The effect of spatial arrangement on cell determination during mouse development

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SUMMARY

The effect of cell position on cell determination was studied in mouse embryos. Embryos and parts of embryos were combined during early preimplantation development. The differentiation of cells in these composites was followed either by prelabelling some cells with tritiated thymidine or by combining cells which synthesized different electrophoretic variants of glucose phosphate isomerase.

It was found that each blastomere of a 4-cell embryo could form both the trophoblast and the inner cell mass of the blastocyst. However, when blastomeres of a 4-cell embryo were placed on the outside of other 4-cell embryos then (a) they tended to form the outside layer of the blastocyst, (b) they tended to develop into the trophoblast and the yolk sac on the 10th day of pregnancy, (c) they tended not to form the coat colour of the foetus. Four- to eight-cell embryos which were completely surrounded by other blastomeres had lost the capacity to form vesicles at the blastocyst stage.

We could find no evidence for the segregation of morphogenetic factors at the 4- and 8-cell stages of mouse development and concluded that at these stages cell position could determine the development of blastomeres.

INTRODUCTION

The cells of the 4-cell stage mouse embryo develop into the trophoblast, the extra embryonic membranes, and the foetus. Three hypotheses have been advanced to explain how this differentiation occurs.

In the first hypothesis, differentiation is thought to be the consequence of pre-existing heterogeneity in the fertilized egg. It is proposed that fertilized egg cytoplasm contains morphogenetic factors which segregate as the cytoplasm is cleaved by division (Dalcq, 1957).

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The second hypothesis suggests that there are predetermined reference points in the embryo (e.g. sources of gradients), and that differentiation occurs because daughter cells differ in their relationship with these points (e.g. Child, 1941; Wolpert, 1969). The heterogeneity may exist in the fertilized egg.

According to the third hypothesis, the differentiation depends solely on the position which the cells occupy, and it is not dependent on pre-existing heterogeneity (Tarkowski & Wroblewska, 1967). It is proposed that the cells on the outside of the morula give rise to the trophoblast and the cells on the inside of the morula give rise to the inner cell mass (ICM) of the blastocyst, as a consequence of their position. This proposal was foreshadowed by the suggestion of Mintz (1965) that the micro-environment may influence the fate of cells.

If the third hypothesis is correct, it is nevertheless possible that within an intact embryo the fate of the blastomeres is fixed. It is known that cleavage is a regular process; the outside cytoplasm of 4-cell stage blastomeres is usually included in the trophoblast and the inside cytoplasm of these cells regularly contributes to the ICM of the blastocyst (Wilson, Bolton & Cuttler, 1972). It is therefore likely that certain parts of the intact 1-cell egg may regularly be exposed to the condition ‘inside’ and other parts to the condition ‘outside’ as development proceeds to the morula. Since this regularity might be disturbed by manipulation it is difficult to prove experimentally that the cells are determined by their position within the intact embryo. This aspect of the ‘inside–outside’ theory has therefore been referred to as ‘cryptic preformation’ (Graham, 1971a).

It is possible to test the ‘inside–outside’ hypothesis by constructing composite embryos from isolated blastomeres or groups of morulae. If cell position is an important determinant of cell fate, then it should be possible to channel all the cells of the embryo to form a particular tissue by changing their position in the composite embryos. If, on the other hand, the original heterogeneity of the fertilized egg is responsible for cellular differentiation then the fate of the donor cells should not be greatly altered by changing their position.

In the present investigation, autoradiographic, genetic, biochemical and cell function studies provide no experimental evidence to support the first two hypotheses. They do provide evidence that the fate of all the cells of an embryo can be similarly determined by altering their position at the 8-cell stage.

MATERIALS AND METHODS

1. Supply of embryos

Female mice were either from a randomly breeding closed colony of Swiss albino animals (strain PO) or they were from inbred strains. The inbred mice mated normally while PO females were superovulated (Runner & Palm, 1953). Eight i.u. of PMSG (pregnant mare serum gonadotrophin) and HCG (human chorionic gonadotrophin) (Gestyl and Pregnyl, Organon Laboratories, U.K.) were injected intraperitoneally 48 h apart. Mating was detected by the presence
2. Culture of embryos

Embryos were cultured in various media contained in 0.05 ml drops under liquid paraffin (Boots Pure Drug Co., U.K.) in siliconized glass dishes (siliconized with Repelcote, BDH, U.K., using some precautions; Graham, 1971b). The gas phase varied with the medium in use.

3. Labelling of embryos

Embryos were labelled in Whitten's medium (1971) for approximately one S phase (terminology of Howard & Pelc, 1953). The concentration of [6-³H]-thymidine (specific activity 26 Ci/m-mole; Radiochemical Centre, Amersham, U.K.) was either 0.5 or 0.25 μCi/ml. Two- and four-cell embryos were dissected from the oviducts of superovulated females between 47-53 h and 58-62 h after HCG injection respectively. They were labelled for approximately 12 h. It is known that a 10 h labelling period in tritiated thymidine is sufficient to label all nuclei during these time intervals (Barlow, Owen & Graham, 1972).

After labelling, the embryos were rinsed three times in Mintz's medium, which contains 50% foetal calf serum (Mintz, 1964). They were cultured for several hours in this medium to dilute out unincorporated radioactive thymidine and then cultured in Whitten's medium up to the cell stages listed in the Results. Subsequently the zona pellucida was removed with Pronase (Sigma, technique of Mintz, 1967), and the embryos were then reincubated in Mintz's medium. These labelled embryos were next placed in Whitten's medium and dissociated by sucking with a flame polished micropipette.

In experiments with genetic markers, naturally mated inbred mice were used and 4- and 8-cell embryos were obtained in the early morning of the third day of pregnancy.

4. Reassociation of embryos

The labelled embryos were combined with unlabelled embryos in various ways (Fig. 1). Their position was checked every 2 h for the first 10 h after they had been put in place. The cells usually adhered rapidly.

For several experiments it was necessary to totally enclose intact labelled 8-cell embryos with unlabelled embryos. To accomplish this, the labelled embryo was first surrounded in one plane by 5 or 6 unlabelled embryos (Fig. 3c, d, e). The central labelled embryo was next capped by floating a raft of four adhering unlabelled embryos on to it. When the embryos of this composite had firmly adhered, the composite was inverted and the exposed surface of the labelled embryo covered with another raft of four adhering unlabelled embryos.

All the composite embryos were cultured for 48 h to the blastocyst stage. They were either fixed or transferred to the uterus of a foster mother on the evening...
of the third or the morning of the fourth day of pseudopregnancy (McLaren & Michie, 1956).

5. **Sectioning and autoradiography**

Embryos were fixed in Heidenhain's fixative (Tarkowski & Wroblewska, 1967) and embedded in melted 2% agar. As the agar solidified, small air bubbles were blown beneath the surface and were filled with phosphate buffered saline (PBS, solution A of Dulbecco & Vogt, 1954). The embryos were pipetted into these and the agar containing the embryos was subsequently treated as a large specimen for embedding in paraffin.

Serial sections were cut at 3–4 μm. Slides were dipped in Ilford K 5 emulsion and exposed for 1–2 weeks. The embryos were stained with either Mayer's haemalum or with Ehrlich's haematoxylin.

In the autoradiographs 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. We think that we have detected the majority of the labelled division products of the originally labelled cells. The total number of labelled cells found in the blastocysts which develop from composites containing all the blastomeres of one originally labelled embryo is similar to the cell number of intact unlabelled PO blastocysts grown in culture from the 2-cell stage (Graham, 1971b). It therefore appears that blastomere dissociation and labelling does not reduce cell viability.

In this study, we define the trophoblast of the blastocyst as that group of cells with membranes in contact with the outside of the embryo (outside cells); the ICM is defined as that group of cells with no visible contact between their membranes and the exterior of the embryo (inside cells) (Fig. 2A, B, C).

6. **Identification of glucose phosphate isomerase (GPI-1)**

Composite embryos were removed from the recipient uterus on the 10th and 11th days of pregnancy. They were dissected into three fractions. These consisted of the trophoblast layer dissected from the uterine wall and including Reichert's membrane (trophoblast), the yolk sac with the exclusion of that part closely bound to the definitive placenta (yolk sac), and the foetus with amnion and allantois excluded (foetus). Each fraction was placed in PBS (phosphate-buffered saline) in a small test-tube and sedimented at 2000 rev/min for 5 min (Sherman, 1972). The PBS was discarded and approximately 50 μl of distilled water was added to the pellet. The tissues were then frozen at -20 °C, thawed, and shaken until the suspension was viscous. Unbroken cells and debris were pelleted by centrifugation at 2000 rev/min for 5 min. The supernatant was drawn off and saved and 50 μl of water was again added to the pellet. The freeze-thaw step was repeated, and the supernatants from the two centrifugations were pooled and used as a source of enzyme. The supernatant was absorbed on to cellulose acetate paper (Shandon Celagram, U.K.) and the paper inserted into a horizontal
**Cell arrangement in mouse embryos**

Fig. 1. Cell arrangements. (a) One labelled blastomere of a 4-cell embryo with an unlabelled 4-cell embryo. (b) One labelled blastomere of a 4-cell embryo between the dissociated blastomeres of another 4-cell unlabelled embryo. (c) One labelled blastomere of an 8-cell embryo with one unlabelled 6-cell embryo. (d) One labelled blastomere of a 4-cell embryo between the two halves of either an unlabelled 4-cell embryo or an unlabelled 2-cell embryo. (e) A pair of labelled blastomeres of an 8-cell embryo on the outside of an 8- to 16-cell unlabelled embryo whose embryonic surface has begun to flatten; the cells are not distinct in such a morula.

gel. The isoenzymes were separated by the technique of Chapman, Whitten & Ruddle (1971) and Chapman, Ansell & McLaren (1972) and the position of the isoenzymes was detected by the staining method of Carter & Parr (1967). The gels were stained for up to 60 min to detect small amounts of the enzyme.

The use of isoenzymes to detect the presence of cells in a particular tissue must be carefully controlled. It is important to be able to detect small quantities of one form of the isoenzyme in the presence of large quantities of the other. Using this assay system, a rare form with only 1% of the total GPI-1 activity can be detected (Chapman et al. 1972). However, it remains difficult to detect small quantities of the enzyme formed by cells of the homozygous genotype in the presence of excess enzyme formed by cells of an heterozygous genotype.

It is essential to show that the isoenzymes detected in the trophoblast fraction are not the consequence of contamination from maternal decidual cells. In some cases we can exclude this possibility; embryos transferred to the same mother have completely different isoenzymes in the trophoblast (Table 3).

**RESULTS**

A. **Position of cells in the blastocyst after cell arrangements made during cleavage**

1. *Outside experiments*

Labelled blastomeres from 4- and 8-cell embryos were disaggregated and placed on the outside of 4- to 16-cell embryos (Fig. 1). These composites developed into blastocysts and the position and the number of the labelled cells was recorded (Table 1, arrangements a, c and e).
Table 1. Contribution of labelled blastomeres to the inside and the outside of the blastocyst

<table>
<thead>
<tr>
<th>Cell arrangement from Fig. 1</th>
<th>Labelled donor cell stage</th>
<th>No. combined and position</th>
<th>Unlabelled recipient cell stage</th>
<th>No. of combinations</th>
<th>Percentage labelled cells in</th>
<th>Average from one blastomere</th>
<th>Range from one blastomere</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>4</td>
<td>1 outside</td>
<td>4</td>
<td>11</td>
<td>3 inside 97 Outside 8-3</td>
<td>8-3</td>
<td>4-13</td>
</tr>
<tr>
<td>c</td>
<td>8</td>
<td>1 outside</td>
<td>4-8</td>
<td>20</td>
<td>8 inside 92 Outside 4-1</td>
<td>4-1</td>
<td>2-8</td>
</tr>
<tr>
<td>e</td>
<td>8</td>
<td>2 outside</td>
<td>8-16</td>
<td>40</td>
<td>8 inside 92 Outside 4-2</td>
<td>4-2</td>
<td>2-7</td>
</tr>
<tr>
<td>b or d</td>
<td>4</td>
<td>1 inside</td>
<td>4</td>
<td>8</td>
<td>40 inside 60 Outside 7-25</td>
<td>7-25</td>
<td>2-17</td>
</tr>
<tr>
<td>d</td>
<td>4</td>
<td>1 between</td>
<td>2</td>
<td>4</td>
<td>4 inside 96 Outside 6-5</td>
<td>6-5</td>
<td>2-14</td>
</tr>
</tbody>
</table>

More than 90% of the labelled cells were found in the outside layer of the blastocysts. In no case were the labelled cells found only in the ICM. The development of all the blastomeres from 12 labelled donor embryos was recorded. All the labelled cells derived from three of these donor embryos were found only in the outside layer of the blastocyst.

2. Inside experiments

Labelled blastomeres from 4-cell embryos were placed between the blastomeres of unlabelled 2- and 4-cell embryos (Fig. 1). The position and the number of the cells were recorded in the blastocysts which developed (Table 1, arrangements b and d).

Only 60% of the labelled cells were found in the outside layer of the blastocysts when labelled blastomeres were placed between the blastomeres of unlabelled 4-cell embryos. However, when the labelled blastomeres were placed between the two halves of an unlabelled 2-cell embryo, then the majority of the labelled cells were found in the outside layer of the blastocysts. The development of all the blastomeres of three labelled donor embryos was recorded. In one case, each of the labelled blastomeres from one donor embryo contributed cells to both the I.C.M. and the outside layer of the blastocysts (Fig. 2).

Experiments were designed to force all the cells of an embryo to form the I.C.M. of the blastocyst. Initially attempts to perform this manipulation were unsuccessful (Fig. 3a, b). It was possible to arrange single blastomeres from 4-cell embryos in such a way that half of them contributed only to the ICM of the blastocysts (Table 2, cell arrangements a and b). However, this did not provide a method for testing the developmental capacity of all the blastomeres from a single embryo.

In subsequent experiments the whole of a labelled embryo was covered with unlabelled embryos (Table 2, cell arrangements c, d and e). When a single embryo
was covered with 14 other embryos on all its surfaces (Fig. 3e), then labelled cells were in some cases found only in the ICM of the large blastocysts which developed. It might be supposed that these giant blastocysts were highly abnormal. However, when six of these composite blastocysts were transferred to pseudopregnant recipients then four developed into apparently normal day-13
Fig. 3. Cell arrangements designed to place labelled cells consistently in the ICM of the blastocyst. (a) A single labelled blastomere of a 4-cell embryo between three unlabelled 4-cell embryos. (b) A single labelled blastomere of a 4-cell embryo between three unlabelled 8- to 16-cell stages whose embryonic surface has begun to flatten. (c) A labelled 8- to 16-cell embryo surrounded by six unlabelled 8- to 16-cell embryos. (d) As in (c), with the addition of a cap of four adhering 8- to 16-cell unlabelled embryos. (e) As in (d), but in this case another cap of four adhering 8- to 16-cell unlabelled embryos was placed on the surface of the composite which had previously lain on the glass substrate.

embryos (Fig. 4, 5). None of these large embryos came to term and they appeared to be resorbed by the 16th day of pregnancy.

In the normal blastocyst, outside cells are able to pump and form vesicles, while inside cells are unable to do so (Gardner, 1971; Gardner & Johnson, 1972). The ICM was dissected from the large blastocysts formed by enclosing a single labelled embryo with 14 unlabelled embryos. The ICM was dissected from seven of these composites and it was found that as in the normal blastocyst the ICM was unable to form vesicles. In four of these cases all the cells derived from the labelled embryo were found only in the ICM.
Table 2. Placing labelled cells and embryos in an inside position

<table>
<thead>
<tr>
<th>Cell arrangement from Fig. 3</th>
<th>Number of experiments</th>
<th>Labelled cells found in:</th>
<th>Labelled cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ICM</td>
<td>Trophoblast</td>
</tr>
<tr>
<td>(a)</td>
<td>9</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>(b)</td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>(c)</td>
<td>22</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>(d)</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(e)</td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

This table records those cases in which labelled cells were found only in the ICM, in the trophoblast, or in both.

Fig. 4. Autoradiograph of a giant composite. The blastocyst contains a group of labelled cells in the ICM. Despite the high grain density, labelled cells can still divide at this time and a labelled cell in anaphase is in the outside layer (arrow).

B. Position of cells in embryos on the 10th day of pregnancy after cell arrangement made during cleavage

1. Outside quartet experiments

It was known that blastomeres placed on the outside of morulae contributed mainly to the outside part of the blastocyst (Table 1, cell arrangement e). It was decided to find out if blastomeres placed on the outside of morulae contributed mostly to the trophoblast and yolk sac on the 10th day of pregnancy.

The contribution of the original blastomeres to these embryonic tissues could be studied by using isoenzyme markers. The strains C57BL and PO differ at the
glucose phosphate isomerase locus (GPI-1). C57BL is homozygous for GPI-1 B and PO is polymorphic for GPI-1 (V. Chapman, personal communication). In a sample of the PO stock there were 14 GPI-1 A, 12 GPI-1 AB, and 1 GPI-1 B. The low frequency of the Gpi-1 b locus prevented any confusion with the C57BL contribution.

Four cell PO embryos were disaggregated and each blastomere was allowed to divide once. The blastomere pairs were placed on the outside of four 8- to 16-cell C57BL embryos. These composites developed into blastocysts and each quartet of blastocysts was transferred to one uterus of a pseudopregnant foster mother. Four quartets of blastocysts were made in this way.

The results of this experiment are in Table 3 (Exp. A). Twelve embryos developed to the 10th day of pregnancy from the 16 blastocysts which had been transferred to the uterus. In each of these the originally outside blastomeres formed part of the yolk sac or part of the trophoblast or part of both. In four cases the outside blastomeres also formed part of the embryo. It therefore appeared that an outside position at the morula stage predisposed a cell to form part of the trophoblast or yolk sac on the 10th day of pregnancy.
Table 3. Distribution of isoenzymes in the foetus, yolk sac, and trophoblast on the 10th day of pregnancy after arrangements made at the morula stage

**Quartet experiments**

A. Pairs of blastomeres from 8-cell PO (A or AB) placed outside C57BL (B) embryos

<table>
<thead>
<tr>
<th>Donor embryo</th>
<th>Embryo number</th>
<th>Embryo</th>
<th>Yolk sac</th>
<th>Trophoblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>B</td>
<td>A + B</td>
<td>A + B</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>B</td>
<td>A + B</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>A + B</td>
<td>A + B</td>
<td>A + B</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>B</td>
<td>B</td>
<td>A + B</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>B</td>
<td>A + B</td>
<td>A + B</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>A + B</td>
<td>A + B</td>
<td>A + B</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>B</td>
<td>B</td>
<td>A + B</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>B</td>
<td>A + B</td>
<td>A + B</td>
</tr>
</tbody>
</table>

**Whole-embryo experiments**

B. All the cells of an 8-cell C57BL (B) embryo outside a PO (A or AB) embryo

<table>
<thead>
<tr>
<th>Donor embryo</th>
<th>Embryo number</th>
<th>Embryo</th>
<th>Yolk sac</th>
<th>Trophoblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>13</td>
<td>AB</td>
<td>AB</td>
<td>AB + B</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>AB + B</td>
<td>AB + B</td>
<td>AB + B</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>AB + B</td>
<td>AB + B</td>
<td>AB + B</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>AB + B</td>
<td>AB + B</td>
<td>AB + B</td>
</tr>
</tbody>
</table>

C. All the cells of an 8-cell PO (A or AB) embryo outside a C57BL (B) embryo

<table>
<thead>
<tr>
<th>Donor embryo</th>
<th>Embryo number</th>
<th>Embryo</th>
<th>Yolk sac</th>
<th>Trophoblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>17</td>
<td>B</td>
<td>B</td>
<td>AB + B</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>B</td>
<td>AB + B</td>
<td>AB + B</td>
</tr>
<tr>
<td>11</td>
<td>19</td>
<td>AB + B</td>
<td>AB</td>
<td>AB</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>13</td>
<td>21</td>
<td>AB</td>
<td>AB</td>
<td>AB</td>
</tr>
<tr>
<td>14</td>
<td>22</td>
<td>A</td>
<td>A + B</td>
<td>A + B</td>
</tr>
</tbody>
</table>

2. *Outside/whole-embryo experiments*

In the previous experiment the failure of some of the blastocysts to develop following transfer made it impossible to study the fate of all the cells of an embryo placed in an outside position. Four-cell C57BL embryos were disaggregated and each blastomere was allowed to divide once. All the blastomeres were then placed around one PO 8- to 16-cell embryo (Fig. 6). These composites developed into blastocysts and were transferred to pseudopregnant recipients.
Fig. 6. The whole of one embryo outside another. A 4-cell embryo was disaggregated and each blastomere was allowed to divide once. All these blastomeres were then arranged on the outside of another 8- to 16-cell embryo whose embryonic surface had begun to flatten.

Fig. 7. The isoenzymes of an embryo dissected from the uterus on the 10th day of pregnancy. This embryo was formed by placing the whole of an 8-cell PO embryo outside a C57BL embryo. Samples were as follows: (1) a marker, (2) embryo 17, (3) yolk sac 17, (4) trophoblast 17. Embryo number 17 contains only the PO contribution (AB) in the trophoblast.

The results of this experiment are in Table 3 (Exp. B). In one case the blastomeres of the whole outside embryo formed only part of the trophoblast (embryo 13). In the other three cases the foetus, yolk sac, and trophoblast were chimeric.

This experiment was repeated with a disaggregated PO embryo outside a C57BL embryo (Table 3, Exp. C). Six composites were analysed on the 10th day of pregnancy. It was found that the outside blastomeres might form only the
Cell arrangement in mouse embryos

trophoblast (embryo 17) (Fig. 7), or the yolk sac and the trophoblast (embryo 18). In one case the inside embryo formed only part of the foetus (embryo 19). In two other cases the only isoenzyme found was that of the outside cells which appeared to have overgrown the inside embryo by the 10th day of pregnancy (embryos 20 and 21). In the last case the result was exactly the reverse of that expected; the inside embryo contributed cells to the yolk sac and trophoblast only (embryo 22).

Except for this latter case, it appeared that all the cells of an 8-cell embryo if placed on the outside of another 8- to 16-cell embryo were predisposed to form yolk sac and trophoblast. However, in half the composites the outside cells also formed part of the foetus.

C. Contribution of cells to the new born mouse after cell arrangements made during cleavage

It was known that the blastomeres of one embryo when placed outside another tended not to contribute to the foetus on the 10th day of pregnancy. It was decided to find out if these outside cells also tended not to contribute to the coat colour of the new born mouse.

$F_1$ CBA/PO 4-cell embryos were disaggregated. Each blastomere was allowed to divide once and all the cells of this embryo were arranged on the outside of an 8- to 16-cell PO embryo. These composites developed into blastocysts and were transferred to psuedopregnant recipients. Four mice were born and of these two were white (PO) and two were brown and white ($F_1$ CBA/PO + PO).

The experiment was repeated except that in this case $F_1$ CBA/PO embryos were surrounded by disaggregated PO embryos. Six of these composites came to term and all of these were brown ($F_1$ CBA/PO). It appeared that all the cells of an embryo when placed outside another during cleavage were predisposed not to contribute to the coat colour of the mouse which was born.

DISCUSSION

Several theories have been advanced to explain cell determination in mouse and rat embryos (see Introduction).

1. Segregation of morphogenetic factors – Theory 1

It is not known how the cytoplasm of the 1-cell egg is apportioned to the inside and outside cells of the blastocyst or to the precursor cells of the trophoblast, the yolk sac and the foetus. It is therefore difficult to predict the stage of development at which cytoplasmic determinants might be segregated into separate cells.

The present experiments tend to exclude an important effect of morphogenetic factors which have segregated by the 4-cell stage (Dalcol, 1957). Each cell of the 4-cell stage can form part of the ICM and of the outside layer of the blastocyst.
Each cell of the 4-cell embryo can form either trophoblast or yolk sac on the 10th day of pregnancy (donor embryo 4, Table 3).

It is also unlikely that morphogenetic determinants have segregated at the 8-cell stage. Twenty labelled blastomeres from 8-cell stage embryos were combined with unlabelled embryos and in no case were labelled cells found only in the ICM of the blastocysts which developed.

2. **Reference points in the embryo – Theory 2**

These experiments do not exclude the possibility that there are reference points in the embryo. If such reference points do exist then the manipulated cells behave as if one of these reference points is at the centre of the embryo.

3. **Cell position – Theory 3**

These experiments demonstrate the following effects of cell position on cell determination. If blastomeres are placed on the outside of other embryos during early preimplantation development then they develop in the following ways.

(a) They continue to divide at a similar rate to cells in an intact embryo. On average each dissociated 4-cell blastomere gives rise to eight labelled cells in the composite blastocysts and on average each dissociated 8-cell blastomere gives rise to four labelled cells in the composite blastocysts (Table 1).

The number of times that a blastomere may divide in the composite is very variable (Table 1, Range). This variation may be caused either by damage during blastomere dissociation or by radiation effects. However, cell cycle lengths are known to vary in the intact preimplantation embryo, and part of the variation which is observed may be the consequence of processes which operate during normal development (Barlow et al. 1972).

(b) They contribute more daughter cells to the outside layer of the blastocyst than to the ICM In Table 1 it can be seen that in all the outside experiments more than 90% of the progeny of these blastomeres were found in the outside layer of the blastocyst. In a normal blastocyst approximately 75% of the cells are in the outside layer (Barlow et al. 1972), and so this distribution shows that the contribution of these cells to the outside layer has been increased. In three cases all the cells derived from a 4-cell embryo contributed only to the outside layer of the blastocyst. This probably shows that all the cells were forced to develop outside layer properties.

(c) They tend to form part of the trophoblast and part of the yolk sac rather than the foetus on the 10th day of pregnancy (Table 3). This tendency is clearly demonstrated by the fact that in ten of the composites the outside blastomeres only formed parts of the trophoblast and yolk sac and in no case did they only form part of the foetus (Table 3).

However, the outside blastomeres may contribute to the foetus on the 10th day of pregnancy. This may be due either to cell mixing during the formation of the composites or to overgrowth of one embryo by another.
(d) They tend not to form part of the coat colour of the foetus. This point was demonstrated by a reciprocal experiment; in one experimental series the blastomeres of $F_1$ CBA/PO embryos were placed outside PO embryos and in the second experimental series the positions were reversed. The result that the coat colour of the newborn tended to be that of the inside cells was therefore not the consequence of any inherent difference in growth rate or viability of the embryos of the two strains.

The development of blastomeres placed outside other embryos may be contrasted with that of blastomeres surrounded by the cells of other embryos. If blastomeres are surrounded by other cells during early preimplantation development then they develop in the following ways.

(a) They contribute a large number of daughter cells to the ICM of the blastocyst. In Table 1 it can be seen that 40% of the progeny of these blastomeres are found in the ICM. It is also rare for originally surrounded blastomeres to form only the outside part of the blastocyst.

(b) They are unable to form vesicles after being surrounded for 2 days by 14 other embryos. This involves the loss of an inherent capacity because each blastomere of an 8-cell embryo can develop vesicles (Tarkowski & Wroblewska, 1968). It would be difficult to argue that they had lost the capacity to pump because they were dead; it is known that some of these giant composites can form apparently normal embryos on the 13th day of pregnancy (Fig. 5).

(c) They may only form part of the foetus. In one case it appeared that all the cells of an inside embryo had been prevented from developing either into part of the trophoblast or into part of the yolk sac (Table 3, embryo 19).

**CONCLUSION**

This evidence supports the view of Tarkowski & Wroblewska that cell position effects are a sufficient explanation of the early differentiation of the mouse egg. If it is the case that cleavage is a regular process (Wilson et al. 1972), then it must be supposed that certain parts of the 1-cell egg are regularly exposed to the condition 'inside' and other parts to the condition 'outside' as development proceeds. It is now necessary to discover the essential features of these conditions.

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