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Minimal Nutrient Requirements for Culture of One-Cell Rabbit Embryos

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ABSTRACT

The minimal nutrient requirements of one-cell rabbit embryos for cleavage during in vitro culture were investigated. One-cell rabbit embryos were cultivated in a simple salt solution supplemented with the macromolecule polyvinylalcohol (PVA) either alone or with bovine serum albumin (BSA), amino acids, or one of a number of potential energy sources. At the end of 48 h culture, the embryos were stained with aceto-orcein HCl and the number of nucleated cells per embryo counted. One-cell embryos in medium with PVA but without an exogenous, fixed nitrogen source or energy substrate cleaved to a mean of 10.4 cells per embryo. Addition of the putative energy substrates—phosphoenolpyruvate, malate, acetate, and lactate—resulted in nonsignificant increases in cleavage rate. Glucose, pyruvate, a group of 20 amino acids from Ham's F-10 medium, and BSA gave a statistically significant doubling of the cleavage rate. These results indicate that the one-cell rabbit embryo, unlike the mouse embryo, has significant endogenous energy sources and that an exogenous, fixed nitrogen source is not essential for cleavage.

INTRODUCTION

Culture studies with preimplantation mammalian embryos have been dominated by the discovery of Whitten (1957) that lactate, as the sole exogenous energy source, would allow development of 2-cell mouse embryos to blastocysts. This work was extended by Brinster and Biggers and their collaborators to determine precisely the energy substrates that supported development of 1-cell and 2-cell mouse embryos to blastocysts. Brinster (1965) showed that cleavage of the 2-cell mouse embryo was supported by any one of a group of four substrates—pyruvate, lactate, phosphenolpyruvate, and oxaloacetate. Biggers et al. (1967) later reported that only pyruvate and oxaloacetate allowed cleavage of the 1-cell stage. While this work was and still is of profound significance for preimplantation embryo culture, in general, it has been applied relatively uncritically to other species. Comparable data are not available for any other mammalian species.

Information is also lacking for species other than the mouse on the fixed nitrogen requirements of embryos in culture. Development of 2-cell mouse embryos to blastocysts has been reported in the presence of either polyvinylpyrrolidone (PVP) or polyvinylalcohol (PVA) by Cholewa and Whitten (1970) and Kuzan et al. (1983). In contrast, however, Wales and Whittingham (1973) reported that the substitution of PVP for albumin did not allow the development of 8-cell embryos to blastocysts.

In previous work with rabbit embryos, we have shown that either pyruvate or long- or short-chain fatty acids in the presence of charcoal-treated defatted bovine serum albumin (BSA) supported development of 1-cell embryos to viable morulae. This implicated these compounds as energy substrates for the cleavage-stage rabbit embryo. However, these studies could not exclude the possibility that the amino acids of the BSA might be acting as energy substrates.

The present study investigated the role of a fixed nitrogen source (in the form of BSA or amino acids) and a number of potential energy substrates in supporting cleavage of 1-cell rabbit embryos in culture.

MATERIALS AND METHODS

Embryos

One-cell embryos were collected from the oviducts of superovulated New Zealand White does 20–21 h

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after luteinizing hormone (LH) injection and insemination, as described previously (Kane, 1983). Embryos were washed free of oviducal components with repeated changes of the flushing medium (basic culture medium with PVA but without any potential energy substrates) and were placed in culture in 0.5-ml droplets under paraffin oil in plastic tissue culture dishes. Culture was carried out at 38°C in a gas phase of 5% CO_2 in air for 48 h. At the end of culture, the embryos were fixed and stained according to the method of Grayson (1978), and cell numbers counted at 400× magnification with the aid of an eye-piece micrometer grid.

Culture Medium

The composition of the basic culture medium to which addition were made was 0.1% PVA, 108 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, and 25 mM $NaHCO_3$. The following compounds were added to this basic medium: (1) pyruvate and a group of compounds metabolically related to it (malate, oxaloacetate, lactate, phosphoenolpyruvate) and (2) a short-chain fatty acid—acetate; a carbohydrate—glucose; a group of 20 amino acids of Ham's F-10 medium (Ham, 1963); and a protein—BSA (Fraction V from Sigma Chemical Co., St. Louis, MO).

Statistical Considerations

Treatments were compared in one experiment that was replicated five times. Embryos collected on one day were pooled and allocated randomly among treatments to form one replicate. Analysis of variance was carried out, both on the untransformed embryo cell counts and on those counts transformed logarithmically. Individual treatments were compared with the control treatment without added substrate by Dunnett's procedure, using both transformed and untransformed cell counts. Conclusions were similar in both cases.

RESULTS

While all treatments were examined in one experiment, for clarity results are presented in two tables. Table 1 presents the mean cell counts for embryos cultured either in medium without added substrate or in medium with pyruvate or substrates related to pyruvate. The most striking fact to emerge from this experiment was that the mean number of cells per embryo in the medium without any added energy source was 10.4, indicating that 1-cell rabbit embryos had sufficient endogenous energy sources to support, on average, more than 3 cleavage divisions. All of the compounds in this group gave nonsignificant increases in cleavage rate, except pyruvate, which gave a significant doubling of cell number.

The effects of acetate, glucose, amino acids, and BSA are presented in Table 2. There was no significant effect of acetate, but glucose, amino acids, and BSA gave a significant doubling of the rate of cell division. The highest number of cells per embryo was obtained in the treatment with BSA.

DISCUSSION

The most surprising fact to emerge from these experiments is that the 1-cell rabbit embryo has sufficient endogenous energy sources to allow up to 3 or more cleavage divisions in culture in the absence of any possible added energy substrate. This is in striking contrast to the mouse embryo, which requires specific exogenous energy sources in the form of pyruvate and oxaloacetate to support cleavage at the 1-cell stage (Biggers et al., 1967) and for which careful manipulation of the energy substrates in the culture

TABLE 1. Effects of pyruvate and related substances on cleavage of 1-cell rabbit embryos.

	Substrate added					
Item	None	Phosphoenol pyruvate (5×10^{-4} M)	Malate (5×10^{-4} M)	Lactate (10^{-3} M)	Oxaloacetate (5×10^{-4} M)	Pyruvate (5×10^{-4} M)
No. of embryos	28	27	27	26	25	26
Mean cell no. per embryo after 48 h culture	10.4	12.0	12.9	14.5	14.9	20.0[a]

[a]Significantly different from control (no added substrate) treatment; ($p<0.01$; Dunnett's procedure).

TABLE 2. Effects of acetate, glucose, amino acids, and bovine serum albumin (BSA) on cleavage of 1-cell rabbit embryos.

Item	Substrate added				
	None	Sodium acetate (10^{-3} M)	Glucose (10^{-3} M)	Ham's F-10 amino acids	BSA (1.5%)
No. of embryos	28	27	26	26	27
Mean cell no. per embryo after 48 h culture	10.4	13.3	19.6[a]	19.1[a]	25[a]

[a]Significantly different from control (no substrate added) treatment; ($p<0.01$, Dunnett's procedure).

medium is necessary to allow culture of 1-cell embryos across the 2-cell block (Cross and Brinster, 1973). This may shed some light on the reasons why there is no 2-cell block in the rabbit embryo and why cleavage-stage rabbit embryos, in contrast to those of most other species, are easy to culture in vitro (Maurer et al., 1969; Kane, 1972, 1979).

The fact that pyruvate and glucose gave a doubling of the cleavage rate is consistent with work in the mouse showing that pyruvate is an important energy source for the early cleavage stages (Brinster, 1965; Biggers et al., 1967) and glucose can be utilized at the 8-cell stage (Brinster and Thomson, 1966). Quinn and Wales (1973) showed clearly, using ^{14}C-labeled glucose, that rabbit embryos could use glucose for energy at the early cleavage stage. Part of the usefulness of exogenous glucose may be an energy-sparing effect by the provision of other essential metabolic intermediates. The early rabbit embryo is known to be capable of utilizing considerable amounts of glucose via the pentose-phosphate shunt (Friedhandler, 1961; Brinster, 1968).

Malate, phosphenolpyruvate, lactate and oxaloacetate all resulted in nonsignificant increases in cleavage rate. This is particularly surprising in the case of lactate because of its high concentration in the rabbit oviduct (Hamner and Fox, 1969) and its importance in mouse embryo culture (Brinster, 1965). A reason for the failure of lactate and some of the other substrates used in this experiment to demonstrate a significant growth promoting effect may have been that the levels used were not optimal.

The failure of acetate to give a significant increase in cleavage rate was surprising in view of previous results showing that, in the presence of charcoal-treated defatted albumin, it supported growth of 1-cell rabbit embryos to viable morulae (Kane, 1979). It is possible that the presence of albumin in the medium is necessary as a carrier for acetate to be utilized by the embryos.

The ability of the group of 20 F-10 amino acids to cause a significant doubling of the rate of cell division could be due to their functioning either directly as energy substrates or indirectly to spare energy by providing intermediates that otherwise would have to be synthesized by the embryos. Bae and Foote (1975) provided evidence that glutamine may act as an energy source for maturation of rabbit follicular oocytes in vitro. Carney and Bavister (1985) showed the importance of glutamine to development of 8-cell hamster embryos in culture.

The greatest increase in cleavage rate in these experiments was produced by the addition of BSA. Much of this effect probably is due to the presence of low-molecular weight energy substrates, such as fatty acids, etc., which are normally bound to albumin (Fredrickson and Gordon, 1958). However, the effect of BSA is superior to all of the other compounds tested. This may result from a favorable physical effect of the BSA or it could be due to the presence of a low-molecular weight embryotropic factor present as a contaminant on the BSA that has been shown to stimulate growth of rabbit blastocysts (Kane, 1985; Kane and Gray, 1985).

It is interesting that a fixed nitrogen source is not essential even at the 1-cell stage for cleavage of rabbit embryos. A major advantage of PVA is that it abolishes the stickiness of the embryos usually found in protein-free media, and, as a result, manipulation of the embryos is as easy as it is in media containing protein. We have not found this to be the case for rabbit embryos using either polyvinylpyrrolidone or Ficoll.

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