The effects of inhibitors of energy metabolism on the growth of one-cell rabbit ova to blastocysts *in vitro*

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Summary. Fertilized 1-cell rabbit ova were cultured in the presence of three oxidative phosphorylation inhibitors (cyanide, 2,4-dinitrophenol and oligomycin), two tricarboxylic acid (TCA) cycle inhibitors (malonate and fluoroacetate) and one glycolytic inhibitor (2-deoxyglucose). All three oxidative phosphorylation inhibitors killed ova at the 1-cell stage and the damage caused by each was similar. Malonate was non-toxic at all concentrations whereas some concentrations of fluoroacetate stopped growth at the 1-cell stage. This toxic effect could, in some circumstances, be reversed by the presence of acetate but not of glucose. 2-Deoxyglucose blocked only the transition from morula to blastocyst, and this was prevented by the addition of glucose to the medium; pyruvate, ribose, glycerol and L- α -glycerol phosphate were ineffective. An active oxidative phosphorylation system and tricarboxylic cycle appear to be present and essential in the rabbit embryo from the 1-cell stage, but glycolysis may not be essential until blastocyst formation.

Introduction

Knowledge of the patterns of energy metabolism in preimplantation mammalian embryos is confined mainly to the mouse, and to a much lesser extent, the rabbit. Whitten (1957) and Brinster (1965) reported that lactate, pyruvate, phosphoenol pyruvate and oxaloacetate supported growth of 2-cell mouse ova to the blastocyst stage, but that glucose was not effective until the 8-cell stage. Subsequent research (reviewed by Wales, 1975) indicates that in the mouse ovum there is a functional tricarboxylic acid (TCA) cycle from the 1-cell stage, but a block to glycolysis for the first two or three cleavage divisions.

The work of Fridhandler and his associates (Fridhandler, Hafez & Pincus, 1957; Fridhandler, 1961, 1968; Fridhandler, Wastila & Palmer, 1967) indicated that the ability of rabbit embryos to carry out glycolysis developed only at the morula stage. Quinn & Wales (1973), however, found clear evidence for some glycolytic capacity in cleavage-stage rabbit embryos, although this capacity was greatly increased at the blastocyst stage. The presence of a very active hexosemonophosphate shunt up to the morula stage has been indicated by carbon-labelling studies (Fridhandler, 1961; Brinster, 1968). An active TCA cycle was found in rabbit blastocysts (Fridhandler *et al.*, 1967) but not in rabbit ova (Fridhandler *et al.*, 1957), although Quinn & Wales (1974) found evidence for TCA activity in the cleavage and blastocyst stages.

The purpose of the present study was to elucidate the importance of some of the different pathways of energy metabolism in rabbit ova by studying the effects of different metabolic inhibitors on the growth of 1-cell ova in culture. A similar study has been carried out by Thomson (1967) on 2-cell mouse ova.

Materials and Methods

New Zealand White and Dutch-belted does, 5–20 months of age, were induced to superovulate by treatment with FSH (twice daily s.c. injections for 3 days; 0.5 mg/injection) followed 1 day later by simultaneous injection of HCG (150 units, intravenous) and artificial insemination. Fertilized ova (1-cell) were flushed from the oviducts 20–21 h after injection of HCG. The ova were washed to re-

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move oviductal debris and all those collected on one day were pooled and divided among the treatments of that day's experiment. The ova collected on one day constituted one replicate of an experiment or, in the case of Exps 1 and 2, one replicate of one inhibitor study. Usually at least three or more replicates were carried out per experiment or inhibitor study.

The eggs were cultured in 0.5 ml droplets of medium under liquid paraffin in plastic tissue-culture dishes and were maintained in culture for 5 days at 38°C under a gas phase of 5% CO₂ in air. Ovum development was assessed microscopically every day. In all experiments the basic culture medium contained 1.5% crystallized bovine serum albumin (Sigma), 108 mM-NaCl, 4.78 mM-KCl, 1.71 mM-CaCl₂·2H₂O, 1.19 mM-KH₂PO₄, 1.19 mM-MgSO₄, 25 mM-NaHCO₃, and 100 units penicillin G and 50 µg streptomycin per ml, and was supplemented with the amino acids, vitamins and trace elements of Ham's F10 medium (Ham, 1963; Kane & Foote, 1970). This medium was used as a control treatment for all experiments and also as a basic medium to which the various inhibitors and substrates were added. All inhibitors were supplied by Sigma, except for potassium cyanide, obtained from May & Baker.

Experiment 1 was a study of the effects of different concentrations of the oxidative phosphorylation inhibitors, potassium cyanide, 2,4-dinitrophenol and oligomycin. Cyanide inhibits cytochrome oxidase and other components of the respiratory chain (Mahler & Cordes, 1971). Dinitrophenol uncouples oxidative phosphorylation and oligomycin inhibits the formation of ATP (Slater, 1963).

Experiment 2. Ova were cultured in the presence of TCA cycle inhibitors, sodium monofluoroacetate and sodium malonate. Malonate blocks the TCA cycle by inhibiting succinate dehydrogenase (Quastel, 1963). Fluoroacetate is first metabolized to fluorocitrate which then inhibits aconitase (Quastel, 1963).

Experiment 3. To determine whether the toxic effects of fluoroacetate were substrate reversible, ova were pretreated for 1 h at the 1-cell stage, either in fluoroacetate alone (10⁻³ M), in fluoroacetate plus sodium acetate (5 × 10⁻² M) or in fluoroacetate plus glucose (10⁻² M). The eggs were then transferred to control medium and cultured for 5 days.

Experiment 4. The effects of different concentrations of the glycolytic inhibitor, 2-deoxyglucose, were examined. This is metabolized to 2-deoxyglucose-6-phosphate which blocks the enzyme glucose phosphate isomerase (Hochster, 1963).

Experiment 5. The stage of embryo growth at which the toxic effects of 2-deoxyglucose were exerted was determined. Ova were either cultured to the morula stage in control medium for 2 days and then transferred to medium with 2-deoxyglucose (10⁻⁴ M) for 3 days, or cultured in medium with 2-deoxyglucose for 2 days and then transferred to control medium for 3 days. The development obtained after these two treatments was compared with that of ova grown for 5 days in control medium, and ova grown for 5 days in medium with 2-deoxyglucose.

Experiment 6. The substrate reversibility of the 2-deoxyglucose effect was examined. Ova were cultured either in 2-deoxyglucose medium (10⁻⁴ M) or in media with 2-deoxyglucose and glucose, pyruvate, glycerol or ribose (all at 10⁻² M).

Results obtained in experimental treatments were compared statistically with those in control treatments by the χ^2 test.

Results

Oxidative phosphorylation inhibitors (Exp. 1)

All three oxidative phosphorylation inhibitors stopped ovum growth at the 1-cell stage (Table 1). The inhibitor concentrations at which ovum death occurred correspond to those normally used to inhibit oxidative phosphorylation in other tissues (Mahler & Cordes, 1971). The resulting cell damage affected almost every ovum and consisted of the formation of a clear space under the vitelline membrane but surrounding the cell cytoplasm, the 'pericytoplasmic space' (compare Pl. 1, Fig. 2 with Pl. 1, Fig. 1). The formation of this pericytoplasmic space first became obvious as early as 20 min after placing ova in 10⁻³ M-cyanide.

Table 1. Effects of oxidative phosphorylation inhibitors on growth of 1-cell rabbit ova

Potassium cyanide					
Inhibitor conc. (M)	0	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
No. of ova	34	36	34	34	34
% Blastocysts	47	0†,***	0†,***	56	65
2,4-Dinitrophenol					
Inhibitor conc. (M)	0	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
No. of ova	30	33	32	35	43
% Blastocysts	70	0†,***	34*	60	47
Oligomycin					
Inhibitor conc. (µg/ml)†	0	1	0.2	0.04	
No. of ova		31	29	33	29
% Blastocysts		52	0†,***	0†,***	14**

Significantly different from 0 concentration of inhibitor: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

† All these ova were killed at the 1-cell stage.

‡ Given in µg/ml because the oligomycin used was a mixture of A and B isomers (1 µg/ml approximately = 2.5×10^{-6} M).

Table 2. Effects of Krebs cycle inhibitors on growth of 1-cell rabbit ova

Sodium monofluoroacetate					
Inhibitor conc. (M)	0	10 ⁻²	3×10^{-3}	10 ⁻³	10 ⁻⁴
No. of ova	70	69	65	58	64
% Blastocysts	63	0†,*	0†,*	0*	28*
Sodium malonate					
Inhibitor conc. (M)	0	10 ⁻²	10 ⁻³	10 ⁻⁴	
No. of ova	27	29	23	27	
% Blastocysts	46	45	43	67	

Significantly different from 0 concentration of inhibitor: * $P < 0.001$.

† All these ova were killed at the 1-cell stage.

TCA cycle inhibitors (Exps 2 and 3)

There was no sign of any toxic effect of sodium malonate (Table 2). However, sodium monofluoroacetate completely prevented development at concentrations of 10^{-2} M to 10^{-3} M and resulted in a significant drop in blastocyst formation at 10^{-4} M (Table 2). Growth was stopped at the 1-cell stage in 10^{-2} and 3×10^{-3} M-sodium monofluoroacetate, concentrations comparable to those which have been used by previous workers to block the TCA cycle and cause citrate accumulation in other tissues (Liebecq & Peters, 1949; Buffa, Peters & Wakelin, 1951). This effect of fluoroacetate was specifically prevented by acetate but not by glucose (Exp. 3, Table 3). Preliminary experiments in which ova were pretreated for 6 h with fluoroacetate before transfer to inhibitor-free media showed that 5×10^{-2} M acetate would not protect ova against 10^{-3} M-fluoroacetate for that length of time. This may be partly due to a toxic effect of high concentrations of acetate, because other preliminary trials showed that ova cultured for 5 days in 5×10^{-2} M-acetate did not develop to morulae or blastocysts. This effect of acetate was not due to osmolarity effects because the osmotic effect of the added sodium acetate was compensated for by the omission of equimolar quantities of NaCl.

Glycolytic inhibitor (Exps 4, 5 and 6)

There was a complete failure of blastocyst formation when concentrations of 10^{-2} to 10^{-4} M-2-deoxyglucose were used (Exp. 4, Table 4). Most of the ova reached the morula stage but none progressed to the blastocyst stage. A concentration of 2.4×10^{-3} M has been shown to inhibit growth of embryonic chicken heart fibroblasts in tissue culture (Hochster, 1963). The inhibitory effect of 2-deoxyglucose was specific to the period of blastocyst formation (Exp. 5, Table 5): ova cultured to the

Table 3. Effects of 5×10^{-2} M-acetate and 10^{-2} M-glucose on the inhibition by 10^{-3} M-fluoroacetate of growth of 1-cell rabbit ova

	Control medium	Fluoro-acetate	Fluoro-acetate + acetate	Fluoro-acetate + glucose
No. of ova†	46	44	47	43
% Blastocysts	61	4*	60	0*

Significantly different from control medium, * $P < 0.001$.

† Pretreated for 1 h at the 1-cell stage and then transferred to control medium for 5 days.

Table 5. Effect of 10^{-4} M-2-deoxyglucose added at different stages of culture on growth of 1-cell rabbit ova to blastocysts

	Control medium (5 days)	Deoxy-glucose medium (5 days)	Deoxy-glucose + control medium (3 days)	Control medium + deoxy-glucose (3 days)
No. of ova	36	40	29	32
% Blastocysts	67	0*	59	0*

Significantly different from control medium, * $P < 0.001$.

Table 4. Effect of a glycolytic inhibitor, 2-deoxyglucose, on growth of 1-cell rabbit ova

Inhibitor conc. (M)	0	10^{-2}	10^{-3}	10^{-4}	10^{-5}
No. of ova	41	44	40	41	36
% Morulae	56	50	68	49	64
% Blastocysts	44	0*	0*	0*	25

Significantly different from 0 concentration of inhibitor: * $P < 0.001$.

Table 6. Effects of different substrates on the inhibition by 10^{-4} M-2-deoxyglucose of growth of 1-cell rabbit ova to blastocysts

	Control medium	Deoxy-glucose	Glucose	Pyruvate	Glycerol	Ribose
No. of ova	27	30	25	28	27	19
% Blastocysts	37	0**	64	0*†	0*	0*

Significantly different from control medium: * $P < 0.01$; ** $P < 0.001$.

† Two embryos began to form a blastocoele cavity but then rapidly degenerated.

morula stage in medium with inhibitor developed to blastocysts when transferred to control medium, whereas ova cultured in control medium to the morula stage were arrested at that stage when transferred to medium with inhibitor.

The inhibitory effect of 10^{-4} M-2-deoxyglucose on blastocyst formation was completely reversed by the addition of 10^{-2} M-glucose but was not prevented by pyruvate, glycerol or ribose (Exp. 6, Table 6), L- α -glycerol phosphate, or by ribose, pyruvate and glycerol in combination (data not shown, separate trial).

Discussion

The results with cyanide, 2,4-dinitrophenol and oligomycin provide strong evidence for an essential role for the process of oxidative phosphorylation in normal growth of the fertilized 1-cell rabbit egg. These findings are in agreement with the work of Fridhandler *et al.* (1957) who found, using Cartesian diver techniques, that cyanide reduced to near zero the oxygen uptake of 2–8-cell rabbit ova and advanced blastocysts. Pincus (1941) reported that cyanide inhibited growth of 3-day rabbit embryos

PLATE 1

Fertilized 1-cell rabbit ova, collected about 20 h after HCG injection and placed in medium containing 10^{-3} M-KCN. Ova were photographed directly from the culture dish.

Fig. 1. Two ova at the start of treatment with KCN. $\times 310$.

Fig. 2. Five ova after 6 h of treatment with KCN. The vitelline membranes of each egg appear to have separated from the cytoplasmic contents to form a 'pericytoplasmic space' (PC). $\times 250$.

(Facing p. 264)

cultured in serum and Thomson (1967) found that cyanide and 2,4-dinitrophenol caused degeneration of 2-cell mouse ova during the first 24 h of culture.

The gross appearance of the damage caused by these three inhibitors in the present study was the consistent formation of a 'pericytoplasmic' space or vacuole, apparently because of a rapid breakdown of the normal relationship of the cytoplasmic contents to the vitelline membrane. We have observed similar types of damage from other causes but never with anything like the same frequency. The toxicity of oligomycin and dinitrophenol is unlikely to be due to effects on cation transport because (i) all three oxidative phosphorylation inhibitors caused the same type of damage and (ii) ouabain, a specific inhibitor of a Na^+/K^+ -activated ATPase system (Post, Merritt, Kinsolving & Albright, 1960), does not cause similar damage and does not stop growth at the 1-cell stage, although it does prevent growth to the blastocyst stage (N. J. Buckley & M. T. Kane, unpublished data).

The TCA cycle inhibitors, fluoroacetate and malonate, gave contradictory results in that fluoroacetate stopped ovum growth at the 1-cell stage whereas malonate was not toxic even at concentrations as high as 10^{-2} M, a finding similar to that of Thomson (1967) for 2-cell mouse ova. We feel that the failure of malonate to exert a toxic effect may be partly due to failure to penetrate the ovum in sufficient quantities because of the dicarboxylic nature of the malonate molecule. There is some evidence that the entry of malonate into mitochondria is dependent on a carrier system (Pressman, 1970). Evidence for the presence of such a carrier system for rabbit embryo mitochondria is lacking but Wales & Biggers (1968) have shown that malate, another dicarboxylic acid, appears to enter mouse ova by an active process.

The fluoroacetate results when taken in conjunction with those of the oxidative phosphorylation experiments strongly indicate that the TCA cycle is both present and essential for fertilized 1-cell rabbit eggs. This conclusion is strengthened by the fact that acetate prevented the toxic effects of pretreatment with fluoroacetate whereas glucose did not. A number of studies have shown the presence of TCA cycle metabolism in the mouse ovum as early as the 2-cell stage (Wales & Whittingham, 1970; Quinn & Wales, 1971; Barbehenn, Wales & Lowry, 1974) and TCA cycle activity in the early cleavage stages of the rabbit ovum has also been demonstrated (Quinn & Wales, 1974). The fact that acetate overcame the toxic effects of pretreatment with fluoroacetate, whereas glucose did not, suggests that even if glycolysis is active during early cleavage insufficient glucose is being metabolized to provide enough energy for ovum growth when the TCA cycle is blocked by fluoroacetate. This idea is supported by the results obtained with 2-deoxyglucose. Ova cultured in concentrations of 2-deoxyglucose as low as 10^{-4} M developed to the morula stage but remained arrested at that stage. Similar results were obtained for 2-cell mouse ova by Thomson (1967) and Lutwak-Mann, Hay & New (1969) reported that treatment of 6-day rabbit blastocysts *in vitro* with 2-deoxyglucose resulted in damage to the embryonic disc and the trophoblast. Our experiments (Exp. 5) showed that the block to development exerted by 2-deoxyglucose is specific to the stage of blastocyst formation. Fridhandler (1961), who measured CO_2 evolution from radiolabelled glucose, found negligible rates of glycolysis under anaerobic conditions in rabbit embryos until the blastocyst stage was reached. Quinn & Wales (1973) found that while glucose was metabolized to acetate, pyruvate and lactate by cleavage stages of the rabbit embryo, accumulation of these compounds was greatly increased at the blastocyst stage. All these results indicate a relatively minor role for glycolysis until the blastocyst stage.

The essential role of glucose at the time of blastocyst formation is not clear. It can hardly be for amino acid formation because a full range of amino acids was present in the media used in our experiments. This role is also unlikely to be related solely to energy production, pentose formation or the production of L- α -glycerol phosphate for triglyceride formation, because pyruvate, ribose and glycerol, alone and in combination, and L- α -glycerol phosphate alone were unable to protect against the effects of 2-deoxyglucose. It is, however, possible that the failure of these substances to act protectively may have been due to an inability of one or more of them to permeate the zygote. Another possible explanation is that the glucose may be used itself or in the form of other hexoses for the production of glycoproteins or of glycolipids which are involved in cell-to-cell interactions (Hughes, 1975). It is possible that 2-deoxyglucose may be incorporated into some of these compounds and interfere with their function by distortion of their structure. The finding of Van Blerkom, Manes &

Daniel (1973) that the appearance of tight cell junctions in the rabbit embryo is coincidental with the onset of blastulation may be relevant here.

The general picture of energy metabolism in the rabbit embryo to emerge from these studies and from those of other workers is that even at the 1-cell stage an active TCA cycle and oxidative phosphorylation system are present and essential for ovum growth, but glycolysis does not appear to become essential until the onset of blastulation. These findings appear to be valid for the mouse and may also apply to other species.

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