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Studies on the Development of Mouse Embyros *in Vitro*:

II. THE EFFECT OF ENERGY SOURCE

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Abstract

The effect of various possible energy sources on the development *in vitro* of two-cell mouse ova into blastocysts was examined. Energy for development of two-cell mouse ova could be supplied by lactate, pyruvate, oxaloacetate, or phosphoenolpyruvate. Compounds such as glucose, fructose, ribose, glucose-6-phosphate, fructose-1, 6-phosphate, acetate, citrate, α-ketoglutarate, succinate, fumarate, and malate could not provide energy for development of two-cell mouse ova. The optimum concentrations at pH 7.38 for those compounds which would supply energy was 5.00×10^{-2} *M* lactate, 3.16×10^{-4} *M* pyruvate, 3.16×10^{-4} *M* oxaloacetate, and 1.00×10^{-2} *M* phosphoenolpyruvate.

The possibility that interactions existed between the effects of osmolarity, pH, and energy source was examined in several experiments. There was no interaction between the effects of osmolarity and pH or osmolarity and the four possible energy sources. However, there was a significant interaction between energy source and pH. The result of this is that an increase in pH of the medium results in an increase in the optimum concentration of the compound supplying energy to the developing ova.

The relative importance of various energy sources to the early mammalian embryo has received little attention. In the past, media used to cultivate rabbit embryos *in vitro* have in most cases contained 50% or more serum (Austin, 1961). This makes the determination of the compounds supplying energy impossible. In contrast, mouse embryos from the eight-cell stage to the blastocyst have been cultivated in relatively simple salt solutions supplemented with protein and glucose (Hammond, 1949; Whitten, 1956). In 1957 Whitten reported that the energy for development of eight-cell mouse ova to blastocysts could be supplied by glucose or mannose, but could not be supplied by fructose, galactose, maltose, or lactose. Similarly, energy could be supplied by lactate, pyruvate or malate, but could not be supplied by acetate, propionate, citrate, glycerol or glycine. Unfortunately, details of the exact experimenttal procedures were not presented. Whitten (1957) was also able to obtain the development of some late two-cell mouse ova into blastocysts in a medium containing lactate.

The recent development of a method to cultivate early cleavage stages of the mouse ovum completely *in vitro* and the ability of the method to produce quantitative results, allows critical examination of possible energy sources of early mammalian embryos (Brinster, 1963). A number of possible energy sources were examined for their ability to support the development of two-cell mouse embryos *in vitro*.

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MATERIALS AND METHODS

The general experimental procedures for obtaining two-cell ova, culturing the ova, measuring the response of the ova and statistical treatment of the results have been described previously (Brinster, 1963, 1965; Biggers and Brinster, 1965). In all experiments the experimental unit was a single drop and each drop contained 12 two-cell ova at the start of the experiment. In preparing media, the compounds to be tested were substituted for NaCl to maintain the desired osmolarity. Media of different osmolarities and pH were prepared as described by Brinster (1965). The osmolarity employed in the experiments was either 0.270, the approximate optimum osmolarity for *in vitro* development (Brinster, 1965), or 0.308, which is the osmolarity of the salts of blood serum. The pH of the culture medium was either 6.82, the approximate optimum pH for *in vitro* development (Brinster, 1965), or 7.38, which is the pH of most culture media. The composition of the basic salt solution is shown in table 1.

RESULTS

Possible energy sources

Employing the optimum pH (6.82), the optimum osmolarity (0.270), and the best purified compounds available, a number of experiments were performed to determine possible energy sources for the developing two-cell mouse ovum. The compounds tested and the results are summarized qualitatively in table 2. Each compound was tested at 10^{-2} *M*, 10^{-3} *M*, 10^{-4} *M* and 10^{-5} *M* except glucose and fructose which were tested at 2.78×10^{-2} *M* (5 mg/ml), 5.56×10^{-3} *M* (1 mg/ml), and 2.78×10^{-3} *M* (0.5 mg/ml). Twelve two-cell mouse ova were cultivated in all concentrations of every compound, and all experiments had control treatments (1×10^{-2} *M* lactate) to show that the ova were viable and would develop into blastocysts. There was a sharp contrast between those compounds which supported growth and those which did not. In those compounds which did not support growth, the majority of the ova failed to cleave and began to degenerate in 24 hours, whereas in those compounds where blastocysts developed, many of the ova which did not become blastocysts underwent some cleavage and maintained a normal appearance. For those compounds which supported growth, the range of concentration in which the ova would develop into blastocysts was about ten-fold, and the range of concentration in which the ova would cleave and maintain normal appearance was about one hundred-fold.

Optimum concentration of compounds supporting development

Those compounds which supported growth of two-cell mouse ova were examined more closely to determine their optimum concentration. For these experiments the optimum pH (6.82) and osmolarity (0.270) as determined by earlier experiments were used. The range of concentrations of lactate, pyruvate, oxaloacetate, and phosphoenolpyruvate, and the dose interval that was used were also based on previous work. The concentrations employed and the results of the experiments are shown in table 3. The optimum concentration to support growth of two-cell mouse ova for each compound was lactate 2.00×10^{-2} *M*, phosphoenolpyruvate 3.16 10^{-3} *M*, oxaloacetate 3.16×10^{-4} *M*, and pyruvate 1.00×10^{-4} *M*. The analysis of variance for the treatment effect of each compound was significant (P <

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0.001). The experiments with the compounds which supported growth were repeated at pH 7.38 and osmolarity 0.308. These results are shown in table 4. In this case, the optimum concentration to support growth of two-cell mouse ova for each compound was lactate 5.00 × 10^{-2} *M*, phosphoenolpyruvate 1.00 × 10^{-2} *M*, oxaloacetate 3.16 × 10^{-4} *M*, and pyruvate 3.16 × 10^{-4} *M*. The analysis of variance for the treatment effect of each compound was significant ($P < 0.001$).

Interaction of energy source and hydrogen ion concentration

The series of experiments on the individual compounds which support development indicated that in each case except oxaloacetate the optimum concentration of the compound was raised when the pH and osmolarity were raised. This seemed to indicate an interaction between the effects of the energy source, pH and osmolarity. In order to examine this interaction more closely, several experiments were performed, each being a 2^3 factorial design.

The first two experiments were designed to examine the joint effects of pH, osmolarity, and lactate. The experimental treatments, the results, and the analysis of variance of the results are shown in tables 5 and 6. The only significant interaction was between pH and lactate ($0.05 > P > 0.01$). The lactate × pH two-way tables showing the mean angular responses strikingly demonstrate this interaction. The other interactions were not significant. The calculations of the main effects of pH and lactate indicated that they were significant, but no weight can be given to these apparently significant values because of the significant interaction that occurred between the effects of these two compounds. The main effect of osmolarity was not significant in the first experiment, but it was significant in the second experiment ($P < 0.001$). The direction of the effect was for lower osmolarity to decrease the number of blastocysts developing from two-cell ova. The reason for this deviation from earlier experiments on osmolarity is unknown. However, this main effect of osmolarity does not influence the primary objective of these experiments, which was to determine interactions between energy source, pH and osmolarity.

The third experiment was designed to determine interactions between the effects of pH, osmolarity, and pyruvate. Table 7 shows the experimental treatments, the results and the analysis of variance of the results. There was a significant interaction between pyruvate and pH ($P < 0.001$). The effect of this interaction is summarized in the pyruvate × pH two-way table. No other interactions were significant. Although the calculations indicated that the main effect of pyruvate was significant, no weight can be given to this because of the significant interaction between pyruvate and pH. The effect of osmolarity was not significant, but the response was greater at 0.270 osmols than at 0.308 osmols. This is the result expected on the basis of the experiments on the effect of osmolarity on development of two-cell mouse ova *in vitro*. In view of the significant interaction between pH and lactate or pyruvate, and the lack of interaction of osmolarity and lactate or pyruvate, it seems safe to assume that the change in optimum concentration of phosphoenolpyruvate with a change in osmolarity and pH is due to the change in pH and not to the change in osmolarity. It also seems probable that pH affects the response of the ova to oxaloacetate in the same manner as with the other three compounds, but the effect is not as marked. In the case of oxaloacetate,

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the optimum concentration is the same at the high and low pH, but 1×10^{-4} *M* oxaloacetate results in poor development at pH 7.38 and good development at pH 6.82.

DISCUSSION

Possible energy sources

From the compounds listed in table 2 which were examined for their ability to support the development of two-cell mouse ova, it appears that the ova respond only to a special group of compounds, namely lactate, pyruvate, oxaloacetate, and phosphoenolpyruvate. These compounds represent a group in that lactate, oxaloacetate and phosphoenolypyruvate are each separated from pyruvate by one reaction. None of the other normal intermediates of the Krebs cycle, Embden-Meyerhof pathway, or pentose phosphate pathway which were examined, would support development of two-cell mouse ova for 24 hours. The possibility exists that the ovum is impermeable to some of the compounds, such as the phosphorylated substrates, listed in table 2, but it seems unlikely that the ovum is permeable to oxaloacetate and at the same time impermeable to compounds such as malate, fumarate, and acetate. Two compounds, glucose and malate, which will support development of eight-cell mouse ova (Hammond, 1949; Whitten, 1957), will not support development of two-cell mouse ova. Either there are changes in cell permeability to these two compounds, or the necessary enzyme systems for their utilization develop in mouse embryos between the two-cell and eight-cell stage. The appearance of new enzyme systems during early development has been demonstrated in *Arbacia, Fundulus*, and *Ascaris* (Moore and Villee, 1963; Wilde and Crawford, 1963; Oya, Costello and Smith, 1963).

The energy metabolism of the early mammalian embryo is poorly understood. In the rabbit there is good evidence that the Embden-Myerhof cycle is functional only after the blastocyst stage is reached; however, there is also evidence for the existence of the pentose phosphate pathway of glucose oxidation in the early cleavage stages (Fridhandler, 1961). After blastocyst formation, glucose appears to be oxidized by the Embden-Meyerhof pathway and the tricarboxylic acid cycle. Direct comparisons between Fridhandler' s experiments and the experiments reported here are quite difficult because the phenomena studied in the two investigations are quite different. In the studies reported here, normal blastocyst development was the criterion of positive response, whereas in Fridhandler' s work normal growth was not part of the experiment, and in fact, normal growth does not occur in the media in which he performed his experiments. Despite this difference, consideration should be given to the possibility that there exist similarities in the energy requirements and energy metabolism in rabbit and mouse embryos and perhaps in all mammalian embryos. Such a possibility has been given more weight by the recent success of Biggers and Moore (personal communication) in culturing one-cell rabbit ova through to the morula stage in the basic medium containing lactate designed for mouse ova (table 1).

In considering the metabolism of the early embryo, it should be pointed out that the development up to the morula stage primarily consists of making many small cells from one large cell. Since the pentose phosphate pathway is probably the only way the cell can manufacture ribose and deoxyribose for RNA and DNA synthesis, it would not be surprising if this pathway occupied a prominent part in cellular metabolism. The synthesis of

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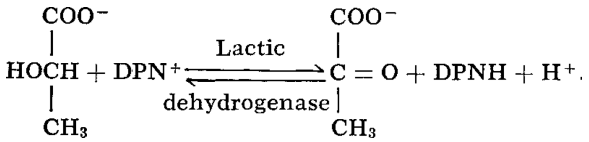
nucleotides could well be one of the limiting processes, since DNA must be duplicated before each mitosis (Brachet, 1960). The pentose pathway also produces TPNH which is critical for synthetic processes, particularly fat synthesis.

Optimum concentration of compounds supporting development

Of the four compounds which were found to support development of two-cell mouse ova to the blastocyst, the one with an optimum closest to the concentration found in blood is pyruvate. The concentration of pyruvate in blood is approximately 1×10^{-4} *M* (Long, 1961). The optimum concentration of lactate appears to be at least ten times the concentration of lactate found in blood (Long, 1961) and rabbit oviduct (Bishop, 1957).

The reason for the differnce in the optimum concentration of the four compounds is not readily apparent. However, if one considers pyruvate as the central compound in this group, it is possible to consider some of the possibilities which may influence the relative optimum concentrations of the other three compounds. It is quite probable that phosphoenolpyruvate is dephosphorylated before entrance into the cell. If this is the case, then the difference in optimum concentration between pyruvate and phosphoenolpyruvate could result if the phosphorylase activity was only sufficient to provide a pyruvate concentration equal to the optimum intracellular concentration of pyruvate. The optimum concentrations of pyruvate and oxaloacetate are as close as might be expected from their structure. However, the optimum concentrations of lactate and pyruvate differ by a factor of approximately 100. Since their structures are so similar, it would be difficult to explain this difference in optimum concentration on the basis of size or permeability. Perhaps the optimum concentration relationship is affected by the lactic dehydrogenase reaction:



The equilibrium constant (*K*) of this reaction is

$$K=\frac{[CH_3CO\,COO^-][DPNH][H^+]}{[CH_3CHOH\,COO^-][DPN]}=1\times 10^{-11.4}. \quad (1)$$

Substituting the values of the anionic form of lactate and pyruvate at their optimum concentration at pH 7.4 into equation (1) yields:

$$K=\frac{[3.46\times 10^{-9}][DPNH][10^{-7.4}]}{[1.51\times 10^{-5}][DPN]}=10^{-11.4}$$

whence

$$\frac{DPNH}{DPN}=\frac{1.51\times 10^{-9}}{3.46\times 10^{-9}}=\frac{1}{2.99}.$$

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The oxidation-reduction potential of the DPN-DPNH system within the ova is completely unknown, but the ratio estimated above is within the range of values (1: 1 to 10: 1) reported for other tissues (Long, 1961). It is interesting that a ratio of DPN-DPNH comparable to that found in other tissues is obtained by the above calculations, but more knowldege must be obtained about intracellular conditions before the influence of the lactic dehydrogenase reaction on the relative optimum concentration of lactate and pyruvate can be established.

Interaction of energy source and hydrogen ion concentration

The information contained in tables 3 and 4 provides evidence for the presence of a pH-metabolite or osmolarity-metabolite interaction. That the interaction is indeed with pH and not with osmolarity is demonstrated in the factorial experiments where osmolarity-pH-lactate interactions and osmolarity-pH-pyruvate interactions were determined. In these three experiments, the only interaction which was significant was pH-lactate interaction and pH-pyruvate interaction. There was no case where osmolarity-pH interaction, osmolarity-lactate interaction, or osmolarity-pyruvate interaction even approached a significant level. On the basis of these experiments, it seems safe to assume that the shift in optimum concentration of phosphoenolpyruvate and the change in the response to oxaloacetate is due to the change in pH and not to a change in osmolarity.

The effect which pH has on determining the optimum concentration of the compounds supplying energy to the developing ova could be a result of a difference in permeability of the ova to the salt and acid form of the compound. In general, the optimum concentration of the compound is increased by a factor of 3.162 ($\sqrt{10}$) when going from the low pH to the high pH. In going from the low to the high pH, the hydrogen ion concentration is decreased by a factor of approximately 3.162.

These four metabolites exist in the media partly in the acid form and partly in the salt form. The quantity in each form can be determined by substitution in the following formula:

$$\mathrm{pH}=\mathrm{pK}+\log\frac{(\text{activity of the salt})}{(\text{activity of the acid})}$$

An increase of 0.50 pH units will cause a decrease in the activity of the acid form relative to the salt form by a factor of 3.162. At a pH near neutrality, the ionizaable group with the lowest pK of each of these compounds is almost completely in the salt form. Therefore, a change in the ratio of salt to acid is predominately a change in the concentration of the acid form. It is not unreasonable to assume that the activity coefficients will not be greatly changed at the dose levels being compared, and one may, therefore, consider molar concentrations in the comparisons made. The relationship of the optimum concentrations for oxaloacetic acid and phosphoenolpyruvate at the two hydrogen ion concentrations is not as exact as the relationship for lactate and pyruvate, but this may be because each of the latter two compounds possess only one ionizable group, whereas the former two compounds each possess more than one ionizable group. It thus appears that the quantity of the metabolite in the acid form is directly related to its ability to support development of two-cell ova. This

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would suggest that the acid form of the metabolite is the form to which the developing ova are permeable.

It should be emphasized that other alternatives to the above explanation for the pH dependency of energy source concentration exist. For instance, transport of the energy compounds across the cell membrane may depend on binding between the energy compound and an ionizable group on the cell membrane. Histidine and imidazole have ionizable groups with a pK in the range of pH studied, and a small change in pH would have a marked effect on the degree of ionization of these groups. If binding and transport through the membrane are dependent on the state of these ionized groups, then the optimum concentration of the energy compounds in the medium could be affected by their degree of ionization.

An outcome of the energy source-pH interaction is that the effect of hydrogen ion concentration on the developing ova cannot be easily separated from the effect the hydrogen ion concentration has on the availability of the metabolite. Since these four compounds are the only energy sources thus far found for the developing two-cell ova, and they all display an interaction with pH, it is not possible at the present time to determine pH effect alone. The results obtained in the pH experiments actually represent a complex effect of changing hydrogen ion concentration and changing metabolite availability (Brinster, 1965).

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TABLE 1

Basic salt solution for ovum culture

Component	Gm/l	M × 10^{-3}
NaCl	6.975	119.32
KC1	0.356	4.78
$CaC1_2$	0.189	1.71
KH_2PO_4	0.162	1.19
$MgSO_4 \cdot 7H_2O$	0.294	1.19
$NaHCO_3$	2.106	25.07
Penicillin G (potassium)	100 U/ml	—
Streptomycin sulfate	50 μg/ml	—
Crystalline bovine serum albumin	1.000	—

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TABLE 2

Possible energy sources for the developing two-cell mouse ova

I. Compounds which will not support growth separately	
A. Krebs cycle intermediates	
1. Malate	5. Citrate
2. Fumarate	6. Acetate
3. Succinate	7. Cis-aconitate
4. Iso-citrate	8. *a*-ketoglutarate
B. Others	
1. Glucose	4. D-glyceraldehyde
2. Fructose	5. Glucose-6-phosphate
3. Ribose	6. Fructose-l,6-diphosphate
(All compounds tested at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} molar except glucose and fructose, which were tested at 2.78×10^{-2}, 5.56×10^{-3}, and 2.78×10^{-3} molar.)	
II. Compounds which will support growth separately	
1. Lactate	3. Oxaloacetate
2. Pyruvate	4. Phosphoenolpyruvate
(See text for details.)	

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TABLE 3

Effect of the concentration of energy source on the development of two-cell mouse ova (pH 6.82, 0.270 osmols)

Treatment	Molar concentration	Response drop number						Mean angular response	Response
		1	2	3	4	5	6		
Lactate									%
1	8.00×10^{-3}	0	2	2	0	5	2	21.52	13.5
2	9.00×10^{-3}	3	4	5	5	4	3	35.17	33.2
3	1.00×10^{-2}	8	6	4	6	6	6	45.00	50.0
4	2.00×10^{-2}	4	6	9	7	4	6	45.07	50.0
5	3.00×10^{-2}	6	6	6	5	6	7	45.00	50.0
6	4.00×10^{-2}	1	3	0	0	0	0	13.33	5.3
7	5.00×10^{-2}	2	2	0	0	1	0	14.98	6.6
Pyruvate									
1	1.00×10^{-3}	0	0	0	0	–	–	8.30	0
2	3.16×10^{-4}	3	5	3	6	–	–	36.30	35.1
3	1.00×10^{-4}	8	7	10	8	–	–	56.28	69.2
4	3.16×10^{-5}	1	2	1	2	–	–	20.45	12.2
5	1.00×10^{-5}	0	0	0	0	–	–	8.30	0
Oxaloacetate									
1	3.16×10^{-3}	0	0	0	0	0	–	8.30	0
2	1.00×10^{-3}	0	1	0	0	0	–	10.00	3.0
3	3.16×10^{-4}	4	7	2	2	6	–	35.66	34.0
4	1.00×10^{-4}	4	4	3	5	1	–	31.52	27.3
5	3.16×10^{-5}	0	0	0	0	0	–	8.30	0
Phosphoenolpyruvate									
1	3.16×10^{-2}	0	1	0	0	–	–	10.43	3.3
2	1.00×10^{-2}	2	1	2	1	–	–	20.45	12.2
3	3.16×10^{-3}	8	6	6	8	–	–	49.85	58.4
4	1.00×10^{-3}	5	6	6	7	–	–	45.00	50.0

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Treatment	Molar concentration	Response drop number						Mean angular response	Response
		1	2	3	4	5	6		
5	3.16×10^{-4}	0	1	0	0	–	–	10.43	3.3

Response is the number of normal blastocysts from 12 two-cell ova after three days cultivation *in vitro*. Two-cell ova obtained 45 hours after HCG injection. The analysis of variance of the treatment effect for each energy source was significant ($P < 0.001$).

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TABLE 4

Effect of the concentration of energy source on the development of two-cell mouse ova (pH 7.38, 0.308 osmols)

Treatment	Molar concentration	Response drop number						Mean angular response	Response
		1	2	3	4	5	6		
Lactate									%
1	1.00×10^{-2}	0	0	0	2	0	0	10.93	3.6
2	2.00×10^{-2}	3	5	3	3	5	3	33.40	30.3
3	3.00×10^{-2}	7	3	5	2	2	2	32.05	28.2
4	4.00×10^{-2}	4	5	6	3	3	3	35.08	33.0
5	5.00×10^{-2}	8	8	7	5	8	9	52.35	62.7
6	6.00×10^{-2}	4	7	3	2	4	7	37.38	36.8
7	7.00×10^{-2}	4	6	5	4	0	2	31.37	27.1
8	8.00×10^{-2}	3	3	3	3	4	2	29.90	24.9
9	9.00×10^{-2}	3	3	1	2	1	1	22.42	14.6
Pyruvate									
1	1.00×10^{-3}	1	1	2	5	–	–	24.48	17.2
2	3.16×10^{-4}	9	10	9	8	–	–	60.15	75.2
3	1.00×10^{-4}	2	0	1	4	–	–	21.13	13.0
4	3.16×10^{-5}	0	0	0	0	–	–	8.30	0
Oxaloacetate									
1	1.00×10^{-3}	0	0	0	0	–	–	8.30	0
2	3.16×10^{-4}	5	8	6	5	–	–	45.03	50.1
3	1.00×10^{-4}	0	0	0	1	–	–	10.43	3.3
Phosphoenolpyruvate									
1	3.16×10^{-2}	0	0	0	0	–	–	8.30	0
2	1.00×10^{-2}	2	5	4	2	–	–	30.93	26.4
3	3.16×10^{-3}	l	o	l	l	–	–	14.68	6.4
4	1.00×10^{-3}	0	0	0	0	–	–	8.30	0

Response is the number of normal blastocysts from 12 two-cell ova after three days cultivation *in vitro*. Two-cell ova obtained 45 hours after HCG injection except in the phosphoenolpyruvate experiment, where the two-cell ova were obtained 43 hours after HCG injection. The analysis of variance of the treatment effect for each energy source was significant ($P < 0.001$).

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TABLE 5

Interaction of osmolarity, pH and lactate Experiment 1

Treatment	Concentration			Response drop number				Mean angular response	Response
	Osmols	pH	Lactate (M)	1	2	3	4		
									%
1	0.308	7.38	5×10^{-2}	2	4	1	6	30.30	25.5
2	0.308	7.38	2×10^{-2}	4	5	2	5	34.95	32.8
3	0.308	6.82	5×10^{-2}	1	0	0	0	10.43	3.3
4	0.308	6.82	2×10^{-2}	3	1	6	2	28.98	23.5
5	0.270	7.38	5×10^{-2}	2	4	4	1	27.88	21.9
6	0.270	7.38	2×10^{-2}	3	4	5	3	33.88	31.1
7	0.270	6.82	5×10^{-2}	0	0	0	2	12.25	4.5
8	0.270	6.82	2×10^{-2}	4	3	2	4	31.18	26.8

Lactate × pH two-way table (mean angular response)

Lactate (M)	pH	
	6.82	7.38
2×10^{-2}	30.08	34.42
5×10^{-2}	11.43	29.09

Analysis of variance

Source of variation	D.F.	MS
Between drops	3	12.091
Between treatments	(7)	(360.561)
Osmolarity (O)	1	0.138
pH	1	975.716 [3]
Lactate (L)	1	1158.008 [3]
O × pH	1	28.312

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Analysis of variance

Source of variation	D.F.	MS
pH × L	1	359.790 [1]
L × O	1	1.488
O × pH × L	1	0.476
Error	21	79.735
Total	31	
Theoretical variance	∞	75.3

Response is the number of normal blastocysts from 12 two-cell ova after three days cultivation *in vitro*. Two-cell ova obtained 45 hours after HCG injection.

[1] $0.05 > P > 0.01$.

[3] $P < 0.001$.

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TABLE 6

Interaction of osmolarity, pH and lactate Experiment 2

Treatment	Concentration			Response drop number				Mean angular response	Response
	Osmols	pH	Lactate (M)	1	2	3	4		
									%
1	0.308	7.38	5×10^{-2}	9	9	8	5	53.73	65.0
2	0.308	7.38	2×10^{-2}	10	9	8	8	58.83	74.7
3	0.308	6.82	5×10^{-2}	4	6	3	5	37.63	37.3
4	0.308	6.82	2×10^{-2}	8	8	6	8	52.28	62.6
5	0.270	7.38	5×10^{-2}	7	7	5	6	46.20	52.1
6	0.270	7.38	2×10^{-2}	6	5	8	6	46.23	52.2
7	0.270	6.82	5×10^{-2}	2	1	0	3	19.80	11.5
8	0.270	6.82	2×10^{-2}	7	5	5	4	41.38	43.7

Lactate × pH two-way table (mean angular response)

Lactate (M)	pH	
	6.82	7.38
2×10^{-2}	46.83	52.54
5×10^{-2}	28.72	49.97

Analysis of variance

Source of variation	D.F.	MS
Between drops	3	76.937
Between treatments	(7)	(584.996)
Osmolarity (O)	1	1193.162 [3]
pH	1	1452.605 [3]
Lactate (L)	1	854.912 [3]
O × pH	1	36.980

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Analysis of variance

Source of variation	D.F.	MS
pH × L	1	483.605 [1]
L × O	1	1.710
O × pH × L	1	72.000
Error	21	41.016
Total	31	
Theoretical variance	∞	75.3

Response is the number of normal blastocysts from 12 two-cell ova after three days cultivation *in vitro*. Two-cell ova obtained 45 hours after HCG injection.

[1] $0.05 > P > 0.01$.

[2] $P < 0.001$.

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TABLE 7

Interaction of osmolarity, pH and pyruvate

Treatment	Concentration			Response drop number				Mean angular response	Response
	Osmols	pH	Pyruvate (M)	1	2	3	4		
									%
1	0.308	7.38	3.16×10^{-4}	6	7	6	4	43.78	47.9
2	0.308	7.38	1.00×10^{-4}	4	1	4	1	26.05	19.3
3	0.308	6.82	3.16×10^{-4}	4	5	2	7	37.35	36.8
4	0.308	6.82	1.00×10^{-4}	6	4	3	5	37.63	37.3
5	0.270	7.38	3.16×10^{-4}	9	8	11	4	55.80	68.4
6	0.270	7.38	1.00×10^{-4}	2	2	5	3	29.60	24.4
7	0.270	6.82	3.16×10^{-4}	4	4	4	2	32.50	28.9
8	0.270	6.82	1.00×10^{-4}	8	6	5	6	46.23	52.2

Pyruvate × pH two-way table (mean angular response)

Pyruvate (M)	pH	
	6.82	7.38
1.00×10^{-4}	41.93	27.83
3.16×10^{-4}	34.93	49.79

Analysis of variance

Source of variation	D.F.	*MS*
Between drops	3	82.585
Between treatments	(7)	(376.563)
Osmolarity (O)	1	186.728
pH	1	1.163
Pyruvate (P)	1	447.753 [1]
O × pH	1	69.915
pH × P	1	1677.652 [3]

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Analysis of variance

Source of variation	D.F.	*MS*
P × O	1	12.375
O × pH × P	1	240.354
Error	21	
Total	31	
Theoretical variance	∞	75.3

Response is the number of normal blastocysts from 12 two-cell ova after three days cultivation *in vitro*. Two-cell ova obtained 45 hours after HCG injection.

[1] $0.05 > P > 0.01$.

[3] $P < 0.001$.