In-vitro incorporation of [^3H]thymidine by diapausing rat blastocysts

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When implantation is experimentally delayed, blastocyst growth and mitotic and metabolic activities cease. A single administration of oestrogen *in vivo* activates the metabolic processes which culminate in implantation of the blastocysts (Prasad, Dass & Mohla, 1968; Psychoyos, 1973, 1974; Holmes & Dickson, 1975). Metabolic activation of 'delayed' blastocysts can also be induced by culturing them in media containing fetal calf serum (Psychoyos & Bitton-Casimiri, 1969; Psychoyos, 1969, 1973, 1974; Weitlauf, 1974a, b; Psychoyos, Bitton-Casimiri & Brun, 1975). Two explanations of the mechanism of embryonic dormancy and activation have been suggested: (1) the uterine environment may be suboptimal because of the absence of an essential component, the replacement of which results in activation; and (2) dormancy may be induced by a 'repressive factor' of uterine origin, the removal of which causes activation (Psychoyos, 1973, 1974; Psychoyos *et al.*, 1975). The present experiments on the activation of DNA synthesis in the 'delayed' blastocyst *in vitro* were undertaken to study these possibilities.

Adult female Wistar rats were mated overnight with males of proven fertility; mating was confirmed by the presence of spermatozoa the next morning which was designated Day 1 of pregnancy. The delay of implantation was induced by ovariectomizing the rats on the morning of Day 4 and giving daily injections of 4 mg progesterone. At autopsy on Day 8 blastocysts were recovered by flushing the uterine horns with Tyrode's solution at room temperature. The blastocysts were randomly allocated to groups and incubated for different times (Table 1) or in different media (Table 2). Groups of 9–10 blastocysts were placed in 0.45 ml medium in watch glasses under an atmosphere of 95% air + 5% CO_2 ; 2 hr before the end of incubation 5 μCi [^3H]thymidine (Tmm 48 B, sp. act. 27 Ci/mm; CEA, France) in a volume of 0.05 ml phosphate-buffered 0.9% (w/v) NaCl were added to each culture dish. At the end of incubation the blastocysts were recovered, washed, placed on slides, fixed with Carnoy's fixative and processed for autoradiography using Ilford L4 emulsion as described by Rath & Prasad (1974).

The blastocysts were stained with methyl green pyronine, and the percentage of cells labelled was calculated in relation to the total number of cells from the cell counts of 20–25 blastocysts for each observation.

The results are shown in Tables 1 and 2. The incorporation of [^3H]thymidine increased progressively to reach a maximum at 18 hr (Table 1). It has been suggested that the presence of serum in the culture medium may act as a stimulating factor for activation of diapausing blastocysts (McLaren, 1973), but in the present experiments blastocysts which were incubated in the medium without serum or in Tyrode's solution alone exhibited augmentation in the incorporation of radioactivity compared with control blastocysts (Table 2). Serum may not therefore be an obligatory factor for activation of diapausing blastocysts *in vitro*. However, when compared with blastocysts cultured in the presence of serum, the blastocysts of Groups 2 and 3 had a lower labelling index and low rate of survival, indicating that serum may be facilitatory. It has been suggested that the microquantities of oestrogen present in fetal calf serum may be the direct cause of activation of blastocysts, but the replacement of fetal calf serum with serum from rats with delayed implantation (Group 4) did not affect the extent of incorporation of [^3H]thymidine.

The cells of the diapausing blastocysts are arrested at the G1 or G2 phase of the cell cycle (Sherman & Barlow, 1972; Surani, 1975; Dupont, Esnault, Duluc & Mayer, 1975). As shown in the present experiments, some factor(s) in the culture conditions enables the cells to overcome this arrest and enter into S phase, with most cells synthesizing DNA by 18 hr after the start of culture. For cells to

Table 1. The incorporation of [^3H]thymidine, added 2 hr before the end of culture, by diapausing rat blastocysts cultured for different times in Medium 199 containing 5% fetal calf serum

Group	Time of incubation (hr)	% cells labelled
1	2	16
2	6	24
3	12	25
4	18	88
5	24	71

Table 2. The effect of various media and incubation for 18 hr of [^3H]thymidine by diapausing rat blastocysts

Group	Medium	% cells labelled
1	Medium 199 + 5% fetal calf serum	88
2	Medium 199	52
3	Tyrode's solution	48
4	Medium 199 + 5% serum from rat with implantation delay	91

reach a peak of DNA synthesis around 18–20 hr of culture, an earlier increase of RNA and protein synthesis is needed at 5–8 hr (Valladares, Alvarez, Tabarés & Alvarez, 1973). Previous studies on the culture of diapausing blastocysts have shown that there is rapid synthesis of RNA and protein within 6 hr of incubation (Bitton-Casimiri, Brun & Psychoyos, 1976; Weitlauf, 1974b).

The extent of activation of diapausing blastocysts *in vitro* was compared with that of blastocysts which had been activated by s.c. injection of 2 μg oestradiol, 22 hr before the rats were killed, and cultured for 2 hr with [^3H]thymidine. The index of labelling in these blastocysts 24 hr after injection was comparable (66%) to that of the blastocysts cultured for 24 hr (Table 1, Group 5). In conclusion, the present study lends support to the suggestion that embryonic diapause is due to some repressive mechanism controlled by the uterus and that the dormant blastocysts are activated when removed from such influence and cultured.

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