changes in the metabolic capacity of mammalian preimplantation embryos with development, and suggested that some regulatory mechanism must function even in these very early stages. Although a great deal of information about the metabolic capabilities of early mammalian embryos has been generated in the 25 years since Whitten's original observations, and some control mechanisms have been elucidated (Biggers, 1971; Wales, 1973,1975), the significance of changes in substrate utilization is still not entirely clear. The purpose of this communication is to review some of the work that has been done with normal and delayed implanting embryos in an attempt to identify questions that remain unanswered.

Abbreviations used: CoA, Coenzyme A; P_i, orthophosphate.

Cleavage stage embryos

Almost ten years after Whitten's original papers, Brinster (1965) reported the results of a systematic study undertaken to evaluate the effects of a wide variety of potential energy substrates on development of 2-cell mouse embryos *in vitro*. He found that development would occur if the embryos were supplied with lactate, pyruvate, oxaloacetate, or phosphoenolpyruvate, but not if they were given acetate, citrate, α-ketoglutarate, succinate, fumarate, malate, or glucose. In further studies by Brinster and his colleagues utilization of O_2 was measured (Mills and Brinster, 1967), and it was demonstrated that lactate and pyruvate could be oxidized equally as well by 2-cell and 8-cell embryos (Brinster, 1967a) while Quinn and Wales (1973a,b) reported that these same substrates would maintain normal levels of ATP in both 2-cell and 8-cell embryos. These findings have been taken as indicating that enzyme systems necessary for citric acid cycle activity and oxidative phosphorylation are intact and functional even in 2-cell embryos. The assumption then, has been that glucose and those citric acid cycle intermediates that do not support development of the early embryos, either cannot enter the cells or cannot be converted to a substrate that will support continued operation of the citric acid cycle.

Embryos develop the capacity to use other substrates between the 2-cell and the 8-cell stages (Brinster and Thomson, 1966) and most attention in this regard has been directed at malate and glucose. Wales and Biggers (1968) were unable to demonstrate either accumulation of labelled carbon or evidence of oxidative utilization of U-[^{14}C]-malate by 2-cell embryos in contrast to 8-cell embryos and concluded that absence of a transport mechanism for malate was, in the early stage, responsible. It should be noted however, that initial rates of uptake of malate were not determined in those studies and therefore, that changes in permeability were not measured directly. Another argument for there being a change in the capacity of embryos to take up malate has been developed from data generated in several different experiments. First, it will be recalled that 2-cell embryos develop *in vitro* with oxaloacetate but not malate as the sole source of energy supplied to the medium (Brinster, 1965); and, second, that 2-cell embryos have NAD^+ dependent malate dehydrogenase activity (Brinster, 1966) and thus should be able to convert malate to oxaloacetate. Although it seems from this that malate should support early embryos if it could enter the cells, one further point must be considered; oxaloacetate cannot be used directly to support continued operation of the citric acid cycle, rather it must be converted to acetyl-coenzyme A presumably through the formation of pyruvate. Although this does not appear to have always been appreciated (Biggers *et al*., 1967), it raises the question of whether the conversion occurs intracellularly and if so, by means of which enzymatic pathway and in which cellular compartment? There are several possibilities (figure 1): Oxaloacetate might enter the cells and be converted to pyruvate in the cytoplasm; this would presumably require formation of phosphoenolpyruvate (*i.e*., *via* phosphoenolpyruvate carboxykinase) and then pyruvate (*i.e*., *via* pyruvate kinase); this scheme seems reasonable although neither of the enzymes necessary for these transformations have been demonstrated at early stages of development. It is also possible that oxaloacetate is reduced to malate in the cytoplasm (malate dehydrogenase activity has been demonstrated at the 2-cell stage; Brinster, 1966; Quinn and Wales, 1971) and then goes on to pyruvate (*i.e*., also in the

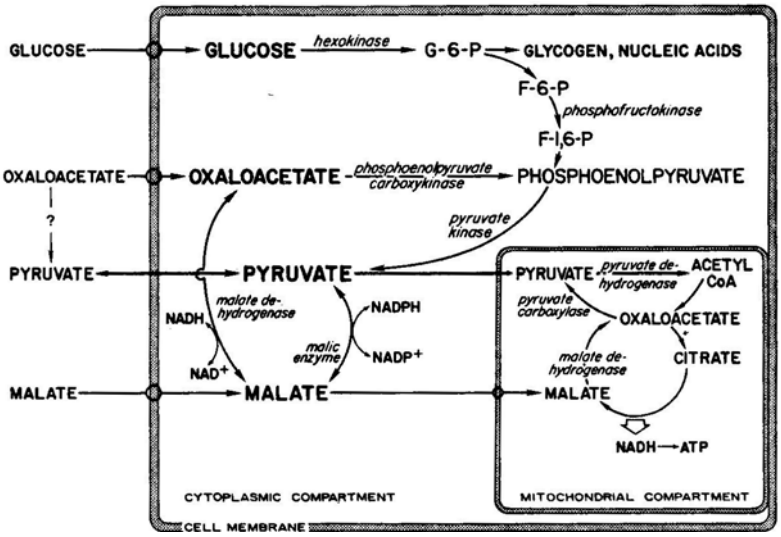

Figure 1. Possible pathways for carbohydrate metabolism in preimplantation embryos.

cytoplasm *via* malic enzyme; the presence of malic enzyme has been demonstrated (Quinn and Wales, 1971). Alternatively, malate from the cytoplasm might enter the mitochondria (malate, but not oxaloacetate, can cross the mitochondrial membrane), be converted back to oxaloacetate, and then on to pyruvate (*i.e.*, by way of the mitochondrial enzyme pyruvate carboxylase). Enzyme activities necessary for these mitochondrial conversions have been demonstrated in early mouse embryos (Quinn and Wales, 1971). Although this scheme might seem unlikely because it would eventually lead to a deficiency of reducing equivalents in the cytoplasm, it is compatible with Thomson's (1967) finding that malonate (an inhibitor of pyruvate carboxylase) will block development of 2-cell embryos supplied with oxaloacetate but not those given pyruvate. Thomson's (1967) observation also argues against the possibility that oxaloacetate is able to support development only because it spontaneously breaks down to pyruvate in the medium. Thus, although it is not clear how oxaloacetate is utilized, it has generally been assumed that it supports the citric acid cycle either directly (Biggers *et al.,* 1967), or after being converted intracellularly to pyruvate by way of one or another of the schemes outlined above. From this assumption it has been argued that there are changes in transport of malate with development. The reasoning is as follows: since the enzymatic capability to convert malate to oxaloacetate is present at the 2-cell stage, the inability of malate to support development at that stage indicates it is not able to enter embryos. That malate is capable of supporting 8-cell embryos then, indicates that capacity for its transport develops at that stage. It must be emphasized that this does not constitute a rigorous proof of a change in permeability, nor has the possibility that malate can enter 2-cell embryos but for some reason is not converted to oxaloacetate or pyruvate (possibly the absence of malic enzyme or a high NADH to NAD^+ ratio) been considered. These questions will have to be resolved before the

problem of why some citric acid cycle intermediates will support early development, and others will not, can be answered definitively.

Utilization of glucose by 2-cell embryos has been examined in detail and although it is apparently involved in synthesis of glycogen and other macromolecules (Stern and Biggers, 1968; Brinster, 1969; Murdoch and Wales, 1973; Wales, 1975), it is not oxidized to any great extent (Brinster, 1967b) and will not maintain normal levels of ATP (Quinn and Wales, 1973b). Glucose will, however, support development of 8-cell embryos (Brinster, 1965) and again it has been proposed that this is because of changes either in enzyme pathways (in this case Embden-Myerhoff scheme), or in the embryo's capability to transport the substrate. The observation by Wales and Brinster (1968) that 2-cell and 8-cell embryos incubated in 5·1 mM U-[^{14}C]-glucose accumulate labelled carbon at equal rates in the first 3 min supports the suggestion that it is regulation of glycolysis rather than uptake that is responsible for the inability of glucose to support the early embryos. However, changes in glucose transport between the 2-cell and 8-cell stage have not been conclusively ruled out. More recently, the various intermediates in glycolysis have been measured in 2-cell, 8-cell, and later stage embryos during starvation and refeeding with glucose or pyruvate (Barbehenn *et al.,* 1974, 1978). Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, ATP, AMP, orthophosphate (P_i), citrate, isocitrate, malate and α-ketoglutarate were measured and it was found that there is a "block" at the phosphofructokinase step in 2-cell embryos. Although phosphofructokinase is allosterically inhibited by high levels of citrate and ATP in many systems, the block is characteristically lost under anaerobic conditions as levels of citrate and ATP fall (i.e., the Pasteur effect). This does not seem to be the case in starved 2-cell embryos where citrate and ATP levels also fall, since phosphofructokinase activity remains low during refeeding with glucose. Furthermore, it appeared that the potential for adequate hexokinase activity was present in the early embryos (*i.e.,* when measured in cell lysates), but maximum levels of the hexosephosphates after refeeding were lower in the early stages than they were in the later stages. This was interpreted as indicating that for some unknown reason glucose phosphorylation is shut off in 2-cell embryos by a low level of glucose-6-phosphate and thus, only a low level of fructose-6-phosphate is attained. Since fructose-6-phosphate can potently release the inhibition of phosphofructokinase, it was proposed that an increase in that compound at a later stage is the major cause of the greater phosphofructokinase activity and hence glycolysis. Although it was concluded that the true cause of the inability of 2-cell embryos to utilize glucose is an "unknown modification" of the initial enzymes in the glycolytic scheme (*i.e*., hexokinase, Barbehenn *et al.,* 1978), no definitive explanation has been offered.

Studies such as these have added greatly to our understanding of metabolism in early embryos but they have not provided satisfactory answers to the original question of whether it changes in transport or enzyme pathways that are primarily responsible for differences in the ability of glucose and various citric acid cycle intermediates to support development at the 2-cell and 8-cell stages of development. Furthermore, although we know that the level of macromolecular synthesis is low in these early embryos, and that as a consequence ATP levels are relatively high, we do not have any information on the significance of putative changes in pathways for various energy intermediates to embryos that are normally in an environment that is rich in pyruvate (Nieder and Corder, 1983).

Delayed implanting embryos

Development of mouse embryos is arrested at the blastocyst stage in mothers that are concurrently lactating (lactational delayed implantation) or are ovariectomized before noon on the fourth day of pregnancy (experimentally delayed implantation). Although it has been known for some time that metabolic and mitotic activity in these embryos decreases until the suckling young are removed or estrogen is injected into the mother (McLaren and Menke, 1971; McLaren, 1973), and many reports have appeared that deal with metabolic activity and synthesis of macromolecules, there has not really been a satisfactory explanation, at the molecular level, for the embryonic quiescence associated with delayed implantation. Typically in these experiments, embryos are recovered from the uteri of mice that have been ovariectomized and treated with progesterone for several days and incubated *in vitro* with radioactive precursor molecules. Utilization or incorporation of label into DNA, RNA, or protein is then estimated by scintillation counting or autoradiographic techniques. The findings can be summarized briefly as follows.

DNA

Incorporation of [^{3}H]-thymidine slows (and finally ceases altogether) as embryos enter the dormant phase associated with lactational delayed implantation (McLaren, 1968). This occurs in the 24–30 h following removal of the maternal ovaries in experimental delayed implantation (Given, 1983; Given and Weitlauf, 1981) and might have been anticipated as the cells become arrested in the G_1 phase of the cell cycle (Sherman and Barlow, 1972); the level of mitotic activity has been shown to decrease over the same interval by direct cell counts (Weitlauf *et al.,* 1979). Furthermore, reactivation of the embryos by injecting estrogen into the mother (or simply removing them from the uterus to appropriate culture conditions), leads to a resumption of [^{3}H]-thymidine incorporation (Given and Weitlauf, 1981, 1982) and cell division (Weitlauf *et al.,* 1979). Taken alone, the changes in incorporation of labelled thymidine might be interpreted as reflecting changes in permeability or specific activities of internal precursor pools. But taken in conjunction with the fact that there are corresponding changes in cell division and the observation that the cells are in G_1 during the dormant period, it seems clear that the reported changes in rates of incorporation of [^{3}H]-thymidine actually reflect changes in synthesis of DNA.

RNA

Several investigators have compared the incorporation of [^{3}H]-uridine by delayed implanting blastocysts with that by embryos implanting normally or undergoing the process of reactivation. Typically, blastocysts were recovered from the uteri of ovariectomized females and incubated *in vitro* with [^{3}H]-uridine and it was observed that the rate of incorporation of label into RNA was lower in delayed implanting embryos than that in normal or reactivated embryos (Weitlauf, 1976; Chavez and VanBlerkom, 1979). However, because the specific activities of the endogenous pools of UTP were not known the actual rates of RNA synthesis could not be determined in those experiments and there has been disagreement over interpretation of the results. More recently a similar series of experiments was performed and the specific activities

of the endogenous pools of UTP were determined and the actual rates of synthesis of RNA could be calculated from the rates of incorporation of [^{3}H]-uridine (Weitlauf and Kiessling, 1980). In this way, after correcting for differences in numbers of cells it was shown that the overall rate of synthesis of RNA in delayed implanting embryos is about one-third of that in reactivated embryos. It was also shown that the increases in synthesis of RNA that occurred with reactivation took place gradually over 12–24 h *in vitro.* A similar change in synthesis of RNA has been shown to occur *in vivo* following the injection of estrogen into the mother (Weitlauf, 1982). Furthermore, in that experiment it was shown that although the bulk of the increase in [^{3}H]–uridine incorporation was into rRNA, there was a transient burst of mRNA synthesis early in the process of reactivation (*i.e.,* 3–4 h after the injection of estrogen). Although these results with RNA are reminiscent of those with DNA, it should be noted that in contrast to that situation, the embryos do not completely stop synthesis of RNA during delayed implantation. Rather, they continue to operate at approximately one-third of the level characteristic of active embryos presumably to support synthesis of 'house keeping' proteins.

Protein synthesis

The rate of incorporation of labelled amino acids into protein by delayed implanting mouse embryos is about half that observed in normal implanting or reactivated embryos; it increases to normal levels within a few hours of the injection of estrogen or removal of the blastocysts from the uterus to a suitable culture medium (Weitlauf, 1974; VanBlerkom *et al.,* 1979). Although the effects of potential differences in specific activities of intracellular amino acid pools have not been rigorously evaluated, it has been shown that total protein content changes proportionately with the incorporation of radioactive amino acids (Weitlauf, 1973) and the estimates of protein synthetic rates from incorporation of label would seem to be valid. Although it is clear from such observations that the rate of protein synthesis in delayed implanting embryos is low and that it increases with reactivation, the unanswered question is whether this involves transcription of specific new mRNA or a more generalized change in metabolic activity and rates of translation. It would be expected that the mechanisms responsible for the developmental arrest associated with delayed implantation will be quite different, depending on which of these strategies has been employed by the embryos. Several relevant observations have been made but the question remains unanswered. Thus, two-dimensional electrophoresis has revealed that some 'new' proteins can be demonstrated in the first few hours of the reactivation process (VanBlerkom *et al*., 1979); unfortunately, it is not clear whether these "spots" are truly new proteins and thus reflect synthesis of new mRNA or simply represent increases in amounts of 'old' proteins that become visible because of increased rates of synthesis. Attempts to block the process of activation *in vitro* with α-amanitin (*i.e.,* at doses that would be expected to block synthesis of new mRNA) do not completely prevent the expected increases in incorporation of amino acids (unpublished results). The difficulty with this approach is, that because it is total incorporated radioactivity that is measured, one cannot determine if small changes in synthesis of specific proteins have occurred. Finally, it has been reported that the amounts of some enzymes increase, others remain the same, or

even decrease with activation (Weitlauf *et al.,* 1979; Weitlauf, 1981; Nieder and Weitlauf, 1983), again suggesting that there are some differential changes in protein synthesis. However, it must be emphasized that such changes could reflect either transcriptional or translational mechanisms. Thus, the question of how macromolecular synthesis is regulated in delayed implanting mouse embryos remains unanswered.

Glucose utilization

In addition to the studies on macromolecular synthesis there have been numerous experiments directed to utilization of glucose as an energy substrate. The original observations were those of Menke and McLaren (1970a,b; Menke, 1972) that demonstrated the production of $^{14}CO_2$ from U-[^{14}C]-glucose was reduced in delayed implanting embryos. In these experiments again, blastocysts were recovered and incubated with labelled substrate and radioactive product generated with time was evaluated by scintillation counting procedures. These studies were followed up with the observation that during activation of the dormant embryos the rate of glucose oxidation increases and that this occurred whether the process was *in vivo* with estrogen or *in vitro* (Torbit and Weitlauf, 1974,1975). Although these studies also suffered from potential questions about permeability and specific activity of endogenous pools, they raised the possibility that decreased energy production might lead to a reduction in synthesis of macromolecules and ultimately, the metabolic quiescence that seems to be responsible for the developmental arrest associated with delayed implantation. This scheme would implicate the regulation of energy production as a prime control in development of preimplantation embryos. Two other observations have tended to reinforce this idea: (i) Nilsson and his co-workers (Nilsson *et al.,* 1982) measured utilization of O_2 by delayed implanting embryos and found it to be decreased. Although this might have been expected from the studies showing reduced oxidative utilization of glucose, it was significant because he measured absolute consumption of O_2 and did not depend on the use of radioisotopes. This approach, then, is not open to questions about specific activity, permeability, etc. In the same report it was also noted that cytochrome oxidase activity was decreased in delayed implanting embyros and suggested that the lower oxidative utilization of glucose is due to a reduced capacity for oxidative phosphorylation and leads to decreased energy production, (ii) Embryos do not develop beyond the blastocyst stage if glucose is missing from the medium (Wordinger and Brinster, 1976; Naeslund, 1979; VanBlerkom *et al.,* 1979). It has been suggested that this may be equivalent to embryonic diapause *in vitro* and that restriction of glucose in the uterine lumen might actually lead to the arrested development in delayed implantation. Again the underlying idea here is that a lack of energy production is responsible, at least in part, for the quiescence associated with delayed implantation.

Although it seems clear from some of this work that utilization of glucose is reduced in delayed implanting embryos, two questions that need to be answered are: (i) what step in the metabolic scheme for glucose is regulated?, and (ii) whether the associated reduction in energy production is responsible for the decrease in macromolecular synthesis?

Glycolysis and oxidative phosphorylation

The observation by Menke and McLaren (1970a,b) that oxidative utilization of glucose is decreased in delayed implantation, along with the report by Nilsson and his co-workers (Nilsson *et al.,* 1982) that there is a reduction in cytochrome oxidase activity, suggests that oxidative phosphorylation is limited in delayed implantation. Further, it has been proposed that it is the resulting inability of the embryos to generate sufficient amounts of energy that leads to the reduction in macromolecular synthesis and the characteristic developmental arrest (Nilsson *et al.,* 1982). To test this hypothesis, experiments were undertaken in our laboratory to measure the capacity for citric acid cycle activity and oxidative phosphorylation in intact delayed implanting mouse embryos (Nieder and Weitlauf, 1983). (i) Dormant and reactivated embryos were recovered and incubated with 2-[^{14}C] -pyruvate; evolved $^{14}CO_2$ was trapped, and the amount of radioactivity was measured. After correcting for differences in embryonic mass, it was found that the ability to utilize pyruvate oxidatively was equal in the two types of embryos (74 ± 11 and 72 ± 9 pmol CO_2/h/μ g respectively). This result demonstrated that failure of delayed implanting embryos to oxidize glucose is not due to deficiencies in the citric acid cycle or oxidative phosphorylation and suggests either uptake of glucose or enzymatic regulation in the Embden-Meyerhoff scheme as possible control points. (ii) To avoid the questions raised by utilization of labeled substrates in testing the glycolytic pathway, embryos were recovered and placed *in vitro* in medium containing non-labelled glucose and the production of lactate (*i.e.*, in the presence of O_2) over 2 h was measured. It was found that active embryos produced more lactate than delayed implanting embryos (92 ± 5, and 45 ± 4 pmol CO_2/h/μg respectively), thus confirming that uptake of glucose or the glycolytic pathway are restricted in the dormant embryos, (iii) The amounts of phosphofructokinase and hexokinase activity were measured in lysed embryos to determine if the levels of these primary regulatory enzymes could be responsible for reduced glycolytic activity. The levels of phosphofructokinase were not different in dormant and reactivated embryos (80·4 ± 8·9, 72·5 ± 6·3 pmol/min/μ g respectively). Interestingly, the level of hexokinase activity in the delayed implanting embryos was nearly 3 times that in the active embryos (*i.e.*, 21·7 ± 2·1, and 6·5 ± l·2 pmol/min/μg respectively), thus demonstrating that absolute amounts of these regulatory enzymes are not limiting in the dormant embryos. (iv) Transport of glucose into blastocysts was examined by measuring the initial rates of uptake and phosphorylation of [^{3}H]-2-deoxyglucose at varying concentrations. The K_m for the substrate was not significantly different in the two types of embryos but surprisingly the V_{max} for dormant embryos was 2·2 times higher than that for the active embryos. This result demonstrates that the capacity for uptake and phosphorylation of glucose are not restricting in delayed implantation, (v) That glucose utilization by delayed implanting embryos is low in spite of the fact that the embryos have sufficient enzymes to maintain glucose uptake and glycolysis, as well as the capacity for normal citric acid cycle activity and oxidative phosphorylation of pyruvate, suggests that there is a negative regulatory control of glycolysis. To pursue this possibility lactate production was examined in anaerobic conditions designed to elicit, if possible; a Pasteur effect that would confirm the presence of negative allosteric feedback in the delayed implanting blastocysts. Embryos were incubated in medium with cold glucose

in the absence of O_2 (i.e., an atmosphere of N_2); lactate production by dormant and active embryos over 2 h was increased several fold over that seen in the presence of O_2 (*i.e.*, confirming a Pasteur effect) and was not different in the two types of embryos (*i.e.*, 862 ± 82, and 906 ± 75 pmol/h/μg respectively). From this result it is clear that the two types of embryos have an equal capacity for glycolysis and that some form of negative allosteric feedback is responsible for regulating the Embden-Meyerhoff pathway, (vi) Several metabolites known to modulate the rate of glycolysis were then measured; ATP levels were found to be approximately 2-fold higher while ADP levels were 3-fold lower in delayed implanting (*i.e.*, compared to activated) embryos (*i.e.*, 16·5 ± 2·5 and 7·9 ± l·0 pmol ATP/μg, and 3·1 ± 3·8 and 9·6 ± l·6 pmol ADP/μg respectively). Hexosephosphates were also measured and found to be much higher in dormant than in activated embryos (*i.e.,* the level of combined glucose-6-phosphate and fructose-6-phosphate was 5·25 ± 0·56 and 1·14 ± 0·22 pmol/μg respectively). The buildup of these metabolites along with the high ATP : ADP suggest that phosphofructokinase is the regulated step in glycolysis and that the regulatory mechanism is the classic negative allosteric feedback described in numerous other systems. These results demonstrate that although the failure of delayed implanting embryos to utilize glucose oxidatively must result in a low rate of energy production, it is not responsible for the characteristic reduction in synthesis of macromolecules. On the contrary, it appears that reduced demand for energy is a result of decreased macromolecular synthesis and that the failure to utilize glucose oxidatively is a consequence of the resulting energy excess.

Comment

Although it has been possible to elucidate some of the control mechanisms for glucose metabolism in 2-cell mouse embryos and delayed implanting blastocysts, their significance is not clear. Indeed, since the embryos at both of these stages have a high ATP : ADP and are probably bathed in pyruvate (Nieder and Corder, 1983) in the reproductive tract anyway, it is not likely that regulation of glycolysis, the availability of glucose, or changes in the capacity to utilize various metabolic intermediates would have important roles in controlling development of preimplantation embryos. If the great question in development is what regulates differentiation, the question in preimplantation biology must be how can the mother cause differentiation of the embryo to stop and restart at a later time as occurs with delayed implantation? The implication of the work reviewed above is that the mechanism probably involves control at the level of embryonic transcription or translation and not inhibition of oxidative phosphorylation or glycolysis, or restriction of energy substrates in the uterine lumen.

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