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The Explanation for the Blockade of Glycolysis in Early Mouse Embryos

(metabolite levels/6-phosphofructokinase/glucose phosphorylation)

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ABSTRACT **The reason for the failure of early-stage mouse embryos to grow on glucose alone was investigated by measurement of glucose-6-phosphate, fructose-1,6-bisphosphate plus triose phosphates, citrate, and malate in individual embryos during starvation and refeeding with glucose or glucose plus pyruvate. The results indicate a block at the 6-phosphofructokinase (EC 2.7.1.11) step at early stages, which is later removed. Although there seems to be no early difficulty in phosphorylation of glucose, maximum glucose-6-phosphate levels (and probably fructose-6-phosphate levels) are much lower at early stages than at later stages. The increase in fructose-6-phosphate with age may be the major cause of the increase in 6-phosphofructokinase activity. Unusually high citrate levels at all ages may help to keep this enzyme strongly inhibited until the increase in fructose-6-phosphate occurs. The changes in metabolite levels also indicate an early defect in mobilization of glycogen and a probably less important defect in the citrate cycle.**

It is known from studies of preimplantation mouse embryos (1–3) that glucose will not serve as sole energy source for development until the 8-cell stage, whereas pyruvate is able to do so. The main approach that has so far been used to study this problem has been to measure the activities of a variety of glycolytic and citrate cycle enzymes. Although a few of these activities show substantial changes with time, the reason for the early block in glycolysis has remained unknown.

This is a report of what we believe to be a successful attempt to locate the site(s) of this early difficulty. The strategy has been to measure some of the metabolites of the glycolytic pathway and citrate cycle in the presence and absence of glucose or pyruvate.

After the experimental procedure, the embryos were freeze-dried and stored at low temperature under reduced pressure. This stabilized the metabolites indefinitely. The embryos were analyzed individually, which meant that a single batch of 100–200 gave enough material for a large experiment.

MATERIALS AND METHODS

Mouse Embryos. Preimplantation embryos were obtained from 8 to 10-week-old random-bred Swiss mice superovulated with 10 international units of pregnant mare serum (Organon), followed 48–52 hr later by 10 international units of human chorionic gonadotrophin (Ayerst). Embryos of the two-cell, eight-cell, and morula/early blastocyst stages were flushed from the reproductive tract 44–48, 66–72, and 94–96 hr, respectively, after injection of the gonadotrophin. The collection medium was a modified Kreb's–Ringer bicarbonate containing 1% bovine-serum albumin, 1 mM glucose, 5 mM D,L-lactate, and 0.5 mM pyruvate. The serum albumin was treated with charcoal by the method of Chen (4) to remove fatty acids and citrate. (All media and reagents used for collecting and culturing embryos were made with H_2O distilled once from glass and twice from quartz.) The collection medium was intended to approximate oviductal fluid. Although no data are available for mice, rabbit oviductal fluid contains 2 mM L-lactate, 0.2 mM pyruvate, and 1 mM glucose (5). That the composition chosen is satisfactory was shown by preliminary experiments in which 90% of 2-cell embryos cultured in this medium consistently developed to the blastocyst stage in 72 hr at 37°.

After flushing, the embryos were transferred immediately with Pasteur pipettes (drawn down to a bore slightly larger than an embryo) into 1 ml of collection medium under oil (at about 26°) and then passed into a second milliliter to free them from blood and debris. Here they were left until 100–200 cells from four to eight mice were accumulated (about 30 min). At this time a baseline sample of embryos was quick-frozen (see *Collection* below). (The mineral oil used throughout was purified by shaking it successively with 2 volumes of 1 N NaOH, H_2O, 0.02 N HCl, and several changes of H_2O.)

Starvation and Refeeding. The remaining embryos were quickly washed by transfer into two successive 1-ml volumes of substrate-free medium in cavity slides, and then into a 30-μl drop of substrate-free medium under mineral oil in a Falcon plastic petri dish. They were finally transferred once more into another 30-μl drop in the same dish and incubated 7.5–60 min at 37° in a humidified jar with continual gassing by humidified 5% CO_2 in air. In refeeding experiments, embryos were transferred after 60 min to another 30-μl drop of medium in the same petri dish containing 5.5 mM glucose or 5.5 mM glucose plus 0.5 mM pyruvate. The use of a higher glucose concentration than in the collection medium was intended to accentuate possible changes in metabolite levels. Glucose has been used at this level in successful embryo culture (3).

Collection. At appropriate times, embryos were transferred in about 1 μl of medium from the petri dish to glass slides, which were then frozen in liquid N_2 and dried under reduced pressure. The time from placement on the slide until freezing was about 5 sec. If too little medium was used, so that embryos projected much above the fluid surface, flattening occurred and embryos were hard to remove after freeze-drying; if too much fluid was used they were likely to fall off the slide after they were dried. The slides were freeze-dried overnight in a vacuum drying tube (6) at −35° at a vapor pressure of 0.01 mm of Hg or less. The slides were stored at −70° before drying and at −20° afterwards under reduced pressure ($\leq$0.05 mm of Hg).

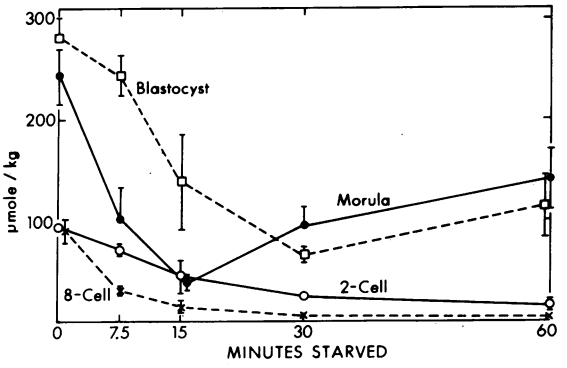

FIG. 1. Glucose-6-P levels during starvation. Each point represents the average of usually three embryos ± SEM.

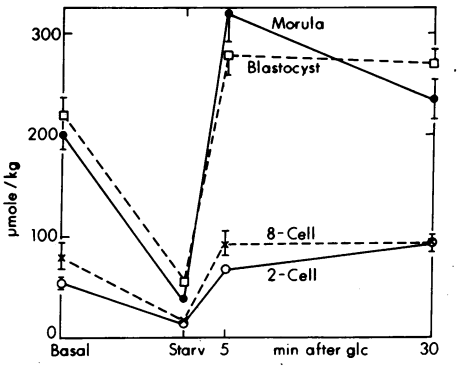

FIG. 2. Glucose-6-P levels before and after 60 min of starvation (*Starv*), and after realimentation in medium containing 5.5 mM glucose (*glc*). Each point represents 9 to 12 embryos for the basal values and 5 to 10 for the rest, except that there were only 2 or 3 embryos in the last three morula points.

Analyses. All measurements were made on single embryos. The amounts of material per assay ranged from 3×10^{-16} mol, for the lowest levels of fructose bisphosphate, to 10^{-12} mol for the highest levels of citrate. To measure these small quantities, we carried out the first analytical steps in droplets as small as 0.05 μl under mineral oil ["oil well technique" (7)]. Enzymatic methods were used that terminated in the oxidation or reduction of NAD^+ or $NADP^+$. The necessary sensitivity was obtained by amplification as much as 25,000-fold by enzymatic cycling (6). The general methodology and each of the specific methods have been described (6), although some of the procedures had to be increased in sensitivity. Glucose-6-P was measured with glucose-6-P dehydrogenase (EC 1.1.1.49) and $NADP^+$, citrate with citrate lyase, malate dehydrogenase (EC 1.1.1.40) and NADPH, malate with malate dehydrogenase and NAD^+ [plus glutamate and aspartate aminotransferase (EC 2.6.1.1) to pull the reaction to completion], and fructose-1,6-P_2 with NAD^+, arsenate, and the enzymes needed to convert fructose bisphosphate to 3-P-glycerate. The standard deviations for standards equal to embryo samples ranged from 4 to 6%.

The method for measurement of fructose bisphosphate includes the triose phosphates. In analyses of adult tissues, the amounts of triose phosphates have ordinarily been found to be much lower than those of fructose bisphosphate. If this were, however, not true in the present case, it would not affect the conclusions drawn from the experimental results.

All values have been calculated as μmol/kg of wet weight. This is based on the assumption of a wet weight of 0.16 μg per embryo (8). There is probably little change in cellular mass from the 2-cell to the blastocyst stage, inasmuch as there is little change in protein content (9). The absolute levels of metabolites can be calculated in femtomoles by multiplying by 0.16.

RESULTS

Individual Variability. One advantage of making the analyses on single embryos is that it gives a measure of variation among individuals. For the freshly collected embryos, the standard deviations were about 25% for glucose-6-P and malate, 35% for citrate (except at the blastocyst stage) and nearly 50% for fructose-1,6-P_2. The embryos were from a number of different mice, which may have contributed to the dispersion of the data. No attempt was made to separate out this factor. For the various experiments, the embryos were randomized as much as possible.

Basal Metabolite Levels (Table 1). In freshly collected embryos, glucose-6-P levels increased 4-fold from the 2-cell to the morula and blastocyst stages. Fructose bisphosphate (plus triose phosphate) amounts were very low at all four stages analyzed. Citrate levels were very high and constant from the 2-cell to the morula stage (5–10 times those in normal brain and liver). At the blastocyst stage much higher and extremely variable levels were found. It is believed that a major portion of the citrate in the blastocyst is in the blastocele because, in contrast to the other stages, there was no consistent decrease during starvation (see below). Individual citrate levels were observed up to 19 mmol/kg (12 times the average at earlier stages). Malate concentrations were a quarter of citrate levels at the 2-cell stage and did not increase with age.

Changes During Starvation and Realimentation. Glucose-6-P fell markedly during starvation (Fig. 1). The decrease was progressive at the 2-cell and 8-cell stages, whereas at the two later stages, after an initial decrease, the levels rose again. This result presumably indicates mobilization of glycogen. [The absolute levels at the end of starvation varied somewhat with different lots of embryos (Fig. 2 compared with Figs. 1 and 3).] The absolute fall in glucose-6-P during the first 15 min increased progressively to the morula stage. This parallels the increased capacity to convert glucose to lactate over this growth period (10, 11).

TABLE 1. *Levels of metabolites in freshly collected embryos*

	Glucose-6-P	Fructose-1,6-P_2	Citrate	Malate
2-Cell	53 ± 4.4(9)	4.4 ± 0.6(7)	1600 ± 150(14)	390 ± 31(15)
8-Cell	81 ± 13(11)	2.0 ± 0.3(8)	1700 ± 130(21)	390 ± 30(12)
Morula	200 ± 14(12)	3.8 ± 0.6(12)	1800 ± 180(19)	380 ± 44(12)
Blastocyst	220 ± 18(9)	5.7 ± 1.3(9)	5300 ± 1300(10)	430 ± 31(11)

Values are μmol/kg of wet weight ± SEM for the number of eggs given in *parentheses*.

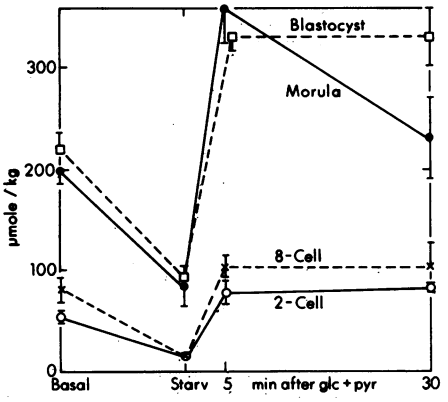

FIG. 3. Glucose-6-P levels after 60 min of starvation (*Starv*) and realimentation in medium containing 5.5 mM glucose (*glc*) and 0.5 mM pyruvate (*pyr*). The basal levels represent the same embryos as in Fig. 2. The other points each represent 5 to 9 embryos ± SEM.

Upon realimentation with glucose alone (Fig. 2) or glucose plus pyruvate (Fig. 3), glucose-6-P levels rose in 5 min to somewhat above basal levels, and then underwent little significant change during another 25 min. The difference between the levels in freshly collected and refed embryos is presumably due to the fact that the collection medium contained 1 mM instead of 5.5 mM glucose.

Fructose-1,6-P_2 (plus triose phosphates) levels did not fall during starvation; in fact there were modest increases in every case, some of them of statistical significance (Figs. 4 and 5). This may be due to the absence of pyruvate (see below). On refeeding with glucose, there was no significant increase in the 2-cell embryos, but there were progressively greater increases in the 8-cell, morula, and blastocyst embryos (Fig. 4). Clearly, as development proceeds, conversion of glucose-6-P to fructose bisphosphate is facilitated. Realimentation with glucose plus pyruvate gave a completely different picture (Fig. 5). Fructose bisphosphate levels fell to zero within 5 min at all but the blastocyst stages. Here it is clear that conversion of glucose-6-P to fructose bisphosphate has been turned off by the presence of pyruvate. Only at the blastocyst stage is there no immediate fall and a distinct rise during 30 min of refeeding.

To test the unlikely possibility that P-glucoisomerase might be responsible for the early block indicated between glucose-6-P and fructose bisphosphate, fructose-6-P was measured in a

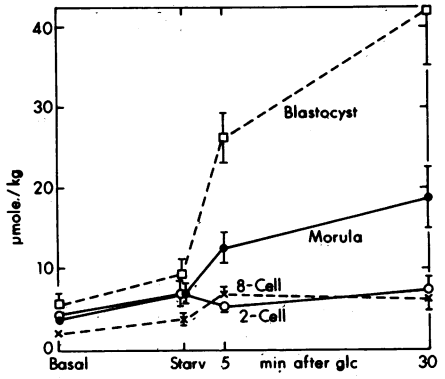

FIG. 4. Fructose-1,6-P_2 levels before and after 60 min of starvation and after realiminentation in a medium containing 5.5 mM glucose. Each point represents 7 to 12 embryos in most cases. There were only three embryos for the starvation point on the blastocyst curve.

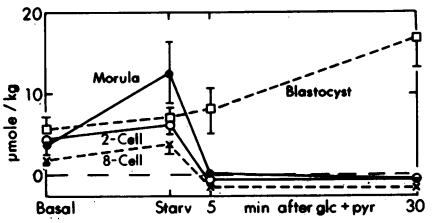

FIG. 5. Fructose-1,6-P_2 after 60 min of starvation and realimentation in medium containing 5.5 mM glucose and 0.5 mM pyruvate. Each point represents 5 to 9 embryos.

small number of embryos taken 5 min after refeeding with glucose or glucose plus pyruvate (data not shown). The levels observed were not far from those expected for equilibrium with glucose-6-P, even at the 2-cell stage. This would seem to eliminate isomerase as a significant hindrance to glycolysis, therefore the block must be attributed to 6-phosphofructokinase (EC 2.7.1.11), which has been shown to be a regulator of glycolysis in many kinds of cells.

Citrate decreased with starvation to 25% of basal levels (Figs. 6–8). In contrast to glucose-6-P, the rate and extent of the decrease was similar at the 2-cell, 8-cell, and morula stages (Fig. 6). In absolute terms the initial change was much greater for citrate than for glucose-6-P (at least 20-fold greater at the 2-cell stage and 10-fold greater at the 8-cell stage). This again demonstrates how much better the early embryo can use the citrate cycle rather than the glycolytic pathway for its energy supply.

The data for the blastocyst stage are not presented because of the wide scatter of the data mentioned above, which obscured any decreases that may have occurred. (If, as suggested earlier, this citrate is located in the blastocele, it may represent a reserve energy supply.)

Refeeding with glucose plus pyruvate caused a return in citrate to basal levels by 30 min, but it was only about half complete at 5 min. This contrasts with the more rapid restoration of glucose-6-P levels after feeding with glucose. However, the *absolute* increases in citrate in the first 5 min were 3 or 4 times greater than the greatest total increase in glucose-6-P. The effects of pyruvate alone (tested with 2-cell and 8-cell embryos) were almost the same as with glucose plus pyruvate (data not shown).

Refeeding with glucose alone did not cause any increase in citrate at the 2- and 8-cell stages (Fig. 8). At the morula stage an increase occurred but it was less than 20% of that produced

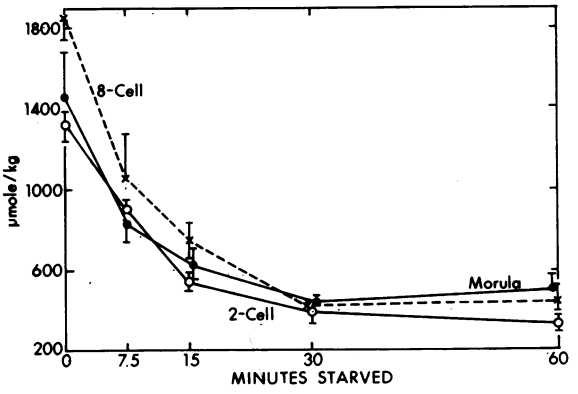

FIG. 6. Citrate levels during starvation. Each point represents the average for at least six embryos.

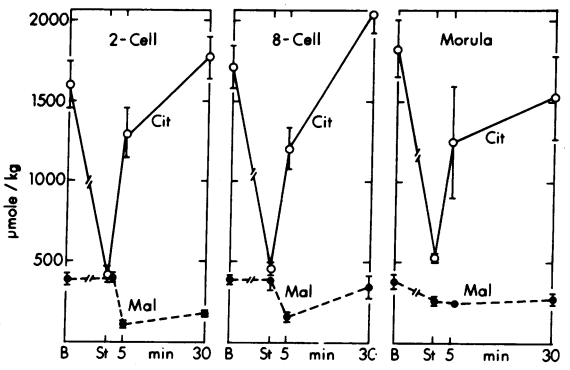

FIG. 7. Citrate and malate levels before (basal = *B*) and after 60 min of starvation (*St*) followed by realimentation in medium containing 5.5 mM glucose and 0.5 mM pyruvate. Each point represents the average of nine embryos at the 2- and 8-cell stages, and six embryos at the morula stage.

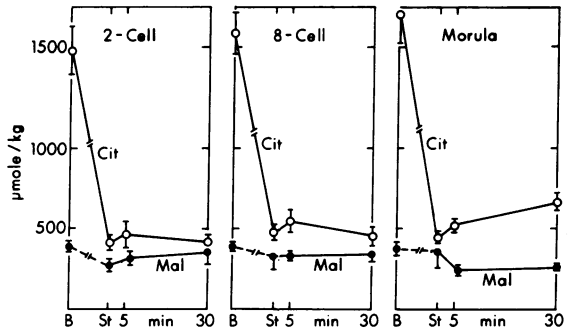

FIG. 8. Citrate and malate levels before (*B*) and after 60 min of starvation (*St*) followed by realimentation in medium containing 5.5 mM glucose. Each point represents the average of six embryos at the 2- and 8-cell stages and 14 embryos at the morula stage.

by glucose plus pyruvate. This is one more sign of the inadequacy of glycolysis at early stages.

In contrast to citrate, malate fell little or not at all during starvation (Figs. 7 and 8). However, when glucose plus pyruvate was given (Fig. 7), or pyruvate alone (not shown), malate fell markedly within 5 min at the 2-cell and 8-cell stages, and then returned, at least partially, to normal by 30 min. No fall occurred at the morula stage (or if it did, recovery had occurred by 5 min). The simplest explanation is that pyruvate caused a flood of acetyl-CoA for citrate synthesis, which at the 2- and 8-cell stages caused oxalacetate (and hence malate) to decrease faster than could be regenerated through the citrate cycle. Once citrate had been restored to normal, regulatory mechanisms turned down the rate of citrate synthesis. Refeeding with glucose alone did not cause significant change in malate (Fig. 8).

DISCUSSION

The results suggest that between the 2-cell and the morula state a significant increase in function of at least four enzymes occurs. These are hexokinase, 6-phosphofructokinase, glycogen phosphorylase, and some enzyme of the citrate cycle between citrate and malate.

Hexokinase (*EC 2.7.1.1*). It is suggestive that the basal glucose-6-P levels, as well as those after refeeding with glucose, increase with age in parallel with the hexokinase content as measured by Brinster (12). It is not clear, however, how this could be cause and effect. On refeeding with glucose, glucose-6-P rises promptly to a level characteristic of each stage and then progresses no further. Moreover, this stationary level is the same whether the glycolytic pathway is almost completely blocked (by the presence of pyruvate) or not. Something must turn off hexokinase when glucose-6-P reaches a low level at the early stages and a much higher level at later stages. It is difficult to see how such complete inhibition can be caused by glucose-6-P alone, even though it is a powerful inhibitor of hexokinase. Possible control of glucose transport into the cell could help explain the observed phenomenon.

In any event, the prompt rise in glucose-6-P on refeeding seems to indicate that neither hexokinase nor glucose entry is a *direct* cause of the failure of the early embryo to survive on glucose alone. (To emphasize this point, note that the *fall* in glucose-6-P when glucose is withdrawn, is many times slower than the *rise* in glucose-6-P when glucose is readministered; i.e., glucose can be phosphorylated at all stages far faster than it can be further metabolized.)

6-Phosphofructokinase. The conclusion that 6-phosphofructokinase is the primary point of glycolysis blockade in the early embryo is surprising in view of the report by Brinster (13) that there is little change from the 2-cell to blastocyst stage in the amount of this enzyme as measured *in vitro*. This must mean that its activity is turned off at early stages. This could be due to higher levels of inhibitors or lower levels of deinhibitors. ATP and citrate are powerful inhibitors in many tissues. ATP has been shown to fall 35% (14) to 60% (15) from the 2-cell stage to the early blastocyst. This could at least partly explain the decreased blockade with increasing age. Citrate levels are very high in the embryo; this would also be expected to greatly depress 6-phosphofructokinase activity. However, since there is no change in citrate from the 2-cell to the morula stage, it cannot be a factor in the increase in 6-phosphofructokinase activity over that age span. Nevertheless, it is probable that the further inhibition of 6-phosphofructokinase caused by pyruvate (Fig. 5 compared with Fig. 4) is due to the 4-fold increase it produced in citrate (Fig. 8 compared with Fig. 7). Similarly, the consistent small increase in fructose bisphosphate during starvation, indicating some 6-phosphofructokinase activation, may be ascribed to the fall in citrate.

Among the deinhibitors of 6-phosphofructokinase, ADP probably increases in this age period (16), whereas P_i does not change significantly (Wales and Barbehenn, unpublished data). Perhaps the most important factor in activation of 6-phosphofructokinase is the rise in the powerful allosteric deinhibitor fructose-6-P itself. Judging from the limited measurements of this metabolite in these embryos, as well as experience with many other tissues, fructose-6-P increases *pari passu* with glucose-6-P, and therefore increases 4-fold from the 2-cell to the morula stage. If changes in fructose-6-P are in fact largely responsible for changes in 6-phosphofructokinase activity, then although the actual block at early stages may be due to 6-phosphofructokinase, the true cause of the block is failure of hexokinase to generate a sufficiently high level of hexose monophosphates.

Glycogen Phosphorylase (*EC 2.4.1.1*). Glycogen is present at high levels even in early embryos (17). The fall in glucose-6-P to very low levels during starvation at the 2-cell and 8-cell stages suggests that glycogen phosphorylase is not able to

mobilize this glycogen. On the other hand, the increases in glucose-6-P during starvation at later stages, after initial decreases, indicate that phosphorylase has become competent to mobilize glycogen. This could be due to an increase in glycogen phosphorylase or activation of enzyme already present.

Citrate Cycle. When pyruvate was restored after starvation, the increase in citrate was even faster than the fall had been, and was independent of the stage of development. 2-Oxoglutarate showed a similar rapid recovery rate (Wales and Barbehenn, unpublished data). Therefore, all steps from pyruvate to 2-oxoglutarate appear to be fully functional at the stages studied. In contrast, the fact that at the 2- and 8-cell stages malate *fell* transiently during refeeding with pyruvate indicates that there is a relatively slow step between 2-oxoglutarate and malate. By the morula stage, malate does not fall under the same circumstances, which indicates the slow step has been eliminated.

Enzyme Changes and the Ability to Grow on Glucose. There is a discrepancy between the time at which mouse embryos can develop on glucose alone (8-cell stage) and the time at which 6-phosphofructokinase appears to be sufficiently active to support metabolism (morula stage). It may be that by the 8-cell stage the less than optimal capacity to use glucose is sufficient to carry over the embryo the few hours during which the necessary enzyme changes take place. Only 24 hr separate the 8-cell and morula stages and there may be substantial enzyme changes in a much smaller time span.

In conclusion, although 6-phosphofructokinase may be most directly responsible for the failure of early embryos to grow on glucose alone, glycogen phosphorylase and more especially hexokinase (or whatever controls glucose-6-P levels) shares this responsibility. In addition, for embryos to grow *in vitro* on glucose alone, pyruvate must be produced fast enough not only to supply energy requirements but also to replace whatever pyruvate or lactate may leak out to the medium. That this leak can be substantial is shown by the fact that earlier studies of embryo glycolysis have in fact depended on measurement of the lactate that accumulates in the medium (10).

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