Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage

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Organization of a mammalian egg at the beginning of development has been the subject of several experimental as well as descriptive cytological and cytochemical investigations.

The regulative capacities of blastomeres isolated at the 2-cell stage have been studied in the rat (Nicholas & Hall, 1942), rabbit (Seidel, 1952, 1960) and mouse (Tarkowski, 1959a, b; Mulnard, 1965a, b). Investigations by Seidel and Tarkowski have shown that although the majority of 1/2 blastomeres can regulate and develop into smaller but otherwise normal blastocysts, some of them give rise to purely trophoblastic vesicles devoid of the inner cell mass. In the mouse, Tarkowski (1959a, b) observed in addition in a few cases multicellular morulae which had not undergone cavitation. The findings of Tarkowski have been recently confirmed by Mulnard (1965a, b) who studied cytochemically mouse 1/2 blastomeres developing in vitro. At least some of the blastocysts developed from 1/2 blastomeres are capable of further normal development—many embryos have been collected and several young were born in both the rabbit (Seidel, 1952, 1960) and the mouse (Tarkowski, 1959a, b). The young proved to be quite normal and fertile.

In the rabbit, single blastomeres of the 4-cell stage develop into blastocysts or trophoblastic vesicles (Seidel, 1956, 1960). However, the incidence of the latter forms is much higher among 1/4 blastomeres than among those originating from the 2-cell stage. The most advanced embryo which developed from a transplanted 1/4 blastomere attained the stage of five somites and was quite normal (Seidel, 1956, 1960). Similar investigations on the mouse have been performed on a very limited scale (Tarkowski, 1959a, b) and allowed only the conclusion that a 1/4 blastomere is still able to develop into a blastocyst composed of a trophoblast and the inner mass.

The conclusions drawn by Seidel and Tarkowski on the basis of their experimental investigations concerning the organization of the mammalian egg were slightly different. Seidel explained his results by assuming the existence in the undivided egg of an organizing centre whose presence, in whole or at least in

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part, in the isolated blastomere is essential for the formation of a typical blastocyst. In a given blastomere lack of the cytoplasm from the organizing centre would lead, according to Seidel, to the development of a trophoblastic vesicle. The fact that trophoblastic vesicles attain higher incidence among forms developed from 1/4 blastomeres than among those developed from 1/2 blastomeres agrees well with this hypothesis.

Tarkowski tried to correlate his findings with the results of cytochemical investigations carried out by Dalcq, Mulnard and their collaborators on rodent eggs (for review see Dalcq (1957), Mulnard (1961); also discussion in this paper) and concluded that the regulative capacities of blastomeres of the 2-cell stage are to a certain degree limited by the character of the inherited cytoplasm. The fact that the normal proportions between the trophoblast and the inner mass are not always reproduced in the resulting forms would seem to confirm the existence in the undivided egg of cytoplasmic territories of different and already determined fate.

However, certain difficulties have been encountered in interpreting the results in terms of Dalcq and Mulnard’s conceptions and an auxiliary hypothesis had to be proposed (Tarkowski, 1959b; see also discussion). In order to clarify the matter and verify the conclusions presented by Tarkowski we have decided to extend the investigations to later stages, i.e. to the 4-cell stage which, so far, has not been extensively studied, and to the 8-cell stage which has not been studied in this respect at all.

The present experiments have been devised in such a way as to allow data to be collected on the developmental capacities of all blastomeres constituting the egg. With the exception of the investigations of Nicholas & Hall (1942) and some of the experiments carried out by Mulnard (1965b) who separated the first two blastomeres from each other, in all other investigations the development of 1/2 and 1/4 blastomeres was studied after the other(s) were destroyed with a needle inside the zona pellucida. Even if one assumes that the material obtained in this way is a random sample, the conclusions concerning the organization of the egg and the mechanisms responsible for the first cellular differentiation in development (inner mass cells versus trophoblastic cells) are inevitably indirect, and can not be as clear-cut as when the development of all blastomeres of the egg can be followed. However, in order to study the development of all the blastomeres of an egg, culture in vitro is indispensable. Such an experiment requires a removal of the zone pellucida and a handling of naked blastomeres. Recovery of naked 1/2 mouse blastomeres transferred to the oviduct is, for unknown reasons, practically null (A. K. Tarkowski, unpublished results). This finding, together with the even smaller size of 1/4 and 1/8 blastomeres, renders transplantation of the latter not worth trying. Consequently, the procedure which we had to adopt consisted in separating the 4- to 8-cell eggs into individual blastomeres and their subsequent culturing in vitro.
MATERIALS AND METHODS

The eggs used in these experiments were obtained from spontaneously ovulating females belonging to inbred strains A, CBA-p, CBA-T6T6 and an outbred albino strain. In one case only ovulation was induced with gonadotropins, the eggs collected being just after the second cleavage.

Autopsy was performed during the third day (first day taken as the day when vaginal plug is found). Depending on the time of day and the strain used the stage of the recovered eggs varied from the late 4-cell stage to the late 8-cell stage, i.e. just preceding the fourth cleavage.

The eggs were washed out from the oviducts with Ringer solution containing 0.1 % of bovine plasma albumin, fraction V (Armour). At least one egg was left in Ringer solution (control egg I) and the others placed in 0.5 % pronase (Calbiochem) in Ringer solution in order to remove the zona pellucida (Mintz, 1962). When it was noticed that the zonae became very thin the eggs were put back into Ringer solution and the zonae removed by gently sucking the eggs into a mouth-controlled micropipette. This last step was introduced in order to prevent the blastomeres from being submitted to direct action of the enzyme. One naked egg (sometimes two or three) was selected as control egg II and left in Ringer solution. The remaining eggs were placed in 0.02 % Versene in calcium and magnesium-free Dulbecco solution. The time required for separation of blastomeres varied from 2 to 30 min and presumably depended mainly on the phase of the intermitotic period of the treated eggs. Blastomeres which have just divided are joined together by a broad cytoplasmic bridge and the separation enforced upon them may lead to their disruption. On the contrary, blastomeres which are just before the next division fall apart very easily. Subtle pipetting of the eggs was usually carried out in order to hasten the separation. Isolated blastomeres were washed in Ringer solution and placed in culture medium.

Culture of blastomeres was carried out in drops of medium under liquid paraffin (Tarkowski, 1961, 1963, 1965—the latter paper describes slight modifications introduced recently). The medium employed was that devised by Brinster (1963). Drops of a few millimetres in diameter were placed on the bottom of a siliconized ('Repelcote', Hopkin and Williams) glass Petri dishes or small plastic dishes. The culture dishes were usually prepared several hours in advance and placed in a desiccating chamber filled with alveolar air. A container with physiological saline was always kept in the desiccating chamber.

Each of the two control eggs (see above) and each of the isolated blastomeres were placed in separate drops. If blastomeres from several eggs were placed in the same dish the two complete eggs served as controls for all sets of isolated blastomeres. All procedures, from autopsy to placing the eggs and blastomeres into the incubator were carried out at room temperature and lasted for 30-45 min. The duration of culture varied from 36 to 48 h. During this period the control eggs can develop into well-formed blastocysts.
The forms developed from isolated blastomeres were fixed in Heidenhein's fixative (saturated aqueous solution of mercuric chloride, 2 parts; distilled water, 2 parts; formalin, 1 part) according to Sembrat (1955). After washing in distilled water the forms were mounted in a drop of egg albumin on a slide, by the method described by Dalcq (1952) and Mulnard (1955). The slide was then processed through 50 % alcohol containing Lugol, 50 % alcohol, stained with Ehrlich haematoxylin, differentiated in slightly acidified 50 % alcohol and subsequently in 50 % alcohol alkalized with ammonium, dehydrated, cleared in xylene and mounted in Canadian balsam.

From control eggs preparations enabling precise counting of nuclei were prepared according to the air-drying method of Tarkowski (1966).

RESULTS

(1) Introductory remarks and classification of the material

In examining the forms developed from isolated blastomeres we have been concerned first with their morphology and, secondly, with the number of constituting cells (i.e. number of interphase nuclei and metaphase plates). The classification presented below comprises all forms irrespective of whether they have developed from 1/4, 2/8, or 1/8 blastomeres.

After the blastomeres which had not cleaved at all or had undergone only one cleavage and degenerated have been excluded, all the remaining forms were divided into completely integrated and non-integrated ones, i.e. those composed of loosely connected cells or vesicles. Among the integrated forms, whose development was in our opinion least disturbed, the following types were discerned:

I. Trophoblastic vesicle (Figs. 3, 4, 12, 28, 36, 50). All cells contribute to the wall of the vesicle and the inner cell mass is absent altogether. The wall of the vesicle is of the same thickness throughout the surface. All cells have undergone differentiation in a trophoblastic direction.

II. False blastocyst (Figs. 2, 5, 8, 11, 18, 25, 26, 27, 33, 34, 35, 43, 44, 46, 48). Among nuclei which are randomly distributed all over the surface of the vesicle, one may discern a group of nuclei lying closely together. The cells to which these nuclei belong contribute to the wall of the vesicle but are usually slightly thicker and not so distended as the remaining cells. When such a vesicle is examined from the side the thickening may imitate an inner cell mass (Figs. 5, 8, 11, 33, 43).

III. Blastocyst—a more or less normal copy of a blastocyst developed from the whole egg (Figs. 1, 6, 7, 9, 10, 13, 14, 16, 17, 22, 23, 24, 32, 39, 40, 41, 42, 49). It possesses a number of inner mass cells (sometimes as few as only one) covered from outside by the overlying trophoblastic cells.

IV. Morula (Figs. 21, 29, 37, 51). In some of these forms very small cavities or intracellular vacuoles are visible.
Among non-integrated forms the following types were discerned:

1. A group of cells attached to a vesicle or a group of vesicles (Figs. 19, 45, 47).

2. A group of small trophoblastic vesicles, each consisting of several cells (Fig. 54), or a group of very strongly vacuolated and loosely connected single cells (Fig. 55). Single cells with one enormous vacuole inside resemble at first sight a vesicle composed of several cells.

3. A group of loosely connected cells (Figs. 15, 38). Small vacuoles can sometimes be seen in the cytoplasm of some or all the contributing cells.

Whether a given form should be considered as integrated or non-integrated did not usually cause much difficulty. Accurate classification of the non-integrated forms into the three above-mentioned types is not of essential importance, since the whole group can be considered as developing rather abnormally. All the available evidence clearly indicates that such an abnormal way of development must be caused simply by a deleterious treatment of blastomeres during manipulations and/or unfavourable conditions of culturing and does not reflect any inherent peculiarities of blastomeres developing in isolation. However, as far as integrated forms are concerned, the proper description of their morphology and consequently an accurate classification is of utmost importance for disclosing the developmental capacities of single blastomeres. This part of the work presented the greatest difficulty. It is obvious that because of the minute dimensions of the collected forms some errors in classifying were inevitable. In several cases it was simply impossible to decide for certain whether a given form should be assigned to one or the other of the two neighbouring types. The main difficulties were as follows: (1) Deciding whether a given vesicle belongs to type I or type II, especially when the supposed false inner mass is viewed from above. (2) Deciding whether some cells are or are not covered from outside by other cells and consequently whether the form should be considered as representing type III or type II, respectively. (3) Deciding whether a form with a very small cavity should be considered as a blastocyst or as a morula. By definition it is already a blastocyst, but if in the same culture other blastomeres have developed large vesicles or into blastocysts, extremely poor cavitation in such a form should not be overlooked. Being aware that in some cases classification must have been inevitably subjective and unprecise we think that in general it does characterize properly both the diversity of material and the main trends of development of isolated blastomeres.

After the whole material had been divided into integrated and non-integrated forms and classified into different types, a selection of poorly developed forms was carried out. We considered as poorly developed all forms originating from 1/4 or 2/8 blastomeres and composed of less than 8 cells and all forms originating from 1/8 blastomeres and composed of less than 4 cells. Since a 4-cell stage of 1/8 form or an 8-cell stage of 1/4 or 2/8 forms correspond to a 32-cell stage of a whole egg, i.e. a stage representing a transition from morula to blastocyst, the
developmental capacity of blastomeres which have reached this number of cells can be conclusively and definitely determined. All data presented in the tables have, therefore, been calculated twice—for the whole material and for the selected material. Since the underdeveloped forms have usually been encountered in those cultures where the development of control eggs (see below) was also extremely poor, it seems sound to assume that the blastomeres from which they have developed must have been damaged in some way during manipulations or have been subjected to very unfavourable conditions during culture.

(2) Development of control whole eggs

The conditions of culturing, and to a lesser degree the effect of harmful treatment during manipulations, can be to some extent evaluated from the state of control eggs. At the end of culturing the appearance of the living control eggs was recorded (large blastocysts, small blastocysts, irregular blastocysts with non-incorporated cells, regular or irregular morulae, early cleavage stages). Next the number of cells was estimated.

Judging from the morphology of control eggs and from the number of cells of which they were composed, the conditions of individual cultures must have varied greatly. The range of variation in the number of cells was very wide (from 8 to 80 cells) and their morphology varied from degenerating cleaving eggs or irregular morulae to large swollen blastocysts. Many of the irregularities observed in the development of isolated blastomeres (for instance, lack of integration into one entity, poor cavitation, or cavitation in forms displaying retarded cleavage) correspond to abnormalities displayed by control eggs and can be thus attributed simply to improper conditions of culturing.

All figures are reproduced at a magnification of \( \times 700 \). The type of blastomere from which a form has developed is given in parentheses.

**PLATE 1**

Figs. 1–4. Four sister forms developed from one egg.
Fig. 1. A 14-cell blastocyst (2/8). The inner cell mass is composed of only two cells.
Fig. 2. A 14-cell false blastocyst (2/8). The false inner mass occupies the upper right part of the wall of the vesicle and lies slightly below the plane of focusing.
Fig. 3. An 8-cell trophoblastic vesicle (2/8).
Fig. 4. A 17-cell trophoblastic vesicle (1/4).
Figs. 5–7. Three sister forms developed from one egg.
Fig. 5. A 16-cell false blastocyst (1/4).
Fig. 6. An 11-cell early blastocyst (1/4).
Fig. 7. A 16-cell blastocyst (1/4).
Figs. 8–10. Three sister forms developed from one egg.
Fig. 8. A 12-cell false blastocyst (2/8).
Fig. 9. A 24-cell very regular blastocyst (2/8).
Fig. 10. A 24-cell very regular blastocyst (2/8) with the inner mass clearly covered from the top by flat trophoblastic cells.
Mouse blastomere development

In calculating the average cell number of control eggs only those eggs were taken into account which developed into blastocysts and reached 16 or more cells. The average cell number of control eggs cultured in zona equals 45.4 (25 specimens), that of control eggs cultured without zona, 44.6 (22 specimens). The difference is negligible and shows that the lack of the zona pellucida has no detectable effect on multiplication of cells during development of such eggs in vitro. It is interesting to note that cleavage of isolated blastomeres was not to any extent inferior in comparison with whole eggs. This can be concluded from the fact that the average number of cells in vesicular forms (types I, II and III) developed from isolated blastomeres and multiplied by a factor of 4 (1/4 and 2/8 blastomeres) or a factor of 8 (1/8 blastomeres) is even higher than the average number of cells in control eggs. The corresponding values for 1/4, 2/8 and 1/8 forms equal 49.7, 51.9 and 54.7 respectively.

(3) Development of 1/4 blastomeres

A total of 42 forms developed from 1/4 blastomeres have been obtained. Eggs from which the blastomeres originated were at the 4- to 7-cell stage. From some of these eggs blastomeres 2/8 or 1/8 were also cultured but these will be dealt with under separate headings. From 4 eggs all four forms are represented by preparations, from the remaining ones only three, two or one form. The results are presented in Table 1.

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Plate 2

Figs. 11–15. Five sister forms developed from one egg.

Fig. 11. A 10-cell false blastocyst (2/8).

Fig. 12. A 6-cell trophoblastic vesicle (2/8).

Fig. 13. An 8-cell blastocyst (1/8).

Fig. 14. A 6-cell blastocyst (1/8) with all nuclei gathered on one pole.

Fig. 15. A 4-cell non-integrated form (1/8) composed of loosely attached cells. Each cell contains one big and several small vacuoles.

Figs. 16–19. Four sister forms developed from one egg.

Fig. 16. A 26-cell blastocyst (1/4).

Fig. 17. A 16-cell blastocyst (1/4). Only two cells are in the inner mass.

Fig. 18. An 18-cell false blastocyst (1/4). The false inner mass is seen from above as a group of nuclei lying in the central part of the wall of the vesicle.

Fig. 19. A 9-cell non-integrated form (1/8) composed of a loose aggregation of eight cells attached to one cell containing an enormous intracellular vacuole.

Figs. 20–23. Four sister forms developed from one egg.

Fig. 20. A very young 14-cell blastocyst (2/8). A group of enveloped cells can be clearly distinguished from flat enveloping cells. The main part of a small blastocoelc cavity lies slightly below the plane of focusing and only a part of it is seen on the figure.

Fig. 21. An 11-cell morula (1/4). Some cells contain small vacuoles.

Fig. 22. An early 15-cell blastocyst (2/8) with a big inner mass and a small cavity.

Fig. 23. A 14-cell blastocyst (2/8).
Of the 42 forms 35 have divided into 8 or more cells. Among 7 poorly developed forms 4 developed into trophoblastic vesicles, 1 into a false blastocyst, 1 into a morula and 1 into a group of loosely connected cells. The majority of the forms collected (88%) are integrated. The average number of cells of the integrated forms is slightly higher than that of the non-integrated ones. The average cell numbers of integrated forms representing types I, II and III are very similar to each other. Among integrated forms blastocysts are most common; they are followed by trophoblastic vesicles and false blastocysts. Morulae are represented in about 13% of cases only. Considering generally the ability of cells descended from 1/4 blastomeres to form vesicles or to accumulate fluid inside their cytoplasm, only one non-integrated form and five integrated ones (morulae) did not display this ability (14-3%).

The different types of forms developed from 1/4 blastomeres are represented in the following figures: trophoblastic vesicle (Fig. 4); false blastocyst (Figs. 5, 18); well-developed blastocyst (Figs. 7, 16, 17); early blastocyst (Fig. 6); morula (Fig. 21).

(4) Development of pairs of 1/8 blastomeres (2/8 blastomeres)

As far as cytoplasmic material is concerned a pair of 1/8 blastomeres corresponds strictly to a 1/4 blastomere. There is no doubt that in each case a 2/8 pair was composed of two sister blastomeres originating from one maternal 1/4 blastomere. However, 2/8 blastomeres should be treated separately for two reasons. First, they originate from a later developmental stage which may be characterized by a higher degree of cellular differentiation. Secondly, because of the more advanced stage of development of the maternal egg, their ability to develop in vitro is less impaired than in the case of blastomeres from younger stages. These factors can express themselves not only in the different rate of cleavage of 1/4 and 2/8 blastomeres but also in different morphology of forms developed from them.

Pairs of 1/8 blastomeres have been obtained from 36 eggs at 5- to 8-cell stage. In some cases both 2/8 and 1/4 blastomeres were cultured, in others both 2/8 and 1/8 blastomeres. From 1 egg all four forms are represented on preparations, from 16 eggs, three forms, from 9 eggs, two forms and from 10 eggs, one form. Altogether 80 2/8 forms were available, but only 61 reached 8 or more cells. All data concerning the development of 2/8 blastomeres are presented in Table 2.

The integrated forms amount to about 80% of the total. Among the integrated forms trophoblastic vesicles are most common; they are followed by blastocysts and false blastocysts. Morulae occur in about 10% of cases. The average cell number of the integrated forms is higher than that of the non-integrated ones. The average cell numbers of the vesicular forms (calculated for the whole material including underdeveloped forms) increase from type I to type III. However, if only those forms which have reached 8 or more cells are taken into account, the average cell number of trophoblastic vesicles becomes close to that
Table 1. Development of 1/4 blastomeres

<table>
<thead>
<tr>
<th>Type of development</th>
<th>No. of forms</th>
<th>No. of cells in particular forms</th>
<th>Average no. of cells</th>
<th>General incidence (%)</th>
<th>Incidence among integrated forms (%)</th>
<th>No. of forms</th>
<th>Average no. of cells</th>
<th>General incidence (%)</th>
<th>Incidence among integrated forms (%)</th>
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<tr>
<td>Trophoblastic vesicle (I)</td>
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<td>2×4, 5, 6, 8, 15, 3×16, 17, 25</td>
<td>12·0</td>
<td>26·2</td>
<td>29·7</td>
<td>7</td>
<td>16·1</td>
<td>20·0</td>
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<td>7, 2×8, 11, 12, 16, 18</td>
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<td>16·7</td>
<td>18·9</td>
<td>6</td>
<td>12·2</td>
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<td>33·3</td>
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<td>9·5</td>
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<td>13·2</td>
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<td>9, 10</td>
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<td>—</td>
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<td>—</td>
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<td>9, 12</td>
<td>—</td>
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<td>2</td>
<td>—</td>
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<td></td>
<td>35</td>
<td>12·8</td>
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of false blastocysts. This is due to the fact that the integrated forms which have cleaved poorly develop most often into trophoblastic vesicles. The average cell number in morulae is clearly lower than in any type of vesicular forms and corresponds rather to the average cell number of the non-integrated forms.

The following figures illustrate forms developed from 2/8 blastomeres: trophoblastic vesicle (Figs. 3, 12); false blastocyst (Figs. 8, 11); blastocyst (Figs. 1, 9, 10, 22, 23); non-integrated form (a group of trophoblastic vesicles) (Fig. 54).

Comparison of data presented in Tables 1 and 2 indicates that the development of 1/4 and 2/8 blastomeres in the same culture conditions differs in two respects. First, 2/8 blastomeres cleave slightly better than 1/4 blastomeres. Secondly, incidence of the three types of vesicles (types I, II and III) is different in each series: while among 1/4 blastomeres blastocysts are most common, among 2/8 blastomeres trophoblastic vesicles predominate. It is quite possible that in vivo or under optimal conditions of culturing in vitro these differences would disappear.

(5) Development of 1/8 blastomeres

Altogether 144 forms were available for study; 122 forms reached 4 or more cells. Data concerning the development of 1/8 blastomeres are presented in Table 3.

The average cell number in the whole of the material equals 6.2, and after excluding 2- and 3-cell forms, 6.9. As far as multiplication of cells is concerned single 1/8 blastomeres develop as well or even better than the whole pairs (compare Tables 2 and 3). It should be emphasized, however, that many of the 1/8 blastomeres failed to divide and degenerated (these are not included in the Tables) and a high proportion have undergone only one cleavage division (2-cell forms). From the morphological point of view development of the 1/8 blastomeres was inferior to that of the 2/8 or 1/4 blastomeres; integrated forms are represented only in 65% of cases (72% in the selected group). Among integrated forms trophoblastic vesicles predominate (about 45%). False blastocysts are also very common (about 30%) while blastocysts account for only slightly more than 15% of cases. Morulae have been encountered in about 8% of cases only. The average cell number in particular types of integrated forms follows the pattern characteristic for 2/8 forms, i.e. it increases from type I to type III, while morulae have the lowest number of cells. Most of the 2-cell and 3-cell forms belong to the non-integrated category. Some of them have formed, however, regular vesicles and were included into type I of integrated forms.

Different forms developed from 1/8 blastomeres are represented in the following figures: trophoblastic vesicle (Figs. 28, 36, 50); false blastocyst (Figs. 25, 26, 27, 33, 34, 35, 43, 44, 46, 48); blastocyst (Figs. 13, 14, 24, 32, 39, 40, 41, 42, 49); morula (Figs. 29, 37, 51); non-integrated forms (Figs. 15, 19, 30, 38, 45, 47, 55).
Table 2. Development of 2/8 blastomeres

<table>
<thead>
<tr>
<th>Type of development</th>
<th>No. of forms</th>
<th>No. of cells in particular forms</th>
<th>Average no. of cells</th>
<th>General incidence (%)</th>
<th>Incidence among integrated forms (%)</th>
<th>No. of forms</th>
<th>Average no. of cells</th>
<th>General incidence (%)</th>
<th>Incidence among integrated forms (%)</th>
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<tr>
<td>Trophoblastic vesicle (I)</td>
<td>24</td>
<td>3, 5 × 4, 6, 2 × 8, 2 × 9, 2 × 11, 12, 13, 2 × 14, 15, 3 × 16, 2 × 17, 20</td>
<td>10.6</td>
<td>30.0</td>
<td>38.1</td>
<td>17</td>
<td>13.0</td>
<td>27.9</td>
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<tr>
<td>False blastocyst (II)</td>
<td>15</td>
<td>5, 6, 8, 10, 11, 4 × 12, 14, 15, 2 × 16, 18, 20</td>
<td>12.5</td>
<td>18.7</td>
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<td>Blastocyst (III)</td>
<td>16</td>
<td>8, 12, 3 × 14, 2 × 15, 4 × 16, 19, 21, 2 × 24, 28</td>
<td>17.0</td>
<td>20.0</td>
<td>25.4</td>
<td>16</td>
<td>17.0</td>
<td>26.2</td>
<td>31.4</td>
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<tr>
<td>Morula (IV)</td>
<td>8</td>
<td>2 × 4, 6, 8, 9, 10, 12, 16</td>
<td>8.5</td>
<td>10.0</td>
<td>12.7</td>
<td>5</td>
<td>11.0</td>
<td>8.2</td>
<td>9.8</td>
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<tr>
<td>Total</td>
<td>63</td>
<td></td>
<td>12.4</td>
<td>78.7</td>
<td>—</td>
<td>51</td>
<td>14.3</td>
<td>83.6</td>
<td>—</td>
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<tr>
<td>Non-integrated forms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Group of cells attached to a vesicle(s)</td>
<td>6</td>
<td>6, 7, 8, 9, 11, 16</td>
<td>9.5</td>
<td>7.5</td>
<td>—</td>
<td>4</td>
<td>11.0</td>
<td>6.5</td>
<td>—</td>
</tr>
<tr>
<td>Group of vesicles</td>
<td>5</td>
<td>2 × 8, 11, 12, 15</td>
<td>10.8</td>
<td>6.3</td>
<td>—</td>
<td>5</td>
<td>10.8</td>
<td>8.2</td>
<td>—</td>
</tr>
<tr>
<td>Loose aggregation of cells</td>
<td>6</td>
<td>4, 5, 6, 2 × 7, 13</td>
<td>7.0</td>
<td>7.5</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>1.6</td>
<td>—</td>
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<tr>
<td>Total</td>
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<td>21.3</td>
<td>—</td>
<td>10</td>
<td>11.1</td>
<td>16.4</td>
<td>—</td>
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<tr>
<td>Total</td>
<td>80</td>
<td></td>
<td>11.7</td>
<td>—</td>
<td>—</td>
<td>61</td>
<td>13.8</td>
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### Table 3. Development of 1/8 blastomeres

<table>
<thead>
<tr>
<th>Type of development</th>
<th>No. of forms</th>
<th>No. of cells in particular forms</th>
<th>Average no. of cells</th>
<th>General incidence (%)</th>
<th>Incidence among integrated forms (%)</th>
<th>No. of forms</th>
<th>Average no. of cells</th>
<th>General incidence (%)</th>
<th>Incidence among integrated forms (%)</th>
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</thead>
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<tr>
<td><strong>Integrated forms</strong></td>
<td></td>
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<tr>
<td>Trophoblastic vesicle (I)</td>
<td>42</td>
<td>4 × 2, 3, 10 × 4, 5 × 5, 3 × 6, 4 × 7, 11 × 8, 2 × 10, 11, 2 × 11</td>
<td>6.0</td>
<td>29.2</td>
<td>45.2</td>
<td>37</td>
<td>6.5</td>
<td>30.3</td>
<td>42.0</td>
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<tr>
<td>False blastocyst (II)</td>
<td>28</td>
<td>4, 3 × 5, 5 × 6, 3 × 7, 9 × 8, 2 × 9, 3 × 10, 2 × 11</td>
<td>7.6</td>
<td>19.4</td>
<td>30.1</td>
<td>28</td>
<td>7.6</td>
<td>23.0</td>
<td>31.8</td>
</tr>
<tr>
<td>Blastocyst (III)</td>
<td>16</td>
<td>2 × 6, 7, 10 × 8, 11, 2 × 12</td>
<td>8.4</td>
<td>11.1</td>
<td>17.2</td>
<td>16</td>
<td>8.4</td>
<td>13.1</td>
<td>18.2</td>
</tr>
<tr>
<td>Morula (IV)</td>
<td>7</td>
<td>4 × 4, 7, 2 × 8</td>
<td>5.6</td>
<td>4.9</td>
<td>7.5</td>
<td>7</td>
<td>5.6</td>
<td>5.7</td>
<td>8.0</td>
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<tr>
<td><strong>Total</strong></td>
<td>93</td>
<td></td>
<td>6.9</td>
<td>64.6</td>
<td></td>
<td>88</td>
<td>7.1</td>
<td>72.1</td>
<td></td>
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<tr>
<td><strong>Non-integrated forms</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group of cells attached to a vesicle(s)</td>
<td>13</td>
<td>3, 4, 5, 2 × 6, 7, 3 × 8, 2 × 9, 10, 11</td>
<td>7.2</td>
<td>9.0</td>
<td></td>
<td>12</td>
<td>7.6</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>Group of vesicles</td>
<td>16</td>
<td>2, 3, 7 × 4, 5, 3 × 6, 2 × 8, 10</td>
<td>5.1</td>
<td>11.1</td>
<td></td>
<td>14</td>
<td>5.5</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>Loose aggregation of cells</td>
<td>22</td>
<td>12 × 2, 2 × 3, 2 × 4, 2 × 5, 2 × 7, 2 × 8</td>
<td>3.5</td>
<td>15.3</td>
<td></td>
<td>8</td>
<td>6.0</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>51</td>
<td></td>
<td>5.0</td>
<td>35.4</td>
<td></td>
<td>34</td>
<td>6.3</td>
<td>27.9</td>
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<tr>
<td><strong>Total</strong></td>
<td>144</td>
<td></td>
<td>6.2</td>
<td></td>
<td></td>
<td>122</td>
<td>6.9</td>
<td></td>
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</tr>
</tbody>
</table>
Mouse blastomere development

(6) Description of cases in which development of all blastomeres of an egg has been recorded

Although in the majority of cases all blastomeres of the egg had been placed in culture, complete sets of developmental forms have been obtained only in a limited number of cases. This was because of the degeneration of some blastomeres or their loss during the making of permanent preparations.

So far, in the description of the material we were concerned separately with 1/4, 2/8 and 1/8 blastomeres and not with the number of forms obtained from each egg. Since from several eggs different types of blastomeres have been obtained and cultured (for instance 2/8 and 1/8, 1/4 and 2/8, 1/4 and 1/8) the number of complete sets obtained is larger than it might appear from the preceding description.

Altogether from 17 eggs all blastomeres have developed and are represented on preparations. In the description which follows the structure and the number of cells (in parentheses) of each form are given.

1. Eight 1/8 forms (Figs. 24-31): one trophoblastic vesicle (7), three false blastocysts (8, 10, 10), one blastocyst (7), one morula (10), a group of vesicles (10) and a 3-cell form with three nuclei and small vacuoles in the cytoplasm.

2. One 2/8 form and six 1/8 forms: 2/8: false blastocyst (11); 1/8: four trophoblastic vesicles (2, 2, 7, 8), one false blastocyst (4), a group of loosely connected cells (8).

3. One 2/8 form and six 1/8 forms: 2/8: morula (16); 1/8: one trophoblastic vesicle (4), two false blastocysts (8, 9), two blastocysts (12, 12), a group of vesicles (4).

4. Two 2/8 forms and four 1/8 forms: 2/8: one trophoblastic vesicle (20), one false blastocyst (20); 1/8: four trophoblastic vesicles (8, 8, 10, 10).

5. Two 2/8 forms and four 1/8 forms: 2/8: one trophoblastic vesicle (16), one blastocyst (16); 1/8: three trophoblastic vesicles (5, 5, 5), one false blastocyst (11).

6. Three 2/8 forms and two 1/8 forms: 2/8: two blastocysts (12, 16), one non-integrated form composed of vesicles and non-vacuolated cells (16); 1/8: two trophoblastic vesicles (4, 8).

7. Three 2/8 forms and two 1/8 forms: two trophoblastic vesicles (11, 17), one blastocyst (28); 1/8: two trophoblastic vesicles (5, 8).

8. Three 2/8 forms and two 1/8 forms: 2/8: one trophoblastic vesicle (13), one false blastocyst (5), one blastocyst (16); 1/8: one trophoblastic vesicle (4), a 2-cell form with enormous intracellular vacuoles.

9. Three 2/8 forms and two 1/8 forms: 2/8: one trophoblastic vesicle (13), two blastocysts (8, 14); 1/8: one trophoblastic vesicle (5), a group of vesicles (8).

10. Three 2/8 forms and two 1/8 forms: 2/8: one trophoblastic vesicle (14), two non-integrated forms composed of groups of vesicles (8, 15); 1/8: one
trophoblastic vesicle (7), a non-integrated form composed of a group of cells attached to one cell with enormous vacuole (7).

(11) Four 2/8 forms: two false blastocysts (12, 12), one morula (9), a non-integrated form composed of highly vacuolated cells (8).

(12) Three 2/8 forms and one 1/4 form: (Figs. 20–23): 2/8: one very young and two young blastocysts (14, 15, 15); 1/4: a morula (11).

(13) Three 2/8 forms and one 1/4 form (Figs. 1–4): 2/8 one trophoblastic vesicle (8), one false blastocyst (14), one blastocyst (14); 1/4: a trophoblastic vesicle (17).

(14) One 2/8 form and three 1/4 forms: 2/8: a trophoblastic vesicle (4): 1/4: one trophoblastic vesicle (4), one blastocyst (16), one morula (11).

(15) Four 1/4 forms: two false blastocysts (8, 8), two morulae (8, 8).

(16) Four 1/4 forms: one trophoblastic vesicle (16), three blastocysts (16, 16, 16).

(17) Four 1/4 forms: two trophoblastic vesicles (16, 16), one blastocyst (8), a group of vesicles (12).

Several sets were also available in which only one or two blastomeres were lacking. Some of these sets are presented in the plates and the details can be found in the description of figures.

In most of the above-described cases all blastomeres of the egg have developed into regular vesicular forms. This demonstrates conclusively that up to the 8-cell stage all blastomeres possess the ability to differentiate into trophoblastic cells. It would seem, therefore, that all cases of sporadic development of

**PLATE 3**

Figs. 24–31. Eight sister forms developed from one egg.

Fig. 24. A 7-cell blastocyst (1/8). The inner mass is covered from the top by one trophoblastic cell.

Fig. 25. A 10-cell false blastocyst (1/8).

Fig. 26. A 10-cell false blastocyst (1/8).

Fig. 27. An 8-cell false blastocyst (1/8). The false inner mass is seen from above.

Fig. 28. A 7-cell trophoblastic vesicle (1/8).

Fig. 29. A 10-cell morula (1/8). One cell contains numerous vacuoles.

Fig. 30. A 10-cell non-integrated form composed of three vesicles (1/8). Only the biggest vesicle resembling a blastocyst is pictured.

Fig. 31. A 2-cell form (1/8) with three nuclei. Small vacuoles are present in both cells.

Figs. 32–38. Seven sister forms developed from one egg.

Fig. 32. An 8-cell blastocyst (1/8).

Fig. 33. An 8-cell false blastocyst (1/8).

Fig. 34. An 8-cell false blastocyst (1/8). It possesses two separate blastocoelic cavities.

Fig. 35. A 6-cell false blastocyst (1/8). Four cells whose nuclei lie closely together (seen from above) constitute supposedly the false inner mass.

Fig. 36. A 4-cell trophoblastic vesicle (1/8).

Fig. 37. An 8-cell morula (1/8).

Fig. 38. A non-integrated form composed of highly vacuolated cells. Eight pycnotic nuclei.
some blastomeres into morulae or non-integrated aggregations of cells may be regarded as resulting from injuries inflicted during manipulations and/or suboptimal conditions of culturing. It should be added that morulae were observed more often among 1/4 and 2/8 forms than among 1/8 forms. According to the hypothesis that the mechanism underlying the formation of inner mass cells and trophoblast is ooplasmic segregation, the reverse should be true. Another conclusion which can be drawn from the above data is that the type of development of blastomeres is extremely variable, both among individual sets and among forms belonging to one set, and that it cannot be predicted a priori.

(7) Abnormal development of isolated blastomeres

Abnormal development displayed by some of the isolated blastomeres has unexpectedly provided useful information about certain peculiarities of cleavage cells.

In normal development of the mouse the blastocoel begins to appear when the

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**Plate 4**

Figs. 39-45. Seven sister forms developed from one egg.
- Fig. 39. An 8-cell blastocyst (1/8).
- Fig. 40. An 8-cell blastocyst (1/8).
- Fig. 41. An 8-cell blastocyst (1/8).
- Fig. 42. A 6-cell blastocyst (1/8) with two cavities separated by a bridge composed of four cells. These cells would presumably have become the inner mass had the two cavities joined together.
- Fig. 43. An 8-cell false blastocyst (1/8).
- Fig. 44. A 6-cell false blastocyst (1/8).
- Fig. 45. A 6-cell irregular non-integrated form with all cells highly vacuolated.
- Fig. 46. A 10-cell false blastocyst (1/8). Six nuclei on one pole, two on the other.
- Fig. 47. A 7-cell non-integrated form (1/8) resembling at first sight a typical blastocyst. In fact this is a group of six cells (two enveloped and four enveloping) attached to one cell containing an enormous intracellular vacuole.
- Fig. 48. A 6-cell false blastocyst (1/8) with a false inner mass seen from above. The nuclei of the two other cells lie slightly below the plane of focusing.
- Fig. 49. An early 8-cell blastocyst (1/8). The inner mass cells are clearly covered from outside by an enveloping cell.
- Fig. 50. An 8-cell trophoblastic vesicle (1/8).
- Fig. 51. A 4-cell morula (1/8) composed of one enveloped and three flattened enveloping cells.
- Fig. 52. A 2-cell vesicle (1/8).
- Fig. 53. A 1/8 blastomere which has not divided in culture and has chromosomes scattered in the cytoplasm. Note numerous vacuoles in the cytoplasm.
- Fig. 54. An 11-cell aggregation of small vesicles developed from a pair of 1/8 blastomeres (2/8).
- Fig. 55. A non-integrated form (1/8) composed of four loosely connected cells, each containing one enormous vacuole.
the 5th cleavage division. Normally, the ability of the cleavage cells to secrete the blastocoelic fluid is not displayed before this stage. It would seem that a certain number of cleavages is required before the cells attain the state of differentiation enabling them to undertake this physiological activity. Examination of the development of isolated blastomeres shows that under unfavourable experimental conditions, mitotic divisions (cleavages) and secretion of the blastocoelic fluid are not impaired to the same degree. Poor culture conditions act in the first instance deleteriously on cell division; many blastomeres divide only once or twice. However, most of the 4-cell forms originating from 1/4 and 2/8 blastomeres have developed into regular trophoblastic vesicles and so did some of the 2-cell forms originating from 1/8 blastomeres (Fig. 52). These blastomeres have undergone only four cleavage divisions and although the 5th cleavage has been inhibited the secretion of the blastocoelic fluid has not been. In one case a 1/8 blastomere has been observed which had not divided at all and had chromosomes scattered in a very highly vacuolated cytoplasm (Fig. 53). Vacuolization of the cytoplasm which in normal development represents a visible sign of secretory activity (see below) can, thus, be displayed by a blastomere which has not gone further than the third cleavage. Secretion of the blastocoelic fluid seems, therefore, to represent a cytoplasmic activity which a cell undertakes after a certain definite period of time and irrespective of the number of nuclear cycles. This puzzling phenomenon certainly merits further investigation.

In histological preparations or in whole permanent mounts of normal blastocysts developing in vivo or in vitro small vacuoles can always be seen in the trophoblastic cells. The contents of these vacuoles are presumably continuously discharged into the intercellular spaces thus leading to the formation of the blastocoelic cavity and preventing the accumulation of large amounts of fluid in the cytoplasm. In our experiments some of the blastomeres developed into non-integrated forms composed of loosely attached cells or groups of cells. In such cases a single cavity could not, of course, be formed, the end product of development being a group of tiny vesicles (Fig. 54) or a group of highly vacuolated cells (Fig. 55). It is quite understandable that the cells whose entire or nearly entire surface is exposed to the surrounding medium cannot secrete the synthesized fluid back into the environment and must accumulate it in the cytoplasm. Consequently, one enormous vacuole can be formed, the cytoplasm and the nucleus being pushed to the periphery of the cell (Figs. 19, 47, 55). The cleavage cells can, thus, start to produce the fluid irrespective of whether they adhere to each other or not and, consequently, whether they have or have not a possibility of discharging it from the cytoplasm. It would seem, therefore, that a prerequisite of a formation of a blastocyst from the whole egg or a vesicular form (of any type) from an isolated blastomere is a close adhesion of cleavage cells and, consequently, the creation of an intercellular environment. Once the egg has about 32 cells, i.e. when all or nearly all blastomeres have completed
opposing surfaces of the cells become exposed to different conditions (inter-
cellular versus external) the cells themselves must inevitably become polarized
and can start secreting the blastocoelic fluid into the interior of the aggregation.

**DISCUSSION**

The results of the experiments described above made it clear that the inter-
pretation proposed by one of us (Tarkowski, 1959a, b) and by Mulnard (1965a)
to explain the development of 1/2 blastomeres cannot be extended to 1/4 and
1/8 blastomeres and that a completely new outlook on mechanisms governing
eye development and responsible for divergent differentiation of cleavage cells
in an embryonic (inner mass) or an extra-embryonic (trophoblast) direction is
needed.

In their work on the development of 1/2 blastomeres of the mouse both
Tarkowski and Mulnard interpreted the results in the light of conceptions
elaborated by Dalcq, Mulnard and their co-workers (for reviews see Dalcq,
(1957) and Mulnard (1961)). On the basis of cytochemical studies on early
mammalian development, the Belgian authors postulate that the uncleaved egg
is characterized by polarity and bilateral symmetry and that the cytoplasm from
the ‘dorsal’ and ‘ventral’ zones of the egg is segregated during cleavage until
it is in the cells of inner mass and trophoblast, respectively. Both Tarkowski and
Mulnard suggested that the great variation in the size of the inner mass (number
of cells) displayed by blastocysts developed from 1/2 blastomeres was due to
variable position of the first cleavage in relation to the plane of bilateral sym-
metry and, consequently, to variable and unequal distribution of cytoplasm
from each zone to the daughter blastomeres. Multicellular morulae and tro-
phoblastic vesicles were considered as representing the extremes of this scale
of variation and originating from eggs in which this distribution was extremely
unequal, i.e. the plane of cleavage deviated very considerably from the plane of
symmetry.

According to a segregation hypothesis, in a random sample of 1/2 forms one
would expect to encounter in similar proportions forms in which trophoblastic
cells predominate and forms composed mainly of ‘dorsal’ cytoplasm, i.e.
morulae or very poorly cavitated blastocysts. However, this was not found to
be true. Multicellular morulae were observed very rarely and the blastocysts, in
comparison with control blastocysts developing from whole eggs, are charac-
terized by a marked preponderance of trophoblastic cells over inner mass cells.
In this situation Tarkowski (1959b) had to propose an auxiliary hypothesis,
mostly that when 1/2 blastomeres develop in isolation the formation of trophoblastic cells is privileged and occurs at the expense of the ‘dorsal’
material.

However, the results of the present work and the recent findings by Mulnard
(1965b) invalidate the above interpretation. Mulnard described the develop-
ment of trophoblastic vesicles from two blastomeres separated from each other at the 2-cell stage. If it were true that the fate of cytoplasm occupying different regions of the uncleaved egg is already determined, then the two sister blastomeres should develop into forms displaying complementary structure. This was not the case. Mulnard himself is very cautious in considering these results as evidence against the conception that ooplasmic segregation plays an important role in early mammalian development. He is inclined to think that the conditions of the experiment itself (separation of blastomeres, sticking of naked blastomeres to the glass, and so on) might modify and disturb the development of blastomeres and lead to the origin of purely trophoblastic structures from the cytoplasm of the whole egg. Although such a caution is certainly justified it seems doubtful whether his results could be ascribed only to the technical inadequacies of the experiment. It is worth stressing that formation of trophoblastic vesicles from 1/2 blastomeres does not occur only for naked blastomeres but has been also observed among blastomeres left inside zona pellucida and developing both in vivo (Tarkowskii, 1959a, b) and in vitro (Mulnard, 1965a).

According to Dalcq and Mulnard's conceptions one would expect that forms developed from blastomeres isolated at later stages should also display a complementary structure and should deviate more drastically from the normal type of development, i.e. from a blastocyst developed from the whole egg. However, in our experiments devoted to stages IV and VIII such a phenomenon has not been observed. The majority of blastomeres isolated at these two stages develop into vesicular forms in which all, or the bulk of, cells behave like trophoblastic cells. Among forms developed from 1/4, 2/8, and 1/8 blastomeres the incidence of morulae is very similar and ranges about 10%. If development into morulae results from the intrinsic properties of certain blastomeres then the incidence of these particular forms should be higher among 1/8 forms than among 1/4 and 2/8 forms because of a more advanced segregation of the cytoplasm of the egg among individual blastomeres. One could also expect that 1/8 blastomeres should develop into forms representing extremes of the scale of variation, such as morulae and trophoblastic vesicles, while intermediate forms like false blastocysts and blastocysts would be very rare or lacking altogether. The picture we observe is not in accord with these expectations. If one agrees that morulae represent badly developing forms and that the occurrence of non-integrated forms also results from poor culture conditions (or injury during manipulations), then it can be suggested that all blastomeres at the 4- and 8-cell stage have an ability to develop into regular vesicular forms (types I, II or III). Such a conclusion seems to be well corroborated by the fact that in several cases all blastomeres of the egg followed this type of development.

On the ground of the results of our present work as well as of those of other authors we want to put forward a hypothesis which seems to explain satisfactorily both normal development (or, strictly speaking, the mechanisms
Mouse blastomere development

responsible for the formation of a blastocyst and the differentiation of blastomers into inner mass and trophoblastic cells), and the development of blastomeres isolated at the 2-, 4- and 8-cell stage.

When a mouse egg passes from morula to blastocyst it is composed of about 30 cells. A certain number of these cells occupy the interior of the morula and are completely separated from outside by other cells. The latter become flattened and represent predecessors of the trophoblastic cells. It seems reasonable to assume that the conditions in which external and internal cells find themselves are diametrically different. The internal cells, being completely cut off from the exterior, develop in a micro-environment created by the external cells. In our opinion, the position of a cell in the morula and, as a consequence of the position, the different environmental conditions play a decisive role in the differentiation of cells in one of the two directions (trophoblast versus inner mass). For the formation of the inner mass it is necessary that certain blastomeres should become isolated from the exterior before the moment when blastocoelic fluid starts to accumulate between the cells.

As far as development of isolated blastomeres is concerned, the later the stage from which the blastomere has been taken, the smaller the number of cells at the time of cavitation. Consequently the number of blastomeres which can become enveloped by other blastomeres is more and more reduced. While in the case of a 1/2 blastomere the number of cells in the morula at the beginning of blastulation is still large enough for the formation of nearly normal blastocysts in the majority of cases (although the number of embryonic cells is clearly reduced in comparison with blastocysts developing from the whole egg), development of blastocysts from 1/4 and 2/8 blastomeres takes place only in about 30–40 % of cases, and from 1/8 blastomeres only in about 15 % of cases. This decrease in the incidence of blastocysts is accompanied by an increase in the incidence of trophoblastic vesicles and false blastocysts. It would seem, therefore, that all blastomeres at the 4- and 8-cell stage possess the ability to differentiate into trophoblastic cells. Differentiation into inner mass cells would not be inherent in any blastomere but would represent an alternative route of development requiring an intercellular environment. Putting this the other way round, up to the 8-cell stage any blastomere of the egg can give rise to inner mass cells if at the proper time the descending cells find themselves shielded by other cells.

It is worthwhile to refer in this connexion to the experiments carried out by Seidel (1956, 1960), who found that in the rabbit trophoblastic vesicles develop more often from 1/4 than from 1/2 blastomeres. This observation confirms our results obtained with mouse eggs and can be explained in a similar way. Seidel’s material consisted of blastomeres left inside zona pellucida and developing in vivo. Neither lack of zona nor development in vitro can be made responsible, therefore, for the results which we have obtained in our experiments.

An experimental approach therefore provides evidence that at least up to the
8-cell stage the fate of blastomeres is still labile and that it becomes fixed at later stages. Mintz (1964a, b, 1965) came to a similar conclusion on the basis of her experiments on fusion of eggs and claims that this lability extends to even later stages (see also Tarkowski, 1965). She has shown that when morulae are fused together there is no selective sorting out of cells in the resulting aggregations and, consequently, those of the originally external cells which occupy the 'sticking surface' become secondarily internal and differentiate into inner mass cells.

It is rather strange that the precocious cytochemical differentiation of the mammalian egg, described by Belgian authors, should play a decisive morphogenetic role in normal development and have no effect upon development of isolated blastomeres. However, it should be stressed in this connexion that in spite of the fact that many interesting and important data on cytochemistry of mammalian eggs and early stages of development have been accumulated, clear-cut evidence of cytoplasmic continuity between different regions of the uncleaved egg and the components of the resulting blastocyst is very limited. Perhaps the most convincing proof in this respect is provided by studies of Mulnard (1955) on acid phosphatase in early development of the rat. However, using the same methods the above author was unable to obtain similar results with the mouse, in which acid phosphatase cannot be demonstrated until the time when the egg enters the fourth cleavage division (Mulnard, 1965a). Increase in the content and/or activity of this enzyme which from this stage on is found in the internal cells may be equally well interpreted as a sign and result of their differentiation caught in statu nascendi. Although some technical modifications introduced recently by Dalcq (1966) allowed him to reveal acid phosphatase in oocytes and early cleavage stages of the mouse, these investigations too do not provide a clear picture of the continuity of 'dorsal' and 'ventral' cytoplasm through cleavage and thus do not fill the gap between the uncleaved egg and blastocyst.

In the opinion of the present authors, the evidence in favour of ooplasmic segregation in mammals is not conclusive, at any rate not as far as the mouse is concerned.

Appearance of bilateral symmetry in mammalian development as early as in the oocyte, as postulated by Dalcq & Mulnard, seems to the present authors very strange and problematical. Why should the mammalian egg display such a type of symmetry? Early appearance of bilateral symmetry is understandable in the case of amphibians or other animals in which the plane of symmetry of the egg becomes the plane of symmetry of the embryo. However, even in the amphibian egg the visible signs of bilateral symmetry do not appear until after fertilization. In mammals, where early development is subordinated, first of all to the elaboration of the embryonic and extra-embryonic material and where the embryo proper is formed much later from the part of the embryonic shield, the bilateral symmetry of the egg would have nothing in common with the bilateral symmetry of the embryo itself. One wonders at the biological sense of an early
appearance of bilateral symmetry in the mammalian egg, a symmetry of very short duration and of no meaning in later development.

Another aspect of the structure of the egg is its polarity. This is an universal peculiarity of all eggs and it is sound to assume *a priori* that the cytoplasm of the mammalian egg also displays some differences along the polar axis. Has this gradient any influence on the fate of individual blastomeres originating from different ‘levels’ of the egg? There is no evidence either for or against such a dependency simply because in isolation experiments we do not know from what part of the egg a given blastomere originates. From our experiments it is evident that up to the 8-cell stage each blastomere can develop into a vesicular form. It is only the structure of the vesicle (trophoblastic vesicle, false blastocyst, blastocyst) that is variable. What causes the development of a given blastomere into one or another type of vesicle? Is it possible that the variable structure of the resulting forms is due to cytoplasmic inequality of blastomeres associated with the polar differentiation of the egg? At present we are not able to offer a conclusive answer to this question, the available evidence speaking rather against such a dependency.

A normal or a false blastocyst developed from an isolated blastomere, as well as a blastocyst developed from a whole egg, display polarity, as on one pole there is an inner mass. This polarity manifests itself for the first time when a small cavity (or cavities) appears on one side of the morula. Is this polarity primary or secondary? In the case of a blastocyst developed from a whole egg it would be tempting to assume that this polarity develops against a background of a pre-existing primary polarity of the egg. However, there is no evidence either for or against such an assumption. The only trace of the animal pole is the position of the second polar body. It is very doubtful, however, whether at this stage the polar body still occupies its original position; very often it is no longer present. No data are available on the relation between the polar axis of the blastocyst and the polar axis of the undivided egg and specially devised experiments would be necessary to elucidate this question.

A blastocyst developed from an isolated blastomere imitates a blastocyst developed from a whole egg and in its formation similar mechanisms must be involved to those of normal development. A trophoblastic vesicle does not display any polarity. This type of vesicle must originate from a group of cells which at the moment when they start secreting blastocoelic fluid through their innermost surfaces are exposed to the exterior to the same degree. With the increase of the cavity the cells are pushed out to the peripheries of the aggregation, which thus becomes a trophoblastic vesicle. Cell divisions which follow can only increase the number of cells constituting the wall of the vesicle but cannot lead to the formation of the inner mass. The most interesting and obscure situation is represented by a false blastocyst (type II) which like a normal blastocyst displays also polarity. In these forms some of the cells, though they contribute to the wall of the vesicle, are packed more tightly together and are
less distended than the remaining ones. It may be that the shape and behaviour of these cells is a result of their lower activity in secretion of the blastocoelic fluid. One could speculate that when the cavity first appeared in these forms, the cells, or just a cell, in question were partially covered by other cells and consequently their character and behaviour became ultimately intermediate between the external (trophoblastic) and internal (inner mass) cells. This seems a probable, although completely hypothetical explanation of the origin of such forms.

This proposed origin of trophoblastic vesicles, false blastocysts and blastocysts would imply that at the time when blastocoelic fluid starts to accumulate the forms are composed of differing numbers of cells—the lowest in presumptive trophoblastic vesicles, the highest in presumptive blastocysts. If a blastomere is able to divide into a relatively high number of cells before starting secretory activity, the possibility that some cells will become enveloped completely by others is increased. If at this crucial moment the number of cells is still low a trophoblastic vesicle will inevitably be formed. No data on the cell number at the time of cavitation are available. However, we know the cell number of already developed forms. As can be seen from Tables 1, 2 and 3 in forms originating from 2/8 and 1/8 blastomeres the average cell number is lowest in trophoblastic vesicle, higher in false blastocyst and highest in blastocysts, which confirms our hypothesis. Among 1/4 forms the average cell number in the three types of vesicles is similar. However, this sample is small, hence the significance of averages is limited.

The crucial problem of why blastomeres develop into one of the three types of vesicles remains unresolved. We are inclined to think that the factors involved are twofold: first, the size of blastomere related to the stage of development at which it is separated and, secondly, some unknown factors, varying from experiment to experiment, in treating and culturing the blastomeres. The differences in the development of blastomeres originating from different developmental stages can be explained satisfactorily in the way proposed by us. Variation in the structure of forms originating from the same stage is, however, more difficult to elucidate. Evidence that external rather than internal (i.e. primary differences in developmental capacities) factors play a major role is provided by the observation that while in some cases all or nearly all sister blastomeres develop into trophoblastic vesicles, in others blastocysts predominate. Generally speaking the incidence of various types of vesicles among sister blastomeres is not constant and changes from egg to egg.

**SUMMARY**

1. Mouse eggs at stages from 4- to 8-cell were disaggregated into individual blastomeres; the blastomeres were subsequently cultured *in vitro* for 36–48 h.

2. The material collected consisted of 42 forms developed from 1/4 blastomeres, 80 forms developed from pairs of 1/8 blastomeres (2/8 blastomeres) and
144 forms developed from 1/8 blastomeres. In 17 cases development of all sister blastomeres constituting the egg has been observed.

3. The majority of 1/4, 2/8 and 1/8 blastomeres develop into vesicular forms. Three types of vesicles have been discerned: (a) trophoblastic vesicle, a purely trophoblastic structure deprived of the inner mass cells; (b) false blastocyst, a trophoblastic vesicle whose wall is thicker on one pole (false inner mass); (c) blastocyst, a small copy of a blastocyst developed from the whole egg.

4. Development of some blastomeres into irregular non-integrated forms or into morulae results presumably from harmful treatment during manipulations and/or suboptimal conditions during culturing.

5. Incidence of blastocysts decreases from about 40% among forms developed from 1/4 blastomeres to about 30% among 2/8 forms and to about 15% among 1/8 forms. This decrease is accompanied by a slight increase in the incidence of false blastocysts and a very marked increase in the incidence of trophoblastic vesicles.

6. A hypothesis is formulated according to which the differentiation of blastomeres into trophoblastic and inner mass cells is achieved epigenetically and depends in the first instance on the position occupied by blastomeres in the morula. Up to the 8-cell stage all blastomeres possess the ability to differentiate in a trophoblastic direction. Differentiation into inner mass cells is not inherent and represents an alternative route of differentiation requiring an intercellular environment.

7. With the decreasing size of the blastomere at the time of separation (i.e. advancing stage of development) the number of cells attained by it by the time of cavitation decreases. Consequently the number of cells which can become enveloped by other cells becomes smaller and smaller. This leads to the decrease in the size of inner mass and finally, in extreme cases, to the development of purely trophoblastic vesicles.

8. It remains obscure why blastomeres originating from the same stage develop either into trophoblastic vesicles or into false blastocysts or into blastocysts. The available evidence does not suggest the existence of a primary developmental inequality of blastomeres and seems to indicate that the type of development is governed to a great extent by some unknown conditions of culturing, varying from experiment to experiment. It seems that a decreasing rate of cleavages increases the chances of development of trophoblastic vesicles.

9. Production of blastocoelic fluid is a cytoplasmic activity which a cleavage cell undertakes after a certain definite period of time and irrespective of the number of the nuclear cycles. Suppression of cleavage does not necessarily lead to the suppression of this activity. If the cleavage cells do not adhere closely to one another blastocoelic fluid cannot be discharged from the cells and accumulates in large amounts in the cytoplasm.
Développement de blastomères d’œufs de souris, isolés aux stades à 4 et 8 blastomères

1. Des œufs de souris, aux stades 4 à 8 blastomères, ont été désagrégés et les blastomères isolés ont été ensuite cultivés in vitro pendant 36 à 48 h.

2. Le matériel obtenu consistait en 48 germes provenant de blastomères 1/4, 80 germes provenant de paires de blastomères 1/8 (2/8), et 144 germes provenant de blastomères 1/8. Dans 17 cas, on a observé le développement de tous les blastomères frères formant l’œuf.

3. La majorité des blastomères 1/4, 2/8 et 1/8 se développent en formes vésiculaires. On a distingué trois types de vésicules: (a) vésicule trophoblastique — structure purement trophoblastique privée des cellules de la masse interne; (b) faux blastocyste, — une vésicule trophoblastique dont la paroi est plus épaissie à un pôle (fausse masse interne); (c) blastocyste — une copie réduite d’un blastocyste provenant d’un œuf entier.

4. Le développement de quelques blastomères en germes irréguliers non intégrés, ou en morulas, résulte probablement d’un traitement lésant au cours des manipulations et (ou) de conditions sub-optimales au cours de la culture.

5. La proportion de blastocystes obtenus s’abaisse depuis 40 % environ parmi les germes développés à partir de blastomères 1/4 jusqu’à environ 30 % parmi les germes 2/8 et environ 15 % parmi les germes 1/8. Cette diminution est accompagnée d’un léger accroissement de la fréquence des faux blastocystes et d’un accroissement très marqué de celle des vésicules trophoblastiques.

6. On formule une hypothèse selon laquelle la différenciation des blastomères en cellules trophoblastiques et internes est épigénétique et dépend en premier lieu de la position occupée par les blastomères dans la morula. Jusqu’au stade 8, tous les blastomères possèdent la capacité de se différencier dans le sens trophoblastique. La différenciation en cellules de la masse interne n’est pas intrinsèque et représente un mode alternatif de différenciation nécessitant un milieu intercellulaire.

7. Au fur et à mesure que diminue la taille du blastomère au moment de son isolement (c’est-à-dire que le stade de développement est plus avancé) le nombre de cellules qu’il comporte au moment de creusement diminue. En conséquence, le nombre des cellules qui peuvent être enveloppées par d’autres cellules devient de plus en plus faible. Ceci mène à la diminution de la taille de la masse interne et finalement, dans les cas extrêmes, au développement de vésicules purement trophoblastiques.

8. On ne sait pas pourquoi des blastomères issus du même stade se développent soit en vésicules trophoblastiques, soit en faux blastocystes, ou encore en blastocystes. Les résultats acquis ne suggèrent pas l’existence d’une inégalité embryogénique primaire des blastomères et semblent indiquer que le type de développement est dirigé à un degré élevé par quelques facteurs de culture.
inconnus, variables d'une expérience à l'autre. Il semble qu'un taux de segmentation en diminution augmente les chances de développement de vésicules trophoblastiques.

9. La production de liquide blastocelien représente une activité cytoplasmique qu'entreprenant une cellule de segmentation après un certain laps de temps, défini, et sans rapport avec le nombre de cycles nucléaires. La suppression des clivages ne conduit pas nécessairement à la suppression de cette activité. Si les cellules de segmentation n'adhèrent pas étroitement l'une à l'autre, le liquide blastocelien ne peut pas être rejeté des cellules et s'accumule en grandes quantités dans le cytoplasme.

REFERENCES


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