MOUSE BLASTOCYSTS GROWN *IN VIVO* AND *IN VITRO*: CARBON DIOXIDE PRODUCTION AND TROPHOBLAST OUTGROWTH

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(Received 6th October 1969)

Summary. (1) CO₂ production by blastocysts cultured from the eight-cell stage in Brinster's medium was compared with that of uterine blastocysts recovered from females 3½ days *p.c.* The variation of CO₂ output in different experiments was significantly greater for the cultured than for the control blastocysts; sometimes the CO₂ output of cultured blastocysts was as high as that of the controls, but on average it was significantly lower.

(2) Adding foetal calf serum to the medium, or substituting glucose for lactate and pyruvate, did not increase the amount of CO₂ produced by blastocysts cultured from the eight-cell stage, nor did it affect the proportion of eight-cell eggs developing to the blastocyst stage. Eagle's medium with foetal calf serum proved less successful than Brinster's medium in supporting development.

(3) Blastocysts which had developed from the eight-cell stage in culture, compared with control blastocysts recovered directly from the uterus and then cultured, showed a lower percentage attaching to the culture dish and a lower percentage with trophoblast outgrowth. All media tested permitted attachment, but only in those media containing serum did any appreciable number of embryos show trophoblast outgrowth.

(4) When blastocysts were cultured for 24 hr, the level of CO₂ output of those taken from the uterus was not affected by the presence of serum in the culture medium; on the other hand, the blastocysts developed *in vitro* (which had a low initial CO₂ output) showed a significantly higher level of CO₂ production when serum was present. The association between CO₂ output and trophoblast outgrowth suggests that metabolic activity needs to reach a certain level if trophoblast outgrowth *in vitro*, and implantation *in vivo*, are to occur.

INTRODUCTION

Potentially, the culture of pre-implantation mouse embryos (see Mintz, 1967, for a review of techniques) offers a valuable tool for the study of early development. In order to apply the results of experiments *in vitro* to the situation *in vivo*,

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however, one must know how closely cultured embryos resemble normal embryos. Although the cultured blastocyst looks like the uterine blastocyst, there is evidence that normal morphology does not guarantee normal developmental potential on transfer, either to ectopic sites (Billington, Graham & McLaren, 1968), or to the uterus of foster-mothers (Bowman & McLaren, 1970). In the uterus, developmental failure of cultured blastocysts occurred both before and after implantation, and foetal weight was reduced.

An attempt was therefore made to achieve a direct comparison of the metabolic activities of cultured and uterine embryos. Critical metabolic developments occur between the eight-cell and the blastocyst stages. With the onset of cavitation, the rate of protein synthesis accelerates (Greenwald & Everett, 1959; Weitlauf & Greenwald, 1967) and protein content rises (Brinster, 1967b). The pattern of enzyme activity alters (Brinster, 1965b, 1966a, b) to favour respiration by way of the Embden-Meyerhof pathway and the tricarboxylic acid cycle. Studies on inhibitors of carbohydrate metabolism (Thomson, 1967) and of protein and nucleic acid synthesis (Thomson & Biggers, 1966) have indicated that the embryo utilizes the same metabolic pathways *in vitro* as *in vivo*. The accelerated metabolic activity of the blastocyst *in vivo* is reflected in its oxygen consumption (Mills & Brinster, 1967) and carbon dioxide output (Brinster, 1967a). In the present study, CO₂ production was used to compare the metabolic activity of mouse blastocysts cultured from the eight-cell stage in a variety of media with that of blastocysts recovered from the uteri of female mice 3½ days *post coitum* (*p.c.*).

We have also compared the ability of trophoblast from cultured and uterine blastocysts to attach and show outgrowth *in vitro*. During cavitation, the embryo differentiates into two cell populations, the trophoblast and the inner cell mass, which are histochemically (Thomson & Brinster, 1966; Mulnard, 1965) and functionally distinct. *In vivo*, the trophoblast invades the uterine epithelium after loss of the zona. *In vitro*, the fate of the trophoblast depends on the components of the medium in which it is suspended and on the surface available to it. Blastocysts cultured from the two-cell stage show trophoblast outgrowth on the walls of a millipore diffusion chamber in the peritoneal cavity (Bryson, 1964), on glass in a complex medium containing serum (Cole & Paul, 1965), and on glass or plastic in Eagle's basal medium supplemented with foetal calf serum (Gwatkin, 1966a, b). Trophoblast outgrowth *in vitro* is considered to be analogous to trophoblast invasion *in vivo* (Bryson, 1964; Gwatkin, 1966a, b).

MATERIAL AND METHODS

Culture of eight-cell embryos

The oviducts of spontaneously ovulating, random-bred, Q mice were flushed 2½ days *p.c.* with either phosphate-buffered saline or Brinster's medium containing lactate and pyruvate (Brinster, 1965a) and 3 mg/ml albumin (Bowman & McLaren, 1970). The eight-cell embryos were transferred immediately to drops of medium under liquid paraffin (see Brinster, 1963) which had been equilibrated to pH 7.2. Table 1 describes the four media used in the study and

lists the code names by which they will be designated in the remainder of this paper. B-1 and B-2 were made up in the laboratory once every 2 months and stored in 5-ml Bijou bottles at -20°C . Stock B-2 was prepared without an energy source; 1 mg/ml glucose was added before culture. B-1 plus serum was made up for each experiment by adding 1% foetal calf serum (Flow Laboratories) to stock B-1 solution. The media were filter-sterilized before use. Sterile

TABLE 1

A DESCRIPTION OF THE MEDIA USED IN STUDYING THE CO_2 OUTPUT OF EMBRYOS CULTURED FROM THE EIGHT-CELL STAGE TO THE BLASTOCYST STAGE *in vitro*

Code name	Reference	Energy source	Albumin content	Serum content
B-1	Brinster (1965a) modified by Bowman & McLaren (1970)	Lactate and pyruvate	3 mg/ml	—
B-1 + serum	Brinster (1965a) modified by Bowman & McLaren (1970)	Lactate and pyruvate	3 mg/ml*	1% foetal calf serum
B-2	Brinster (1967a)	Glucose	1 mg/ml	—
Eagle's + serum	Eagle (1955) (Eagle's basal medium)	Glucose	—*	1% foetal calf serum

* The foetal calf serum in the medium presumably contained some albumin.

Eagle's basal medium was obtained from Flow Laboratories and stored at 4°C . L-Glutamine and foetal calf serum to 1% were added on the day of culture.

The cultures were placed in an incubator maintained at approximately 36°C and gassed with a continuous flow of 10% CO_2 in air. After 40 to 44 hr, the cultures were examined and the percentage of embryos in the blastocyst stage was noted.

Culture of blastocysts

Uterine blastocysts were flushed from the uteri of Q females $3\frac{1}{2}$ days *p.c.* with stock B-2. Cultured blastocysts were taken directly from the microdrops in which they had developed from the eight-cell stage, and pooled in stock B-2.

The embryos were distributed to drops of B-1, B-1 plus serum, and Eagle's medium plus serum, under liquid paraffin in sterile disposable Escoplastic dishes (Esco Rubber Ltd). The blastocyst cultures were incubated under the same conditions as were the eight-cell cultures. Pre-attachment blastocysts destined for CO_2 determinations were removed after 24 hr. The remaining embryos were examined daily for 4 days. On the 4th day, the percentage of blastocysts showing attachment and outgrowth was recorded and the extent of outgrowth noted. A blastocyst was considered to have attached when it could not be dislodged by vigorously shaking the culture dish. Outgrowth was assessed as described by Gwatkin (1966a, b).

Measurement of CO₂ production

Carbon dioxide output was measured by the liquid scintillation method of Brinster (1967a), in which the blastocyst metabolizes [¹⁴C]glucose to form ¹⁴CO₂, which is then trapped by hyamine hydroxide.

The embryos were washed three times in chemically-defined medium without energy source (Brinster, 1967a) and transferred in a minimal volume of fluid to 50 µl of medium containing [U-¹⁴C]glucose (1 mg/ml) under 50 µl of liquid paraffin in small glass or plastic test-tubes. The number of embryos in each determination was checked by observing the blastocysts in each tube under ×25 magnification. The number of blastocysts per tube ranged from four to fifteen. At least two tubes with no embryos were included in each set of determinations in order to assess background radioactivity.

The tubes were placed in low-background liquid-scintillation vials along with empty decapitated 2-ml ampoules. The vials were sealed with rubber stoppers, gassed with 5% CO₂ in air, and incubated at 36° C. After 4 hr, the embryos were killed and the CO₂ liberated from the medium by injection of 0.1 ml standard M/20-potassium hydrogen phthalate buffer (pH 4.0) into the tubes through the rubber stoppers. Hyamine hydroxide (1 ml) was similarly injected into the empty ampoules to absorb the ¹⁴CO₂. The stoppers were removed 24 hr later, the test-tubes discarded, and 10 ml of standard PPO-POPOP-toluene scintillation fluid mixed with the ¹⁴CO₂-labelled hyamine in the ampoules. The vials were stored overnight in the dark at 4° C, which reduced background radioactivity from chemiluminescence and thermal noise to less than 30 counts/min. They were then counted for 30 min in a Packard Tri-Carb Liquid Scintillation spectograph, Model 3320, at a channel voltage optimal for the level of radioactivity of the ¹⁴CO₂.

The data in counts/min were converted to picomols of CO₂/embryo/hr as follows:

$$P = \frac{kC}{E_m E_h n q t}$$

where: P = picomols CO₂/embryo/hr

k is the constant for converting picoCi to disintegrations/min and mols of glucose to mols of CO₂; $k = 3/(1.1 \times 10^3)$

C = sample counts/min – background counts/min

E_m = machine efficiency and quenching

E_h = hyamine uptake efficiency

n = number of embryos in the sample

q = specific activity of [¹⁴C]glucose

t = incubation time

Machine efficiency and quenching were measured simultaneously using a known amount of benzoic [¹⁴C]acid external standard (5500 disintegrations/min/mg, Packard) dissolved in 1 ml hyamine plus 10 ml scintillation fluid in the presence of a 2-ml glass ampoule. The two factors cut the sensitivity to 63.9%.

The efficiency with which hyamine trapped CO₂ was assessed with a [¹⁴C]-bicarbonate standard (specific activity 33 mCi/mmol, Amersham). A carefully measured amount of radioactive bicarbonate in stock B-2 was added directly

to hyamine. When glass tubes were used, the extraction efficiency was approximately 89%; when plastic tubes were used, 98%.

The specific activity of the [U- ^{14}C]glucose (Amersham) ranged from 2.9 to 3.1 mCi/mmol.

Since the number of eggs/vial varied from four to fifteen, the estimated value for CO_2 /egg/hr was weighted by the number of eggs in the vial before any statistical calculations were performed. All values for means, variances and other statistics are therefore weighted values. When differences between two values were to be combined, each difference was weighted by the factor $n_1 n_2 / (n_1 + n_2)$, where n_1 and n_2 were the numbers of eggs on which the two values were based. This procedure takes account of the fact that differences are more reliable when based on larger numbers of observations, and when the observations are more evenly distributed between the two groups.

To compare the CO_2 outputs of individual cultured and uterine blastocysts more accurately, pairs of embryos were incubated in 20 μl of high-radioactivity medium under 30 μl of liquid paraffin. Previous studies indicated that reducing the volume of oil and medium did not alter blastocyst CO_2 output. The high-radioactivity medium contained approximately 0.1 mg/ml [U- ^{14}C]glucose (specific activity 320 mCi/mmol, Amersham) plus 0.9 mg/ml carrier [^{12}C]glucose. Several estimates of background were included in the experiment. Counts of more than 24 above the background level were considered to have come from $^{14}\text{CO}_2$ of blastocyst origin. Since the exact concentration of radioactive glucose in the culture medium was not measured, the uterine blastocysts were compared with the cultured blastocysts in terms of counts/min rather than CO_2 /hr.

RESULTS

Carbon dioxide production of blastocysts developing from the eight-cell stage *in vitro*, in the B-1 medium, was compared on a number of occasions with that of control blastocysts recovered from the uteri of females 3½ days *p.c.* The results are given in Table 2.

The most striking feature of the data is the significantly higher level of variation between experiments in CO_2 output of cultured as compared with control blastocysts (for Exps. 1 to 10, $F = 10.0$, $P < 0.01$). On some occasions, the cultured blastocysts produced significantly less CO_2 than the controls; on others, there was no difference. In Experiment 12, which cannot be compared directly with the other experiments since a different source of radioactive glucose was used, the cultured and control groups were very similar, both in mean CO_2 output and in variance. Evidently, the conditions of culture varied and were not always able to support metabolic activity at the level *in vivo*.

In an attempt to increase CO_2 production of blastocysts cultured from the eight-cell stage, the medium was modified by adding 1% foetal calf serum (B-1+serum), or by substituting glucose for lactate and pyruvate as an energy source (B-2). As can be seen from Table 3, both modifications depressed CO_2 output, though not significantly.

The three media (B-1, B-1+serum, B-2) proved equally successful in supporting development of eight-cell embryos to the blastocyst stage (Table 4).

However, a significantly lower proportion of eight-cell embryos developed to the blastocyst stage in Eagle's basal medium supplemented with 1% foetal calf serum.

When blastocysts were recovered from the uterus and cultured in B-1, B-1 + serum or Eagle's medium + serum (Table 5), the majority became attached to

TABLE 2
THE EFFECT OF CULTURE ON CO₂ PRODUCTION BY MOUSE EMBRYOS MAINTAINED FOR 48 HR IN B-1 MEDIUM FROM THE EIGHT-CELL TO BLASTOCYST STAGE

Experiment	No. of vials		No. of blastocysts		Mean CO ₂ /egg/hr (picomols)			P*
	Uterine	Cultured	Uterine	Cultured	Uterine	Cultured	Difference	
1	1	2	15	30	7.55	3.91	+3.64	< 0.01
2	2	1	17	14	6.06	6.32	-0.26	> 0.2
3	1	1	8	5	8.07	11.52	-3.45	—
4	1	2	6	16	8.09	3.31	+4.79	< 0.10
5	2	1	10	12	7.48	7.57	-0.09	> 0.2
6	5	6	41	64	7.55	2.37	+5.18 ± 0.51	< 0.001
7	1	2	7	19	6.85	5.05	+1.80	> 0.2
8	1	1	6	8	7.08	3.04	+4.04	—
9	1	1	6	6	9.68	9.49	+0.19	—
10	2	1	10	6	6.06	5.05	+1.01	> 0.2
12	14	12	28	24	29.9†	28.2†	+1.7†	> 0.2

* Significance levels have been estimated where appropriate from the between- to within-treatment variance ratios.

† For Exp. 12, glucose of higher specific activity was used, and CO₂ output is expressed in terms of mean counts/min minus background counts/min (see Methods section).

TABLE 3
THE EFFECT OF ADDING FOETAL CALF SERUM TO THE CONTROL B-1 MEDIUM, OR REPLACING LACTATE AND PYRUVATE BY GLUCOSE (B-2), ON CO₂ PRODUCTION DURING CULTURE OF MOUSE EMBRYOS FOR 48 HR FROM THE EIGHT-CELL TO BLASTOCYST STAGE

Experiment	No. of vials			No. of blastocysts			Mean CO ₂ /egg/hr (picomols)		
	B-1	B-1 + serum	B-2	B-1	B-1 + serum	B-2	B-1	B-1 + serum	B-2
2	1	2	1	14	28	16	6.32	6.70	4.17
3	1	2	1	5	18	11	11.52	4.24	10.73
4	2	2	—	16	19	—	3.31	2.27	—
5	1	1	—	12	9	—	7.57	6.92	—
Combined	5	7	2	47	74	27	7.18 ± 1.28	5.03* ± 0.72	6.84* ± 3.22

* The depression of CO₂ output is not statistically significant for either variant of the medium.

the surface of the Petri dish within 48 hr. Outgrowth of the trophoblast, on the other hand, was almost entirely restricted to blastocysts cultured in the two media containing serum. Blastocysts derived by culturing eight-cell embryos in B-1 were also transferred for 24 hr to media containing serum. In Eagle's

medium+serum, which was the only medium extensively tested, the blastocysts grown in culture showed a significantly lower percentage, both of attachment and of trophoblast outgrowth, than did uterine blastocysts ($P < 0.01$ for each criterion).

We also examined the CO_2 output of blastocysts maintained for 24 hr either in B-1 alone, or in those media (B-1+serum, Eagle's medium+serum) which

TABLE 4
THE EFFECT OF CULTURE MEDIUM ON THE PERCENTAGE OF EIGHT-CELL MOUSE EMBRYOS DEVELOPING TO THE BLASTOCYST STAGE

	Culture medium			
	B-1 (control)	B-1 + serum	B-2	Eagle's + serum
No. of occasions	20	6	5	3
No. of eight-cell embryos cultured	639	125	66	26
% blastocysts	77.8	80.0	80.3	23.1*

* $P < 0.001$ for comparison with control.

TABLE 5
THE EFFECT OF CULTURE MEDIUM ON ATTACHMENT AND TROPHOBLAST OUTGROWTH OF MOUSE BLASTOCYSTS OBTAINED EITHER FROM THE UTERUS $3\frac{1}{2}$ DAYS P.C., OR FROM CULTURE OF EIGHT-CELL EMBRYOS IN B-1

Origin of blastocysts	Behaviour of blastocysts	Culture medium		
		B-1 (control)	B-1 + serum	Eagle's + serum
Uterus	% attaching	72.2 (18)†	88.5 (26)	83.2 (155)
	% outgrowing	5.6	84.6*	80.6*
Culture from eight-cell	% attaching	— (0)	100.0 (8)	56.2 (64)
	% outgrowing	—	50.0	40.6

* $P < 0.001$ for comparison with control.

† Figures in parentheses indicate number of blastocysts.

were known to support trophoblast outgrowth. Both types of blastocyst were tested: those recovered directly from the uterus $3\frac{1}{2}$ days p.c., and those derived from eight-cell embryos by culture for 48 hr in B-1. The results are given in Table 6.

When blastocysts were taken directly from the uterus, CO_2 output after culture for 24 hr was slightly but not significantly lower with serum in the medium than with B-1 alone. By contrast, blastocysts which had developed from the eight-cell stage in culture showed a significantly higher CO_2 output if transferred for a further 24 hr to medium containing serum than if they were

left in B-1. Eagle's medium + serum produced a greater effect than B-1 + serum, but not significantly so. The uterine and cultured blastocysts differed at the 1% level of significance in their response to serum.

TABLE 6

THE EFFECT OF 24-HR EXPOSURE TO SERUM AND CO₂ PRODUCTION BY MOUSE BLASTOCYSTS OBTAINED EITHER FROM THE UTERUS OR FROM CULTURE OF EIGHT-CELL EMBRYOS

Origin of blastocysts	Exp.	Mean CO ₂ /egg/hr (picomols) after a further 24-hr culture in:			Mean effect of serum on CO ₂ output	Difference
		B-1 (control)	B-1 + serum	Eagle's + serum		
Uterus	10	6.36 (8)*	6.15 (8)	4.41 (10)	-0.79 ±0.45	+2.56 ±0.67 (P < 0.01)
	11	8.61 (6)	8.00 (7)	8.50 (7)	(P > 0.2)	
Culture from eight-cell	10	4.51 (11)	5.33 (12)	6.38 (11)	+1.77 ±0.50	(P < 0.05)
	11	3.99 (15)	5.37 (13)	6.97 (15)	(P < 0.05)	

* Figures in parentheses denote the number of blastocysts tested; for the uterine blastocysts, one vial/treatment was used, and for the cultured blastocysts, two vials/treatment.

DISCUSSION

Carbon dioxide production as an indication of the effect of culture

When cultured blastocysts which were morphologically normal were compared with uterine blastocysts in respect of their capacity to give rise to viable foetuses after transfer to foster mothers, all batches of cultured blastocysts proved inferior to the control blastocysts (Bowman & McLaren, 1970). By contrast, our studies on CO₂ production, carried out over the same period of time with medium from the same pool as that used by Bowman & McLaren, detected some batches of cultured blastocysts which differed very little from uterine blastocysts, and others in which CO₂ production was well below the control level. The mean level of the metabolic activity of the cultured blastocysts was significantly lower than that of the uterine blastocysts.

Thus, although CO₂ output is a more sensitive tool than morphology for detecting the deleterious effect of culture, normal CO₂ production at the blastocyst stage does not guarantee that subsequent development will be normal.

Effect of culture medium on CO₂ output

Most of the embryos cultured from the eight-cell to the blastocyst stage in the present study were grown in a medium with a lactate-pyruvate energy source. By the eight-cell stage, the embryo is capable of metabolizing glucose (Whitten, 1956, 1957; Brinster & Thomson, 1966). A small series of CO₂ determinations with blastocysts cultured in a glucose medium indicated that

substituting a six- for a three-carbon energy source at this stage does not influence metabolic activity. The study also showed that those embryos grown in the absence of glucose were at no disadvantage in the culture system used for CO₂ determination, where the only available energy source was [¹⁴C]-glucose.

Of the three media most extensively used in studies of pre-implantation development (Mintz, 1964; Mulnard, 1965; Brinster, 1965a), two contain serum. In a preliminary experiment (unpublished), Bowman & McLaren observed a higher mortality and lower foetal weight among embryos cavitating *in vitro* in the presence of 1% foetal calf serum and transferred to foster mothers, than among those cavitating in the absence of serum. In the present experiment, foetal calf serum did not enhance the development of eight-cell embryos. Neither the proportion of embryos reaching the blastocyst stage, nor the CO₂ output of these blastocysts, was increased by including serum in the medium.

By contrast, blastocysts developed *in vitro* produced more CO₂ after further culture for 24 hr in medium containing serum than in medium not containing serum. Blastocysts developed *in vivo*, in which CO₂ output was already relatively high, showed no further increase on culture in serum-supplemented medium.

The inability of cultured embryos to be stimulated by foetal calf serum until after cavitation is reminiscent of the inability of 2½-day embryos to implant in a 3½-day uterus (McLaren & Michie, 1956; Noyes, Dickmann, Doyle & Gates, 1963). The change in blastocyst metabolism which alters the response to serum must occur during cavitation *in vitro*.

Carbon dioxide production, trophoblast outgrowth and implantation

To bring about implantation, the mouse blastocyst interacts with the uterus in two apparently independent ways (McLaren, 1968a): (1) it sets off the decidual cell reaction by releasing its metabolic products in the vicinity of the uterine epithelium; (2) its trophoblast cells invade the uterine epithelium. The first signs of the decidual cell reaction and trophoblast invasion occur within a few hours of each other, both in normal implantation (Finn & McLaren, 1967) and in delayed implantation (McLaren, 1968b).

The present study suggests that a certain threshold level of metabolic activity (of which CO₂ production may be regarded as one index) is necessary, both for the induction of the decidual cell response and for trophoblast invasion. In a normal pregnancy, this threshold level of activation has probably been reached by the time cavitation is completed. At the blastocyst stage, exothermic metabolic pathways are opened, resulting in the output of markedly increased amounts of the by-products of metabolism, among them CO₂ (Brinster, 1967a). A preliminary experiment (Menke & McLaren, unpublished) has shown that in the 10 hr before the onset of implantation, indicated by localized Pontamine Blue uptake, there is no further sharp rise in blastocyst metabolic activity. If the hormonal state of the uterus makes it receptive, the concentration of metabolic by-products in the vicinity of the embryo may be sufficient to induce the decidual cell reaction.

The outgrowth of trophoblast from blastocysts *in vitro*, which may be

regarded as analogous to trophoblast invasion *in utero* (Bryson, 1964), occurs only under those conditions which we have found to favour metabolic activation, i.e. in medium supplemented with serum. Gwatkin (1966a) obtained outgrowth in Eagle's medium plus serum; we have found B-1 plus serum to be equally effective. B-1 alone neither stimulated CO₂ output, nor supported outgrowth. Evidently some component in foetal calf serum induces trophoblast outgrowth and permits an increase in metabolic activity of cultured blastocysts to a level approaching that of uterine blastocysts. However, uterine blastocysts retained a high rate of CO₂ production in B-1 yet showed no outgrowth, so metabolic activation is evidently not a sufficient condition for trophoblast outgrowth, though it may be a necessary one.

Our results differ from those of Gwatkin in that our blastocysts showed attachment (though not outgrowth) in B-1 alone. This may have been due to the higher concentration of albumin in our B-1, or to the fact that the blastocysts cavitated *in vivo* rather than *in vitro*. The lower percentage of attachment among our cultured blastocysts and the failure of some embryos to progress beyond the attachment phase under conditions optimal for outgrowth (Table 5) parallel the results of egg transfer studies (Bowman & McLaren, 1970), in which cultured embryos suffered increased mortality both before and immediately after implantation.

Blastocysts in implantation delay, whether during lactation or after ovariectomy, resemble cultured blastocysts in that their CO₂ output is generally lower than that of 3½-day uterine blastocysts in normal pregnancy (Menke & McLaren, 1970). Blastocysts in delay lie within 1 µ of the uterine epithelium without initiating the decidual cell response or invading the endometrium (McLaren, 1968b; Martin, Finn & Carter, 1970). Gwatkin (1966b) observed that blastocysts prevented from implanting by ovariectomy early in pregnancy showed trophoblast outgrowth in culture only in medium supplemented by serum, i.e. where metabolic activation is likely to occur, and took longer to outgrow than did blastocysts cultured from the two-cell stage.

Metabolic activation of the blastocyst must be brought about by some factor(s) external to the embryo, since it does not occur *in vitro* in the absence of serum. The stimulus for activation *in vivo* presumably comes from the oestrogen-sensitized uterus. On the basis of his studies on blastocyst attachment and outgrowth, Gwatkin (1966b) suggested that implantation might be controlled by the free amino acid content of the uterine fluid.

ACKNOWLEDGMENTS

We wish to thank Dr John Bishop and his staff for technical advice and for the use of the liquid scintillation counter. One of us (T.M.) was supported by a grant from the U.S.-U.K. Educational Commission. The work was financed in part by the Ford Foundation.

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