Glucose 6-Phosphate-Dehydrogenase Activity in the Preimplantation Mouse Embryo

By RALPH L. BRINSTER

King Ranch Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa., U.S.A.

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1. The activity of glucose 6-phosphate dehydrogenase was determined in mouse embryos during the first 5 days of development from the time of ovulation up to implantation. 2. The activity decreased from 1.39 to 0.19 m μ moles of NADP reduced/hr./embryo from ovulation to implantation. 3. The specific activity of the embryo on the first day was higher than specific activities reported for most other tissues.

Two-cell mouse embryos will develop into blastocysts *in vitro* when the culture medium contains lactate, pyruvate, oxaloacetate or phosphoenolpyruvate as the only energy source. However, when glucose, glucose 6-phosphate, fructose, ribose, malate, α -oxoglutarate or citrate is the sole energy source, the two-cell embryos degenerate within 24 hr. (Brinster, 1965a). When embryos are placed in culture at the eight-cell stage, any of the above compounds in the medium will allow development of the embryos into blastocysts (Brinster & Thomson, 1966). This information suggests that there may be important changes in enzyme activities during the preimplantation stages of the mouse embryo. Information about enzyme activity in early mammalian embryos is limited and the activity of an enzyme in the preimplantation stage of a mouse embryo was measured for the first time only recently (Brinster, 1965b). The lactate-dehydrogenase activity was very high during the first 3 days of pregnancy but decreased markedly during the fourth and fifth days. This information suggests the need for further studies on enzyme activity in the early embryos. Glucose 6-phosphate dehydrogenase was chosen because it changes in activity during the cleavage stages of the sea-urchin embryo (Bäckström, 1959), and because the pentose shunt is very active in the early rabbit embryo (Fridhandler, 1961).

MATERIALS AND METHODS

The embryos were obtained by superovulating 8–10-week-old random-bred Swiss mice. The females received 10 i.u. of pregnant-mare serum (Gestyl; Organon Laboratories, London) followed 48 hr. later by 10 i.u. of human chorionic gonadotrophin (Pregnyl; Organon Laboratories). The hormones were injected intraperitoneally in 0.2 ml. of

0.9% NaCl. At the time of the second injection, the females were placed with males, and on the following morning the females were checked for vaginal plugs as evidence of copulation. Fertilized ova and cleaving embryos were obtained from females that had copulated, and unfertilized ova were taken from females that had not been placed with males. An estimation of the time since ovulation can be made, since ovulation takes place 11–14 hr. after injection of human chorionic gonadotrophin (Edwards & Gates, 1959).

Successive developmental stages of the preimplantation embryo were removed from the mouse at specific times after ovulation by flushing the reproductive tract with culture medium (Brinster, 1963, 1965c). The cumulus cells that surround one-cell embryos were removed by exposure to hyaluronidase at a concentration of 300 units/ml. in phosphate-buffered salt solution, to which had been added 1 mg. of polyvinylpyrrolidone/ml. (Rowlands, 1944; Parker, 1961). In some early experiments, the zona pellucida was removed by treating the embryos with Pronase at a concentration of 2.5 mg./ml. in phosphate-buffered salt solution (Mintz, 1962). The embryos were observed during the action of hyaluronidase and Pronase, and were removed and washed free of enzyme as soon as the cumulus or zona had disappeared. This prevented damage to the embryos, particularly by Pronase. All the embryos were washed at least three times to remove any enzyme, extraneous cellular debris or reproductive-tract fluid from the medium containing the embryos. The wash medium was a Krebs-Ringer bicarbonate solution containing 1 mg. of albumin/ml. and no energy substrates (Brinster, 1965a). The embryos were picked up from the third wash in groups of ten in a volume of 2–5 μ l. and placed in small tubes. The tubes containing the embryos were stored at -70° until activity measurements were made. Embryos were not stored longer than 1 week.

Glucose 6-phosphate-dehydrogenase activity was determined spectrophotometrically from the rate of reduction of NADP at 37° . The reaction mixture consisted of 100 μ l. of 50 mm-tris buffer, pH 7.5, 0.1 μ mole of NADP and 0.1 μ mole of glucose 6-phosphate. In addition, the reaction mixture contained bovine serum albumin (0.05%) and

MgCl₂ (10mm). The reaction was initiated by the addition of glucose 6-phosphate and the linear increase in E_{340} during the first 15min. was used to calculate the activity of the enzyme. The $m\mu$ moles of NADP reduced/hr./embryo were used as total enzyme activity and the μ moles of NADP reduced/min./mg. of protein were used to represent specific activity. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Extinction readings were made in a Beckman model DU spectrophotometer modified with a precision cell positioner (Greenberg & Rodder, 1964) and micro-cells (Pyrocell, Westwood, N.J., U.S.A.). In the absence of substrate, NADP was not reduced at a significant rate by the tissue. In preliminary experiments the concentrations of NADP and glucose 6-phosphate were individually doubled and decreased by one-half. Since these changes resulted in slightly lower activities, the concentrations shown above, which gave maximum activity, were used throughout the experiments.

In addition to experiments on glucose 6-phosphate dehydrogenase, measurements were made on hexokinase and 6-phosphogluconate dehydrogenase. The reduction of NADP in the presence of ribose 5-phosphate was also measured. For these experiments the analysis conditions were the same as for glucose 6-phosphate dehydrogenase except for the following changes: (1) the hexokinase reaction mixture contained no glucose 6-phosphate but contained 0.1 μ mole of ATP and 0.1 μ mole of glucose; (2) the 6-phosphogluconate-dehydrogenase reaction mixture contained 0.1 μ mole of 6-phosphogluconate in place of glucose 6-phosphate; (3) the reduction of NADP in the presence of ribose 5-phosphate was carried out with 0.1 μ mole of ribose 5-phosphate replacing the glucose 6-phosphate.

All chemicals were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), except tris, which was obtained from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.).

RESULTS

The first experiments were designed to determine if removal of the zona pellucida, the inert protective shell of the embryo, had an effect on the measurement of glucose 6-phosphate-dehydrogenase activity. It seemed possible that the zona might prevent the diffusion of the enzyme through

the reaction mixture, since the zona is not completely ruptured by freezing. In addition, the effect of repeated freezing and thawing on enzyme activity was determined. The statistical experimental design was a 2×2 factorial with four replicates for each treatment and the results are shown in Table 1. An analysis of variance showed no interaction between removing the zona and freezing. There was no significant difference between ova with intact zona pellucida and those where the zona was removed. However, those embryos that were frozen five times had a significantly lower activity than those ova that were frozen once ($P < 0.001$). Consequently, subsequent enzyme measurements were made on embryos with the zona intact and that had been frozen once, except in late blastocysts, which normally have shed their zona pellucida at the time of recovery.

Determinations of total glucose 6-phosphate-dehydrogenase activity of preimplantation mouse embryos at various stages are shown in Table 2. The significance of differences between the total activity/embryo of successive developmental stages was tested by Student's t test. There was a slight but significant increase in total activity from the fertilized one-cell ova to the two-cell stage ($0.05 >$

Table 1. *Effect of removal of the zona pellucida and repeated freezing and thawing on glucose 6-phosphate-dehydrogenase activity of one-cell mouse embryos*

The values are means of four determinations. The pooled error mean square is 12.73 (9 degrees of freedom). Further details are given in the text.

	$\Delta E_{340}/30 \text{ min./10 ova}$	
	Frozen once	Frozen 5 times
With zona	0.413	0.318
Without zona	0.389	0.317

Table 2. *Glucose 6-phosphate-dehydrogenase activity in early mouse embryos*

The values are means $\pm$ S.E.M. The protein content was determined by the method of Lowry *et al.* (1951). Further details are given in the text.

Developmental stage	Approx. time after ovulation (hr.)	No. of determinations	Total activity ($m\mu$ moles of NADP reduced/hr./embryo)	Specific activity (μ mole of NADP reduced/min./mg. of protein)
Unfertilized	12	8	1.39 ± 0.04	0.835 ± 0.023
Fertilized	12	8	1.36 ± 0.05	0.817 ± 0.030
Two-cell	36	10	1.51 ± 0.03	0.965 ± 0.020
Eight-cell	60	10	1.40 ± 0.03	0.998 ± 0.022
Morula	84	8	0.87 ± 0.03	0.705 ± 0.025
Blastocyst	84	8	0.60 ± 0.02	0.420 ± 0.013
Late blastocyst	108	11	0.19 ± 0.02	0.145 ± 0.015

$P > 0.01$) but the eight-cell embryo has approximately the same activity as the one-cell stages. Each succeeding developmental stage has a significantly lower total enzyme activity/embryo than the preceding stage ($P < 0.001$). At the late blastocyst stage of development, just before implantation, the enzyme content of the embryo is 13.7% of that at the one-cell or eight-cell stage. In general, changes in specific activity run parallel to changes in total activity (Table 2).

The reduction of NADP is due almost exclusively to glucose 6-phosphate dehydrogenase. Reliable measurements of 6-phosphogluconate-dehydrogenase activity could not be obtained, but its activity must be 1% or less of the activity of glucose 6-phosphate dehydrogenase in the early stages and certainly no more than 10% in the later stages. Similarly, the activities of hexokinase and of the ribose 5-phosphate pathway appear to be 1% or less of the activity of glucose 6-phosphate dehydrogenase.

DISCUSSION

Activities have been determined for three enzymes in the mouse embryo: lactate dehydrogenase, glucose 6-phosphate dehydrogenase and malate dehydrogenase. Lactate dehydrogenase has by far the highest activity (46 μ moles of NADH₂ oxidized/hr./embryo; Brinster, 1965b), and it represents about 5% of the protein of the one-cell stage. The corresponding total activities of glucose 6-phosphate dehydrogenase and malate dehydrogenase (Brinster, 1966) are 1.4 and 0.8 μ moles of NADH₂ oxidized/hr./embryo respectively. Glock & McLean (1954) found glucose 6-phosphate-dehydrogenase specific activities of 0.055 and 0.0019 μ mole of NADP reduced/min./mg. wet wt. at 20° for lactating mammary gland and 10-day-old rat embryo respectively. If the factor 15 is used for correction to 37° and mg. of protein, then lactating mammary gland and the one-cell mouse embryo have similar specific activities. Of the tissues examined by Glock & McLean (1954), lactating mammary gland had the highest specific activity. Krah, Keltch, Walters & Clowes (1955) found the specific activity of sea-urchin eggs to be approx. 2–3 μ moles of NADP reduced/min./g. wet wt. at 23° for unfertilized eggs. By using the conversion factor 15, the enzyme activity in the mouse unfertilized egg is about 20 times the activity in the unfertilized sea-urchin egg.

In addition to its high activity, the second striking feature of glucose 6-phosphate dehydrogenase in the preimplantation embryo is the 86% fall in total activity during the 48hr. period from the eight-cell stage to the late blastocyst. Lactate

dehydrogenase also shows a marked fall in activity from the eight-cell stage to the late blastocyst (Brinster, 1965b). In fact, the total activity of the latter enzyme is about 30 times that of glucose 6-phosphate dehydrogenase at all stages up to the late blastocyst. In contrast, malate dehydrogenase maintains a constant activity up to the eight-cell stage or morula and then increases by 10–15% up to the late blastocyst (Brinster, 1966). This suggests that glucose 6-phosphate dehydrogenase and lactate dehydrogenase play more important roles during the first 3 days of development whereas malate dehydrogenase seems to become more essential at later stages of growth. The embryo passes from the fallopian tube into the uterus at about 72hr. after ovulation and these changes in metabolism may be associated with this marked change in environment (Lewis & Wright, 1935).

The approximate relative activities in rat liver of hexokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and ribose 5-phosphate metabolism are 1:1:1:4 (Glock & McLean, 1954; DiPietro & Weinhouse, 1960). The reason why glucose 6-phosphate-dehydrogenase activity is much higher than the other three in mouse embryos is not known.

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