*THE PATTERN OF ENERGY METABOLISM IN THE MOUSE OÖCYTE AND ZYGOTE**

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In vitro methods for the quantitative culture of cleaving mouse embryos on chemically defined media[1, 2] have demonstrated that their basic patterns of energy metabolism differ from those found in later embryonic stages and the adult. The late 2-cell stage can only use lactate, pyruvate, phosphoenolpyruvate (PEP), and oxaloacetate to support cleavage.[3] As development proceeds, more compounds can serve as energy sources, and at the 8-cell stage such compounds as malate, α-ketoglutarate, and glucose can be utilized.[4] These observations pose a fundamental question: Are the unique patterns of energy metabolism in the early mammalian embryo established prior to fertilization? To answer this question we have compared the energy requirements of the maturing oöcyte and the zygote. This comparison has become possible because of recent advances in the hitherto independently studied fields of oöcyte and zygote culture.

First, Edwards[5] showed that mouse oöcytes, liberated from their ovarian follicles, will mature in medium 199[6] supplemented with bovine serum. The maturation process involves breakdown of the arrested dictyate (germinal vesicle) stage of meiosis, and passage through metaphase I, anaphase I, and telophase I with first polar body extrusion, ending in an arrest of the chromosomes in a metaphase II configuration. We have now shown that maturation of the mouse oöcyte will occur in simple chemically defined media used for the culture of the cleavage stages. Second, Whittingham and Biggers[7] have demonstrated that the mouse zygote will cleave to the 2-cell stage in a simple medium containing both lactate and pyruvate. Although a 2-cell stage produced in this way stays arrested in the simple medium, it will develop into a normal blastocyst in an organ culture of the ampullary region of the fallopian tube. Furthermore, such blastocysts will develop into normal fetuses if they are transferred into uterine foster mothers. All these results demonstrate the normality of the first cleavage division and make possible an investigation of the exogenous energy requirements of the zygote and first cleavage division.

The evidence from results presented in this paper suggests that: (1) the pattern of energy metabolism of the zygote is determined in the oöcyte before fertilization; (2) pyruvate is the main energy substrate which can be used directly by the oöcyte and zygote; (3) the follicular cells can supply the oöcyte and zygote energy requirements by the metabolism of other substrates.

Materials and Methods.—The basic medium was a modified Krebs-Ringer bicarbonate (NaCl, 119.39 mM; KCl, 4.78 mM; $CaCl_2$, 1.71 mM; KH_2PO_4, 1.19 mM; $MgSO_47H_2O$, 1.19 mm; $NaHCO_3$, 25.07 mM) supplemented with crystalline bovine serum albumin (1 mg/ml), penicillin G (potassium) (100 U/ml) and streptomycin sulphate (50 μg/ml). The individual energy substrates were added in concentrations near optimal for the development of late 2-cell or 8-cell stages.[3] The osmolarity was maintained at approximately 0.308 osmols by adjusting the sodium chloride content of the basic medium, and the hydrogen ion concentration was kept within the range of

pH 7.2–7.4. All media were gassed with 5% CO_2 in air and kept in the refrigerator at 4°C before use.

Follicular oöcytes were obtained from the ovaries of 8- to 12-week-old CF1 unbred female mice (Carworth Farms). The mice were killed and the ovaries removed and placed in a Petri dish containing culture medium. The manipulation of the ovaries and the culture of the oöcytes were carried out in a medium containing the substrate being examined, since only one substrate was tested in each experiment. The ovaries were dissected free of adipose and connective tissues and transferred to embryological watch glasses containing 1/2 ml of medium under 1 ml of light-weight mineral oil. The oöcytes and follicular cells were liberated by puncturing the follicles with a 25-gauge hypodermic needle. During this process the follicle cells readily separated from the oöcytes and no treatment with hyaluronidase was necessary. Oöcytes, free of cumulus cells and possessing intact germinal vesicles, were washed once in medium contained under mineral oil and were finally transferred to small microdroplets of medium similar to the method previously described by Brinster.[1] Oöcytes (20–40) were obtained from each mouse and 10–20 oöcytes were placed in each microdroplet. The follicular cells were similarly washed and placed into culture when required. The oöcytes were cultured with or without follicular cells for a period of 16–19 hr at 37°C in an incubator gassed with 5% CO_2 in air. At the end of the culture period, the oöcytes were examined by phase-contrast microscopy. First of all, they were transferred to a glass slide and gently compressed with a cover slip using petroleum jelly to control the amount of compression, then fixed with 45% glacial acetic acid in absolute ethanol and stained with 1% orcein in 50% acetic acid and 50% isotonic saline.

Fertilized 1-cell ova were obtained from the ampullary regions of the fallopian tubes of superovulated 7- to 8-week-old random-bred Swiss mice on the morning the vaginal plugs were found. The mice were superovulated by an intraperitoneal injection of 5 IU of pregnant mare serum gonadotrophin (PMS) (Equinex, Ayerst), followed 48 hr later by an intraperitoneal injection of 5 IU of human chorionic gonadotrophin (HCG) (A.P.L., Ayerst). The medium used for the initial collection of the ova in all experiments contained sodium pyruvate at a concentration of 0.316 mM in the basic medium. The cumulus cells were removed with hyaluronidase. One ml of Dulbecco's phosphate-buffered salt solution[8] (pH 7.2) with 300 units of hyaluronidase and 1 mg of polyvinylpyrrolidone was added to 1 ml of medium containing the ova and cumulus. The final concentration of 150 U/ml of hyaluronidase resulted in the removal of the cumulus cells surrounding the ova within several minutes. The enzyme was made up initially in phosphate-buffered saline to enable its storage at −20°C before use. The ova were washed free of hyaluronidase in two changes of pyruvate medium (2 ml/wash), and then free of pyruvate by washing in two changes of basic medium without an energy source (2 ml/wash). This latter procedure was used so that the different energy sources could be tested simultaneously. In experiments where the cumulus cells and ova were left intact, similar procedures of washing them free of pyruvate were used. The ova were set up in the different substrates 20–24 hr after the injection of HCG, and cultured according to the method previously described by Brinster.[1] Twenty-four hr later the number of ova which had undergone the first cleavage division was noted.

Results.—Table 1 shows that maturation of mouse oöcytes *in vitro* can occur under much simpler chemically defined conditions than those described by Edwards.[5] Furthermore, they demonstrate that mouse oöcytes will not undergo maturation if an energy source is omitted from the medium. Only pyruvate and

TABLE 1

NUMBER OF MOUSE OÖCYTES UNDERGOING MATURATION TO THE METAPHASE II STAGE IN MEDIA CONTAINING PYRUVATE, OXALOACETATE, LACTATE, PEP, AND GLUCOSE

Energy source	Concentrations (mM)	No. oöcytes	No. in metaphase II	Per cent in metaphase II
None	—	63	0	0
Pyruvate	0.25	128	111	87
Oxaloacetate	0.50	27	19	70
Lactate	25.0	51	3	6
PEP	10.0	31	0	0
Glucose	5.60	17	0	0

TABLE 2

NUMBER OF MOUSE OÖCYTES UNDERGOING MATURATION TO THE METAPHASE II STAGE IN THE PRESENCE OR ABSENCE OF FOLLICULAR CELLS IN MEDIA CONTAINING PYRUVATE, OXALOACETATE, LACTATE, PEP, AND GLUCOSE

Substrate	Concentration (mM)	Cumulus	No. oöcytes	No. in metaphase II	Per cent in metaphase II
None	—	—	15	0	0
		+	29	0	0
Pyruvate	0.25	—	19	17	89
		+	24	21	88
Oxaloacetate	0.50	—	19	19	100
		+	15	14	93
Lactate	25.0	—	15	0	0
		+	13	9	69
PEP	10.0	—	23	0	0
		+	13	12	92
Glucose	5.60	—	19	0	0
		+	13	7	54

oxaloacetate supported maturation. In glucose and phosphoenolpyruvate (PEP), none of the eggs reached metaphase II, although in both a small percentage (7–10%) underwent germinal vesicle breakdown. A very slight response was obtained with lactate. A few oöcytes (6%) placed in lactate reached metaphase II; however, some (15%) began to mature but became arrested at various stages, while in the remainder no germinal vesicle breakdown occurred. Table 2 shows that if follicular cells are included in the cultures lactate, PEP, and glucose are also able to support a high level of oöcyte maturation. It seems that the follicular cells are able to metabolize these energy sources and supply the oöcyte with products which permit maturation.

Table 3 shows the effect of culturing zygotes in the four energy sources known to support cleavage of the late 2-cell mouse embryo. Clearly, an energy source is necessary for the first cleavage division, confirming earlier findings.[7] However, only pyruvate and oxaloacetate can support the first cleavage division, lactate and PEP being totally ineffective. The response to oxaloacetate is significantly less than that to pyruvate ($\chi^2_{[1]} = 13.2$, $P < 0.001$). The 75 per cent response to pyruvate is probably close to the maximum possible because of an unavoidable proportion of abnormal and unfertilized eggs.

Table 4 shows the effect of placing cumulus cells and zygotes in the microdroplets of media in the presence of lactate, PEP, and glucose. Under these conditions lactate, PEP, and glucose can support the first cleavage division, but no development was obtained on media devoid of an energy source. The yield of 2-cell embryos in these experiments is somewhat lower than obtained in cumulus cell-free experiments. This may be due either to insufficient cumulus cells being present in the microdrop, or to a rapid exhaustion of the energy source by the large number of

TABLE 3

NUMBER OF MOUSE ZYGOTES WHICH CLEAVE TO THE 2-CELL STAGE IN MEDIA CONTAINING PYRUVATE, OXALOACETATE, LACTATE, AND PEP

Substrate	Concentration (mM)	No. 1-cell	No. 2-cell	Cleaved (%)
None	—	180	0	0
Pyruvate	0.316	180	135	75
Oxaloacetate	0.316	180	98	54.4
Lactate	25.0	180	0	0
PEP	10.0	180	0	0

TABLE 4

NUMBER OF MOUSE ZYGOTES WHICH CLEAVE TO THE 2-CELL STAGE IN THE PRESENCE AND ABSENCE OF CUMULUS CELLS IN MEDIA CONTAINING LACTATE, PEP, AND GLUCOSE

Substrate	Concentration (mM)	Cumulus	No. 1-cell	No. 2-cell	Cleaved (%)
None	—	—	60	0*	0
		+	57	0*	0
Lactate	25.0	—	56	0	0
		+	174	90	51.7
PEP	10.0	—	24	0	0
		+	82	22	26.8
Glucose	5.60	—	60	0*	0
		+	89	29	32.6

* All degenerate after 24 hr cultivation.

cumulus cells in the microdrop. The results indicate that the cumulus cells are able to metabolize those substrates not supporting first cleavage, and produce products which pass to the zygote and allow it to divide.

Discussion.—The energy requirements *in vitro* for oöcyte maturation and the first cleavage division of the mouse are similar, and at the same time unique. Only pyruvate and oxaloacetate can support oöcyte development *in vitro* if no follicle or cumulus cells are present. However, when these cells are included in optimum numbers in the culture system lactate, PEP and glucose are also able to support oöcyte maturation and the first cleavage division. These findings raise several questions of central importance in mammalian reproductive physiology and embryology. These are: (1) What are the mechanisms that determine which exogenous substrates can be utilized at a particular stage of development? (2) What is the role of the fertilizing spermatozoon in the determination of the changing patterns of energy metabolism in early development? (3) To what extent are the observations on the *in vitro* requirements of early mammalian embryos relevant to the natural situation *in vivo?*

Our present information on the utilization of various energy sources at different stages in the mouse, and their metabolic relationships in mammalian cells, is summarized in Figure 1. The combined information strongly suggests that the restricted patterns of energy metabolism in the zygote are maternally determined, arising during the differentiation of the oöcyte.

If pyruvate and oxaloacetate are the only energy sources able to support oöcyte maturation and the first cleavage division, it is feasible that CO_2 fixation may occur, resulting in the conversion of pyruvate to oxaloacetate via the reaction:

$$\text{Pyruvate} + CO_2 + \text{ATP} \xrightleftharpoons{\text{Pyruvate carboxylase}} \text{oxaloacetate} + \text{ADP} + P_i.$$

The oxaloacetate would then be available for the synthesis of PEP, and hence gluconeogenesis, via the reaction:

$$\text{Oxaloacetate} + \text{GTP} \xrightleftharpoons{\text{Phosphoenolpyruvate carboxykinase}} \text{phosphoenolpyruvate} + CO_2 + \text{GDP}.^{9}$$

Furthermore, the oxaloacetate would be synthesized to supply the anapleurotic pathway maintaining the citric acid cycle.[10] The maintenance of both these pathways may be critical in cellular systems, such as the early embryo, rapidly syn-

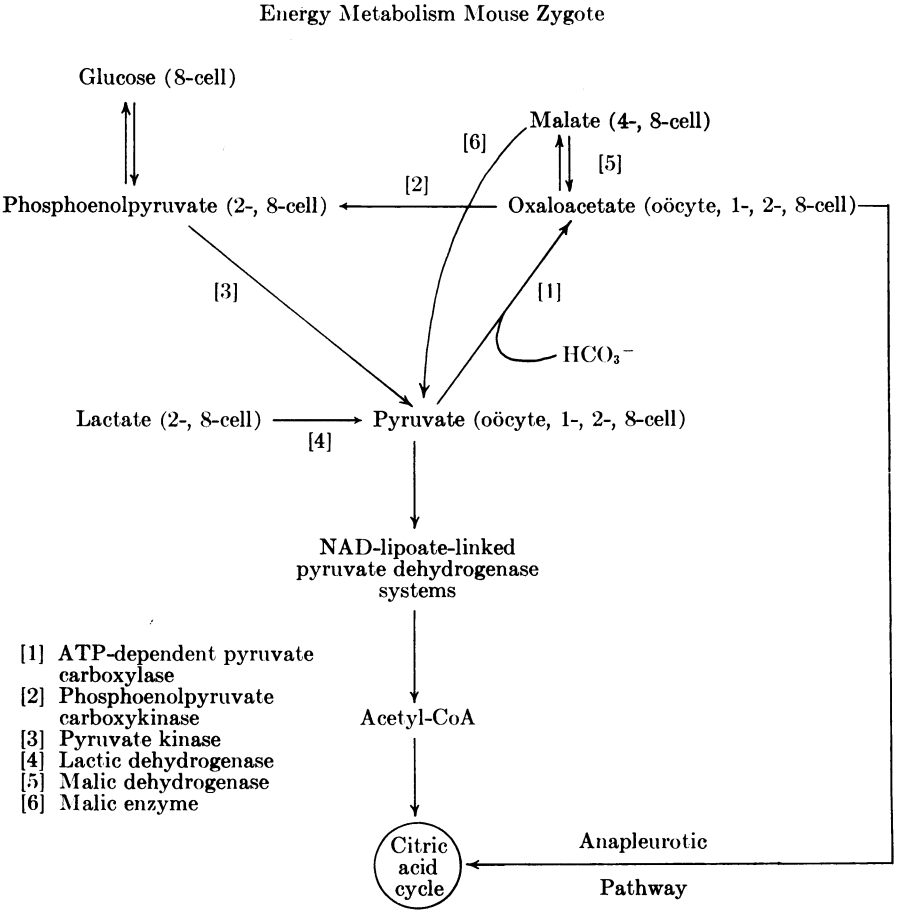

FIG. 1.—The compounds able to act as sources of energy to mouse oöcytes and early cleavage stages, and their metabolic relations. The stages supported by each compound are shown in parentheses.

thesizing a variety of new cellular compounds. Evidence suggesting that the *de novo* synthesis of oxaloacetate does take place comes from the experience of many investigators who have cultured mouse embryos. It is necessary to use a bicarbonate buffer in the medium and carefully gas the system with 5 per cent CO_2 in air. No cleavage occurs, and the embryos rapidly die if the bicarbonate is replaced by a phosphate buffer.

The available knowledge of the mechanisms which regulate the changing patterns of energy metabolism during cleavage of the mouse is only fragmentary. There is evidence, however, which indicates that at least two types of mechanism must be recognized. These are changes in the permeability of the blastomeres to the energy substrates and possibly activation of enzyme systems. Studies with C^{14}-L-malate show that the 2-cell mouse embryo is impermeable to this substrate. However, by the 8-cell stage it is probably taken into the blastomeres by means of active transport.[11] Thus, although there are significant amounts of L-malate

dehydrogenase in mouse zygotes and 2-cell stages,[12] exogenously supplied malate is unavailable to the enzyme. Studies with C^{14}-DL-lactate show that this substrate, in contrast to L-malate, enters the zygote.[13] Nevertheless, it is unable to support first cleavage. What restricts the utilization of DL-lactate is unknown at the present time since lactic dehydrogenase is present in the zygote in high concentration.[14] The substrate may be unavailable as a source of energy because certain enzyme pathways are nonfunctional.

The role of the enzymes which may enter the ovum in the mitochondria of the sperm is not clear at present since the fate of these organelles has not been settled. The only study of the fate of the mid-piece mitochondria of the spermatozoon in the mouse has been made with the light-microscope. It showed that the mitochondria are rapidly dispersed through the ovum and then randomly distributed between the two blastomeres at the first cleavage division.[15] After this stage the sperm mitochondria could not be distinguished from those of maternal origin. More recently, Szollosi[16] has studied the fate of the mid-piece mitochondria in the rat by means of the electron microscope, and has shown that they are not randomly distributed between the blastomeres, and that they swell and degenerate by the 4-cell stage. Thus, the spermatozoa may play no critical role in determining the patterns of energy metabolism in the cleaving mouse embryo.

If the oöcyte, zygote, and first three cleavage stages depend *in vivo* on unique exogenous energy sources, what are they and where do they come from? The oöcyte which undergoes maturation in the ovary is intimately associated with the cells of the Graafian follicle. These cells have long been suspected of having a nutritional role during oögenesis and of being primarily concerned with building up stores in the oöcyte in anticipation of a nutritionally deprived cleavage period. Our results show that the follicle cells can metabolize substrates not available to the oöcyte and presumably provide substances which this specialized cell can use. Thus a much more metabolically active role can be proposed for the follicle cells during the final stages of maturation of the oöcyte. The cumulus cells which are associated with the egg *in vivo* until the first cleavage division are also able to support the development of the zygote in media containing an energy substrate which cannot be used directly. Thus, they may exert an active metabolic function for the zygote similar to that proposed for the oöcyte. At present it is difficult to assess this role, since we have an insufficiently precise estimate of the functional life of the cumulus cells once the ovum has left the Graafian follicle. In the rat, cultured follicle cells have a diminished ability to proliferate after ovulation.[17]

Obvious sources of exogenous energy substrates are the follicular fluid and secretions of the fallopian tube. It must be stressed at once that there are no *a priori* grounds for assuming that substrates such as oxaloacetate and PEP, which are effective energy sources *in vitro*, are the natural sources secreted *in vivo*. Since studies on the composition of follicular fluid and of fallopian tube secretions in the mouse have not been done, it is necessary to consider comparative physiological evidence. In 1957, Bishop[18] described the high concentrations of lactate in the secretions of the fallopian tube of rabbits. Similar high concentrations of lactate have now been reported in the sheep.[19, 20] The possible importance of lactate in early mammalian development was suggested by the report of Whitten[21] that lactate permitted the cleavage of late 2-cell mouse embryos *in vitro* in a simple

medium containing glucose. Brinster[1] subsequently strengthened this view by showing that lactate would support the development of late 2-cell mouse embryos without glucose being present. Recently, however, Holmdahl and Mastroianni[22] have shown that pyruvate as well as lactate rise to equivalent levels shortly after ovulation in the rabbit, whereas glucose levels do not change. The observation that a high percentage of mouse zygotes devoid of cumulus cells will cleave to 2-cell stages in centrifuged bursal fluid (Whittingham, unpublished) suggests that utilizable energy sources, such as pyruvate, are secreted by the upper end of the mouse genital tract. The presence of lactate and glucose in the follicular fluid has been reported in cows stimulated with pregnant mare serum gonadotrophin.[23]

The evidence presented in this paper suggests that the mouse embryo is dependent on its mother during cleavage for an exogenous source of energy. Unfortunately, there are no studies *in vitro* on other species which give unequivocal information on the exogenous energy requirements of cleavage stages since the work was done with biological media (see Biggers, Rinaldini, and Webb[24] for a general discussion of the study of growth factors *in vitro*). Nevertheless, the fragmentary evidence available so far on energy and bicarbonate requirements of cleaving embryos, and their high concentration in fallopian tube secretions, indicates that the metabolism of the embryo and the secretions of the fallopian tube have been evolved to provide special conditions for cleavage. This hypothesis will not be clarified until we have the joint evidence of the nutritional requirements of cleavage and the composition of the fallopian tube secretions of several mammalian species. Closely related to this topic are the mechanisms which determine the very restricted patterns of energy metabolism in the oöcyte during oögenesis. It is clear that in further analyses of the physiology of early pregnancy we must consider not only the separate properties of the early embryo and the fallopian tube, but the possibility of interactions between the two components of a highly integrated system.

Because of the complex relations between a mammalian cleaving embryo and its environment, it is instructive to consider a microlecithal ovum of an oviparous form such as *Arbacia*. The cleaving *Arbacia* embryo is able to develop unretarded in artificial sea water containing no exogenous energy source.[25] Thus, this form is independent of its environment as far as energy is concerned, in striking contrast to the mouse. The independence, however, does not mean that *Arbacia* cleavage stages cannot use glucose or other energy substrates if they are added to the sea water, a fact which has been exploited many times in studies of the biochemistry of fertilization and early development.[26, 27] An appreciation of this difference between a viviparous and oviparous form is nevertheless crucial in deciding whether different control mechanisms, of energy metabolism for example, have been selected during the evolution of these two different modes of development.

Summary.—The maturation of the mouse oöcyte and the first cleavage division of the zygote can occur *in vitro* in a simple chemically defined medium containing pyruvate or oxaloacetate. If follicular cells are present, oöcyte maturation and cleavage of the zygote can also occur with lactate, PEP, and glucose. Such evidence indicates that the follicular cells are able to metabolize the latter energy substrates and play an active role in providing the unique energy requirements of the oöcyte and zygote during *in vivo* development. The similarity between the

energy requirements of the oöcyte and zygote is determined prior to fertilization during differentiation of the oöcyte.

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