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Article *in* Journal of Embryology and Experimental Morphology · May 1972

DOI: 10.1242/dev.27.2.431 · Source: PubMed

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J. Embryol. exp. Morph. Vol. 27, 2, pp. 431–445, 1972
Printed in Great Britain

DNA synthesis in the preimplantation mouse embryo

By P. BARLOW,[1] D. A. J. OWEN,[2] AND CHRIS GRAHAM[3]

From the Paediatric Research Unit, Guy's Hospital Medical School, London, the Institute of Animal Genetics, Edinburgh and the Sir William Dunn School of Pathology, University of Oxford

SUMMARY

Strain PO preimplantation embryos were labelled with [^{3}H]thymidine. The incorporation of the label was studied by autoradiography of air-dried and serially sectioned embryos. DNA amounts were measured with a microdensitometer.

The following observations were made at the 5- to 16-cell stages. All nuclei contained 2*C*–4*C* amounts of DNA and all could eventually synthesize DNA. However, after short labelling intervals, unlabelled nuclei were found with 2*C* and 4*C* amounts of DNA. We concluded that both the G_1 and the G_2 phases of the cell cycle were present at this time. Embryos were found in which the S phase of the 4th and the 5th cell cycles post fertilization overlapped. In 12- to 15-cell embryos which contained inside cells it was found that the inside cells were produced by one of the first four 8-cell-stage blastomeres to divide.

The inside cells of 9- to 256-cell embryos had a significantly higher labelling index than the outside cells and the number of inside cells increased faster than the number of outside cells during development. We concluded that the inside cells were dividing faster than the outside cells.

Blastocysts which had developed *in vivo* or *in vitro* contained nuclei with greater than 4*C* amounts of DNA. We concluded that the development of excess DNA amounts does not depend on the maternal environment. These nuclei which contained greater than 4*C* amounts of DNA were labelled after short exposures to radioactivity. We concluded that it was likely that they were becoming polyploid.

INTRODUCTION

The development of cell populations with distinct cell cycles is usually the earliest sign of functional differentiation within an embryo. We have studied the cell cycle of preimplantation mouse embryos to find out:

(*a*) When the G_1 and the G_2 phase first appear (terminology of Howard & Pelc, 1953).

[1] *Author's address:* Unit of Developmental Botany, University of Cambridge, 181 A Huntingdon Road, Cambridge, CB3 0DY, U.K.

[2] *Author's address:* Institute of Animal Genetics, West Mains Road, Edinburgh, EH9 3JN, U.K.

[3] *Author's address:* Department of Zoology, South Parks Road, Oxford, OX1 3PS, U.K. *Reprint requests to C. F. Graham.*

(*b*) When the cell cycles of different regions of the embryo first become distinct.

(*c*) When the cells of the embryo first contain nuclear DNA in excess of the diploid G_2 amount.

Previous work on this subject has been recently reviewed (Graham, 1971*a*).

MATERIALS AND METHODS

Supply of embryos

The mice were from a randomly breeding closed colony of Swiss albino animals (Strain PO). Six- to ten-week-old virgin females were superovulated (Runner & Palm, 1953). Eight i.u. each of PMSG (pregnant mare serum gonadotrophin) and HCG (human chorionic gonadotrophin) (Gestyl and Pregnyl, Organon Laboratories, U.K.) were injected intraperitoneally 48 h apart. After the injection of HCG each female was placed with one male. Embryos were taken only from females which had mated (indicated by the presence of a copulation plug on the first day of pregnancy), and the time after HCG injection was recorded in all this work. Embryos which appeared degenerate were discarded.

Culture and labelling

Embryos were cultured in Whitten's medium (1971). The medium was placed on 0·05ml. drops beneath liquid paraffin (Boots Drug Co., U.K.; method of Brinster, 1963) and up to ten embryos were cultured in each drop. The embryos were labelled in medium containing either 0·25 μCi/ml (continuous labelling) or 2 μCi/ml (short labelling) of [6-^{3}H]thymidine (specific activity 26 Ci/mM; The Radiochemical Centre, Amersham, U.K.). In the short labelling experiments the embryos were placed in the label within 5 min of the death of the mother. In other experiments the embryos were stored in medium at 37 °C for up to 30 min before labelling.

Cell counting, fixation and autoradiography

Air-dried preparations of the embryos were made by Tarkowski's technique (1966), and embryos for sectioning were fixed in Heidenhain's fixative (Tarkowski & Wroblewska, 1967). The sections were cut at 5 μm. All slides for autoradiography were placed in 5 % trichloracetic acid for 30 min at 5 °C. Air-dried preparations were hydrolysed in 5 N-HCl for 30 min at room temperature (technique of Deitch, Wagner & Richart, 1968), and stained with Feulgen. Sections were stained with Mayer's haemalum and Light Green.

A nucleus was scored as labelled if the number of grains over it exceeded by two or more the number over an adjacent area of equal size in the cytoplasm. DNase treatment removed most of the nuclear label (technique of Darlington & La Cour, 1962).

Cell counts, microdensitometry and autoradiography were performed on the air-dried material. Cell counts, serial reconstructions and autoradiography were performed on sectioned material.

Table 1. *Comparison of cell counts on air-dried and serially sectioned embryos*

Time post HCG dissected from mother (h)	Time in culture (h)	Air-dried counts			Serial section counts			t
		No. of embryos	Mean	S.D.	No. of embryos	Mean	S.D.	
74·50	—	19	12·95	3·205	13	15·31	3·545	1·96
74·50	4·50	7	15·00	1·732	9	14·66	2·179	0·33
74·50	10·00	6	22·83	8·447	18	21·38	8·548	0·36

The number of cells counted by the two methods is not significantly different at the 5 % level (Bailey, 1959, equation 21).

Microdensitometry

Photometric measurements were made over the nuclei with a Barr and Stroud integrating microdensitometer (Richards, Walker & Deeley, 1956). All measurements were made at 565 nm. Liver cells were placed on each slide and stained with the embryonic cells. The nuclei of the liver cells provide an internal standard of the absorbance shown by nuclei containing the 2*C*, 4*C*, and 8*C* amounts of DNA. The air-drying technique involves loosening the cells of the embryo with citrate. To find out if DNA leaked from the nucleus during this process, we measured the absorbance in nuclei from air-dried embryos without prior citrate treatment and in nuclei from liver cells which had been pretreated with citrate. In neither case could we detect changes in the absorbance shown by the nuclei.

RESULTS

Increase in cell number

To establish that our methods of cell counting were accurate, we compared counts obtained from air-dried preparations and from serial sections. Air-dried preparations might be subject to the loss of cells during fixation; counts from serial sections might be overestimated because it is not difficult to count the same cell twice. The results of the comparison between counts made by the two methods are shown in Table 1. The air-dried counts and serial section counts do not differ significantly.

Cells were counted at various times post HCG injection in embryos which had been fixed within 30 min of the death of their mother (Table 2, Fig. 1; see Materials and Methods).

The most important points to notice are:

(*a*) Up to the 4-cell stage, the embryos from many different females divide roughly in time with each other.

(*b*) From the 4-cell stage onwards, the sample variance increases. At a

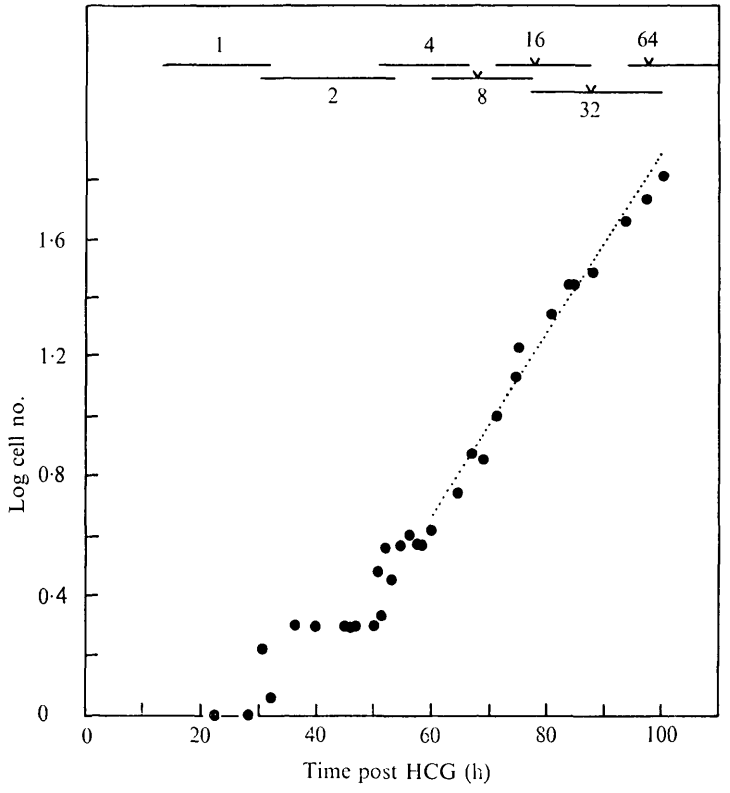

Fig. 1. Log mean cell number of cells within the embryo against time post HCG injection. Data taken from Table 2. The first and the last appearance of embryos with a particular cell number is indicated at the top of the figure by a continuous horizontal line. A small V superimposed on this line marks the time at which the mean cell number equals this particular cell number.

particular time post HCG, the embryos may be at the 8-, 16- or 32-cell stage; this variance is recorded in Table 2, and it is indicated by the overlap of lines representing the first and the last appearance of a particular cell stage in Fig. 1.

(*c*) From the 4-cell stage (at 60 h post HCG) to the 64-cell stage (at 100 h post HCG) there is an almost exponential increase in cell number. The line of best fit to the cell numbers during this time has a correlation coefficient of 0·9856, and it is described by $x = 32{\cdot}23y + 39$, where x is the time post HCG in hours and y is the log mean cell number. The average doubling time between the 8- and 64-cell stage is approximately 10 h.

(*d*) Fig. 1 does not represent a completely accurate description of the doubling time within the embryo. As we will show, there is considerable heterogeneity of cell cycles within the embryo. Also inaccuracies are introduced because the number of degenerating embryos increases towards the blastocyst stage.

Table 2. *Increase in cell numbers with time post HCG*

Time post HCG (h)	Sample	Mean	Sample variance	S.D.
22·00	21	1·00	—	—
28·00	20	1·00	—	—
30·75	34	1·68	0·22	0·47
32·00	36	1·14	0·12	0·35
36·33	32	2·00	—	—
39·55	43	2·00	—	—
44·75	40	2·00	—	—
46·00	20	2·00	—	—
47·00	49	2·00	—	—
50·15	39	2·00	—	—
50·50	68	3·06	0·99	1·00
51·10	35	2·14	0·18	0·43
52·00	28	3·64	0·52	0·73
53·30	124	2·87	0·83	0·92
54·25	41	3·83	0·29	0·54
56·00	45	4·00	—	—
57·75	44	3·89	0·19	0·44
58·50	35	3·83	0·26	0·51
60·00	107	4·17	1·05	1·03
64·50	22	5·59	2·06	1·47
67·00	38	7·65	1·59	1·27
69·00	27	7·20	0·76	0·89
71·15	22	11·45	4·43	2·15
74·50	19	13·68	7·90	2·88
77·00	28	17·29	53·40	7·44
80·50	34	22·29	54·56	7·50
83·40	23	29·65	20·40	4·62
84·60	13	30·84	11·66	3·50
88·00	39	31·50	51·17	7·24
93·75	20	46·60	169·03	13·33
97·25	20	54·80	82·70	9·33
100·00	20	65·80	296·00	17·65

Long labelling experiments

To show that all the cells inside the embryos were capable of synthesizing DNA, we cultured them in tritiated thymidine for increasing times and recorded the increase in the percentage of labelled interphase nuclei and of division figures (Fig. 2).

The results from many experiments are combined, and we group together the data about the embryos with the same cell number at the end of the experiment; these results therefore refer to the cell cycle prior to the counted stage.

We refrain from estimating the length of the G_2 phases from plots of the increase of labelled division figures with time; the results are likely to be inaccurate because of the effects of *in vitro* culture (see Discussion).

Notice that after long labelling times all the cells become labelled. This means

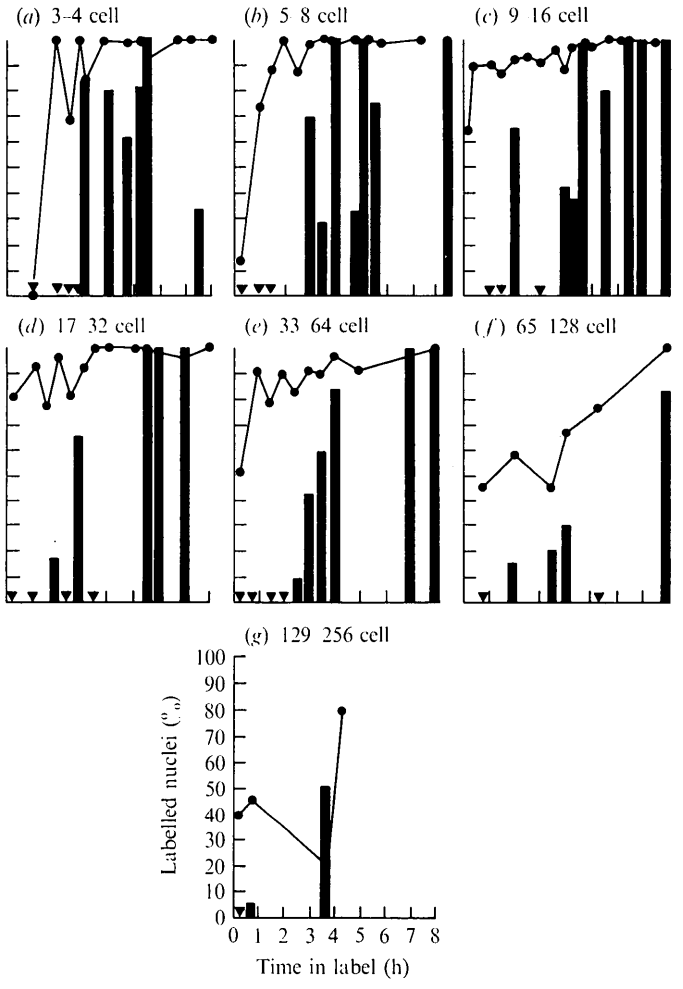

Fig. 2. Increase in labelled interphase nuclei and labelled mitoses, after continuous labelling up to the cell stage indicated on the figure. ●—●, Increase in percentage labelled interphase nuclei; solid bars indicate percentage of labelled mitoses; inverted solid triangles indicate those times at which none of the mitoses were labelled. The information from a number of different experiments has been combined, and each graph in this figure contains data about embryos which were all at a particular cell stage at the end of the labelling period. All axes labels the same as for (*g*).

that they are all capable of synthesizing DNA, and the absence of label in nuclei which have been exposed to radioactivity for a short time cannot be due to their inability to replicate.

Short labelling experiments

We wished to find out if the unlabelled interphase nuclei observed in the long labelling experiments were in the G_1 or the G_2 phases of the cell cycle. The embryos were exposed to tritiated thymidine for 15 min. Subsequently, the

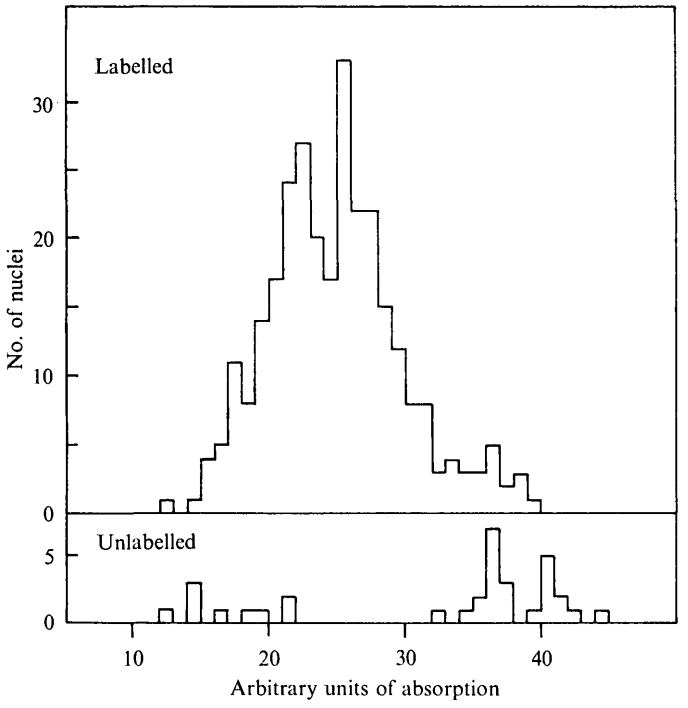

Fig. 3. Microdensitometry and autoradiography of twenty-nine 5- to 16-cell stage embryos. Observations were made on 324 nuclei from five 5- to 8-cell embryos and twenty-four 9- to 16-cell embryos. Microdensitometry was performed on 85 % of the nuclei of the embryos. All the nuclei from the 5- to 8-cell embryos had DNA values greater than the mean in this sample. These embryos were labelled for 15 min after dissection from the mother at 72·00 and 70·50 h post HCG. However, in another sample of 138 nuclei from embryos at 69·00 h post HCG, the unlabelled nuclei of 5- to 8-cell embryos were present at both ends of the range of microdensitometry readings.

nuclear DNA content was measured with a microdensitometer and autoradiographs were prepared.

The results from embryos at the 5- to 16-cell stages are illustrated in Fig. 3. Unlabelled nuclei have bimodal distribution and this observation suggests that both the G_1 and the G_2 phases are present at these stages of development (see Discussion). There appear to be about three times as many G_2 nuclei compared to G_1 nuclei.

In many cases we found evidence that a single embryo could contain cells in the S phase of both the 4th and the 5th cell cycle post fertilization; in other words cells could overlap each other around the cell cycle. Fig. 4 illustrates such an embryo. This shows that cell cycles within an embryo can be of varying lengths.

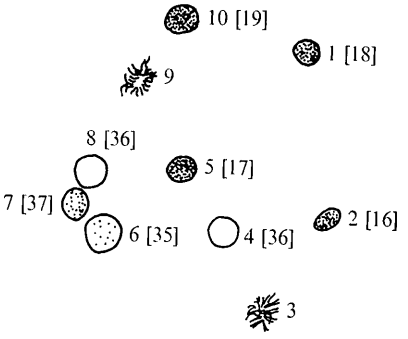

Fig. 4. Evidence for lapping of cell cycles. Drawing of a 10-cell embryo which was labelled for 15 min at 70·50 h post HCG. The grain density over the nuclei is indicated by stippling and the photometric measurements (in square brackets) are on the right of the number of a cell. This embryo is part of the sample of measurements in Fig. 3 and this should be consulted for the G_1 and the G_2 DNA values. Four nuclei are in early S phase (1, 2, 5, 10) and two nuclei are in late S phase (6, 7). Two nuclei are in G_2 (4, 8) and two are in mitoses (3, 9). This embryo therefore contains four nuclei (1, 2, 5, 10) in the 5th cell cycle and six nuclei in the 4th cell cycle after fertilization. The S phases of the 4th and 5th cell cycle overlap.

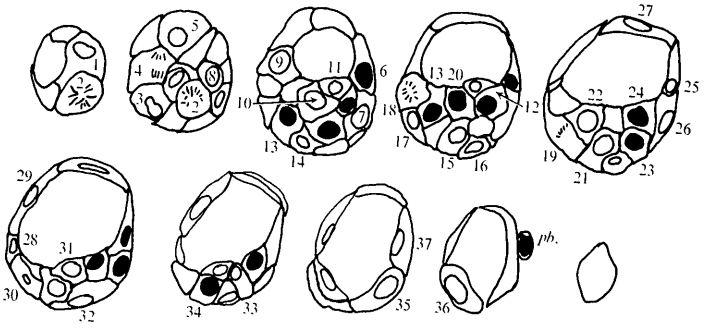

Fig. 5. Example of a serial reconstruction of an embryo. This embryo contains 37 cells and a polar body. It was labelled for 15 min at 84·50 h post HCG. The solid black nuclei are labelled and the open nuclei are unlabelled. The number of a cell is usually written on or beside a cell at the first appearance of its nucleus, looking left to right. In this embryo, the inside cells are 8, 10, 11, 12, 13, 20, 22, 24 and 31. A nucleus may appear unlabelled in one section and labelled in another because the radioactivity in a visible nucleus may not emit far enough to reduce the silver grains in the overlying emulsion. The method of fixation will inevitably influence the exact number of cells which are scored as 'inside'.

Serial sections of continuously labelled embryos

We know from the short labelling experiments that cell cycles within the embryo were of different lengths. To find out if the cell cycles were different in various regions we cultured the embryos in tritiated thymidine for increasing periods, cut serial sections, and prepared autoradiographs. The embryos were reconstructed and we scored the labelling index for cells with membranes in contact with the outside of the embryo (outside cells) and for cells with no part of their membrane in contact with the exterior of the embryo (inside cells). In

Table 3. *Labelling index of inside and outside cells*

Cell stages	Time label (h)	No. of embryos	Total no. of inside cells	Total no. of outside cells	Inside cells labelled (%)	Outside cells labelled (%)	Significantly different (5 % level)
9–16	0·25	3	6	42	100·00	61·40	—
	0·45	15	23	183	92·00	72·67	*
	1·00	8	5	115	100·00	98·26	—
	1·15	8	13	87	84·62	75·86	—
	1·90	5	5	67	100·00	94·03	—
	3·00	7	9	99	88·88	91·91	—
	4·15	4	1	59	100·00	100·00	—
	5·00	8	5	109	100·00	95·41	—
	6·00	7	7	108	100·00	100·00	—
	7·00	10	10	129	90·00	98·44	—
17–32	0·25	14	88	337	84·09	66·46	*
	0·45	5	16	83	87·50	67·49	—
	1·00	1	4	17	75·00	47·06	—
	1·15	6	40	141	95·00	85·81	—
	6·00	3	9	44	100·00	100·00	—
33–128	0·75	6	183	503	77·59	50·09	*
	2·00	6	179	432	70·39	49·76	*
	3·00	7	149	498	74·49	52·61	*
	4·00	5	165	365	81·21	60·54	*
	5·00	9	163	578	88·34	66·26	*

Significance tested for difference in two observed proportions (Bailey, 1959, equation 18).

Table 4. *Ratio of total number of inside cells to total number of outside cells between particular cell stages*

Cell stages	Ratio inside/ outside cells
9–16	0·1070
17–32	0·2534
33–64	0·2768
65–128	0·3390
129–256	0·4330

embryos containing more than 64 cells it was found that the circumference of the blastocyst farthest from the inner cell mass sometimes consisted of several cell layers. These were all scored as outside cells, even though some might have been contaminants from the uterine epithelium.

After short labelling periods (15–25 min) the labelling index of the inside cells is significantly higher than that of the outside cells in all the stages studied (5 % level of significance, Table 3). This difference is blurred after longer labelling periods as the embryos become 100 % labelled. However, the significance of

Table 5. *The number of inside cells at different stages*

Range of cell stages	No. of embryos	Range of no. of inside cells	Mean no. of inside cells
8	4	0–2	0·50
9–16	58	0–3	0·98
17–32	20	2–10	5·50
33–64	7	9–13	10·86
65–128	27	12–37	24·04
129–256	3	44–59	51·00

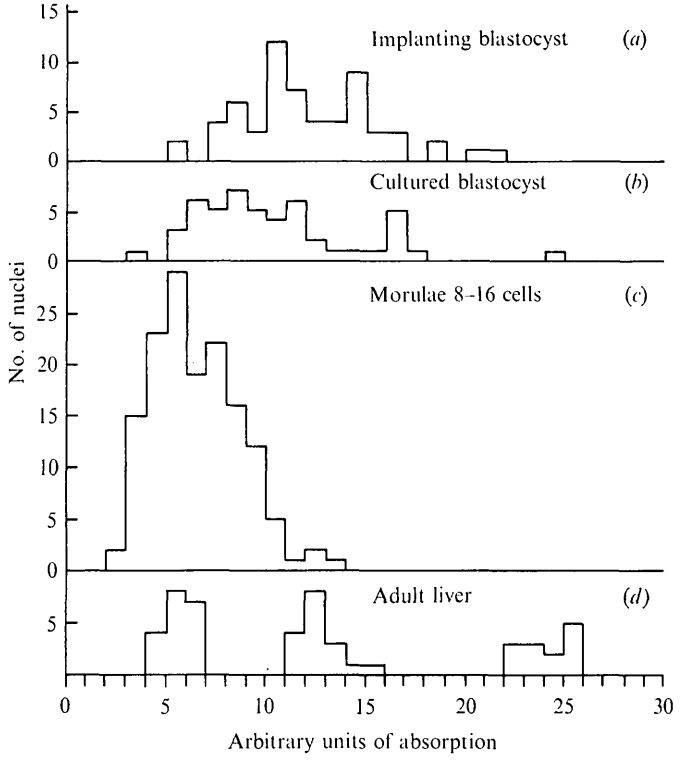

Fig. 6. Microdensitometry of morulae and blastocysts. (*a*) Forty-eight nuclei from three embryos dissected at 126 h post HCG, fixed within 30 min of the death of the mother; (*b*) 50 nuclei from three embryos, dissected at 48 h post HCG and cultured in Whitten's (1971) medium for 72 h; (*c*) 147 nuclei from eleven 8–16 cell embryos at 70 h post HCG; fixed within 30 min of the death of the mother; (*d*) 49 nuclei of a liver smear (absorbance controls). The liver nuclei act as an internal standard of the absorbance shown by 2*C*, 4*C* and 8*C* amounts of Feulgen-stained DNA. Both implanting blastocysts and blastocysts cultured *in vitro* contain nuclei with greater than 4*C* DNA amounts.

the difference does persist in the more slowly dividing blastocysts. The ratio between the number of inside cells and outside cells between various cell stages is shown in Tables 4 and 5.

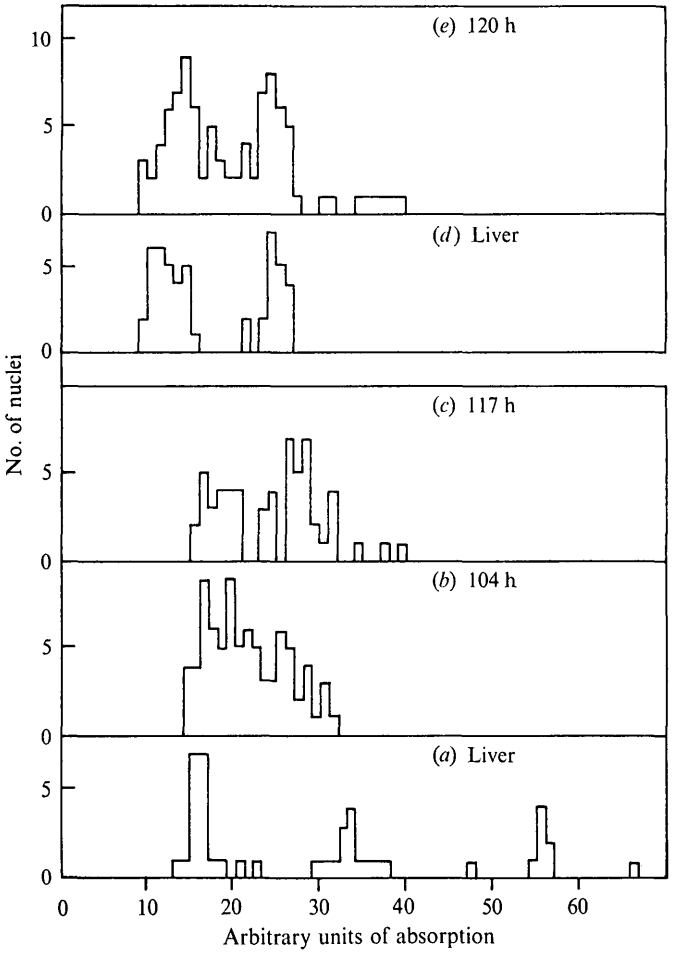

Fig. 7. Microdensitometry of blastocysts. (*a*) Thirty-three nuclei of a liver smear (absorbance controls for (*b*) and (*c*)); (*b*) 81 nuclei from 2 blastocysts at 104 h post HCG (fixed within 30 min of the death of the mother); (*c*) 55 nuclei from 4 blastocysts at 117 h post HCG (these were labelled for 30 min before fixation; the nuclei with greater than 4*C* amounts of DNA were labelled); (*d*) 49 nuclei from a liver smear (absorbance control for (*e*)); (*e*) 89 nuclei from 4 blastocysts at 120 h post HCG (fixed within 30 min of the death of the mother).

Microdensitometry of developing embryos

We know that trophoblast cells may contain greater than 4*C* amounts of DNA inside the nucleus on the 5th day of pregnancy (Graham, 1971*b*). The labelling index will not be related to cell multiplication if many cells are replicating DNA but not dividing. To show that cells were not becoming polyploid, we studied the amounts of DNA in embryos dissected from the uterus and in embryos developing *in vitro* from the two-cell stage.

Fig 6 shows that implanting blastocysts and blastocysts developing in culture from the 2-cell stage may have greater than 4*C* amounts of DNA.

However, at the 8- to 16-cell stages the measured DNA values are consistent with diploid DNA values (2*C*–4*C* amounts).

Fig. 7 shows the time post HCG at which greater than 4*C* values are first observed. This appears to occur in the late blastocyst; thus the S phase appears to be a discrete interval in the normal cell cycle during the stages of development we studied in detail.

DISCUSSION

The G_1 and the G_2 phases of the cell cycle

We show that there are nuclei which do not incorporate radioactivity during 15 min labelling periods; these nuclei also contain 2*C* and 4*C* amounts of DNA (Fig. 3). It is unlikely that these nuclei are unlabelled because the radioactive precursor failed to penetrate the cells; serial reconstructions demonstrate that after an identical time in label, the inside cells incorporate radioactivity into the nucleus as readily as the outside cells at the 8- to 16-cell stages (Table 3). These cells could not be unlabelled because they were dead; all the cells in the embryos we studied were capable of synthesizing DNA (Fig. 2). We conclude that the G_1 and the G_2 phases of the cell cycle are present in some cells of mouse embryos between the 5- to 16-cell stages.

We performed similar studies on the 1- to 4-cell stages. In this case we found that unlabelled nuclei did not always contain the 2*C* and the 4*C* amounts of DNA; it is likely that radioactive precursors penetrate the embryo slowly at these stages of development (Graham, 1971*a*). We are therefore unable to draw any conclusions about these phases in the first two cell cycles after fertilization.

Using similar labelling conditions (20 min), Gamow & Prescott (1970) proposed that the G_1 phase was absent during cleavage. It is likely that they did not study a sufficient number of embryos to detect the brief appearance of this phase. Our results support Samoshkina's deductions (1968) that the G_1 phase is present during early cleavage.

We have not estimated the length of the G_2 phase from plots of the increase of labelled division figures with time. *In vitro* culture slows cell division in mouse embryos (Bowman & McLaren, 1970; Graham, 1971*b*) and these calculations would only provide gross overestimates of the length of the G_2 phase. However, the data in Fig. 2 demonstrate that this phase is present in all the stages studied.

Appearance of distinct cell populations

Origin of inside cells

We show that an average 16-cell embryo contains one inside cell (Table 5). To discover the origin of this cell we studied serial sections of 19 embryos which contained at least one inside cell and which had cell numbers in the range 11–15 cells. In the embryos, the cells which had divided since the 8-cell stage could be distinguished from those that had not; cell volume is approximately halved during the first five cleavage divisions of mouse development. In

those embryos the inside cell was always the product of a division after the 8-cell stage (a small cell). The outside cells contained cells still at the eight-cell stage, as well as the division products of these cells.

We can therefore conclude that the inside cell (at the 11- to 16-cell stages) originates from one of the first four blastomeres to divide away from the 8-cell stage. Of course we do not know if this cell remains on the inside nor do we know that it is a progenitor of all the other inside cells. Since at least some 11- to 16-cell stage embryos contain no inside cells, it must be assumed that some embryos recruit cells to the inside after this time.

Behaviour of inside cells

The inside cell of an average 16-cell embryo has a significantly higher labelling index than the outside cells (Table 3). The inside cells continue to have a significantly higher labelling index than the outside cells throughout development into the blastocyst.

The labelling index will not reflect the rate of cell multiplication if many cells are synthesizing DNA but not dividing. It is unlikely that this occurs; there is no accumulation of nuclei containing 4*C* amounts of DNA (compare Figs. 3, 6, 7*b*). It is only in the late blastocyst that we detected nuclear DNA values in excess of the 4*C* amount (Fig. 7*c*, *e*).

The labelling index will not serve as a guide to the rate of cell multiplication in inside and outside cells unless we make two assumptions. We assume (*a*) that the proportion of the cell cycle occupied by the S phase is the same in these two regions, (*b*) that the S phase occurs in the same relative position in the two cell cycles. If these two assumptions are correct, then the high labelling index of the inside cells suggests that they are proceeding through the cell cycle faster (multiplying faster) than the outside cells. The inside cells become 100 % labelled before the outside cells, which confirms this view. Assuming that the inside cells are a closed population after the 32-cell stage, then they increase faster than the outside cells (Table 4).

The observation that the outside cells have a lower labelling index than the inside cells contradicts the conclusions of Samoshkina (1968) and Zavarzin, Samoshkina & Dondua (1966). Samoshkina distinguished the inside from the outside cells on the basis of their nuclear morphology in air-dried preparations and this is likely to be subject to error. Zavarzin studied the labelling of blastocysts *in vivo* using sectioned material. Errors in this case are likely to be due to the low-grain counts and the failure to reconstruct the embryos from the sections.

Nuclear DNA in excess of the diploid G_2 amount (4C)

Nuclear DNA in excess of 4*C* was first detected in the blastocyst of 117 h post HCG (Fig. 7). These excess values were also found in blastocysts which had developed from the two-cell stage *in vitro*.

The three nuclei with DNA values greater than 4*C* at 117 h post HCG were all

labelled. We therefore think that these large quantities of DNA are the consequence of DNA synthesis and not of cell and nuclear fusion. High DNA values could be due either to polyploidization or to replication of part of the genome (discussed in detail by Barlow & Sherman, 1972). Whichever is the case, it is reasonable to suppose that these nuclei with greater than 4*C* DNA amounts will form part of the primary trophoblast which contains cells with up to 500*C* DNA amounts (Barlow & Sherman, 1972).

In the late blastocyst we show that the histogram of DNA values becomes bimodal for the first time in development (Fig. 7). This observation suggests that the G_1 and the G_2 phases are occupying a greater proportion of the cell cycle in this time compared to earlier stages of cleavage.

CONCLUSION

Our conclusions may only apply to PO strain embryos.

(1) The G_1 and G_2 phases of the cell cycle are present in some cells of 5- to 16-cell embryos.

(2) Nuclei contain 2*C*–4*C* amounts of DNA and can all synthesize DNA during the 5- to 16-cell stages.

(3) During the 5- to 16-cell stages the S phase of the 4th and the 5th cell cycle may overlap.

(4) The inside cell of an average 16-cell embryo originates from one of the first four blastomeres to divide from the 8-cell stage.

(5) The inside cells of 9- to 256-cell stages have a significantly higher labelling index than the outside cells. They also appear to divide faster.

(6) Blastocysts which develop in culture or *in vivo* contain cells with greater than 4*C* amounts of DNA. The development of this character is therefore not dependent on the maternal environment.

(7) The cells with greater than 4*C* amounts of DNA are labelled after short periods. It is therefore likely that they are becoming polyploid.

The experiments were performed while we were all guests of the Sir William Dunn School of Pathology. We wish to thank Professor H. Harris and the Department for their hospitality. The Medical Research Council kindly provided funds for the research, and Miss Z. A. White provided excellent technical assistance.

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(*Manuscript received 13 August 1971*)