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# DEVELOPMENT IN VITRO OF "HALF EGGS" AND SINGLE BLASTOMERES ISOLATED FROM TWO-, FOUR-, AND EIGHT-CELL MOUSE OVA



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In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

## ABSTRACT

The extent of mouse blastomere lability was studied by isolating and growing in vitro blastomeres as various components of two-, four-, and eight-cell ova. The results were in accord with the view that blastomeres of the eight-cell ovum and possibly even later stages have the capability of becoming either trophoblast or inner cell mass, depending on location in the cell aggregate at the time of blastulation. The formation of "trophoblastic vesicles" from single blastomeres of four- and eight-cell ova is a result of an inadequate number of cells at time of cavitation.

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# I. INTRODUCTION

Regulation of development in the embryo has been observed in several invertebrate species, but this notion has not yet been resolved in the mammalian embryo. Seidel[1,2] and Tarkowski,[3] utilizing rabbit and mouse ova, respectively, demonstrated the equipotency of the blastomeres of the two-cell ovum. These experiments consisted of destroying one blastomere of the two-cell ovum by puncturing through the zona pellucida with a glass needle and allowing the continued development of the remaining blastomere. When the latter were transferred to recipient females, the birth of several living young was observed. Attempts to sustain development from four-cell ova with three destroyed blastomeres failed. Tarkowski[3] concluded that the rodent egg was characterized by polarity and bilateral symmetry and that progressive segregation of the cytoplasm into dorsal and ventral sections gave rise to inner cell mass and trophoblastic cells, respectively.

Mintz[4] presented results from studies involving the experimental production of genetically mosaic embryos indicating that the mouse ovum is still developmentally labile at the eight-cell stage and possibly even beyond. Her conclusions were based on the fact that selective sorting out or migration of cells did not occur in chimeric aggregations from mouse ova fused at the cleavage stage just preceding blastulation. There was complete integration with the formation of a single blastocyst after the usual in vitro culture period. This does suggest that early determination of blastomeres has not occurred. Tarkowski and Wroblewska[5] have since spoken against Tarkowski's earlier conclusions and demonstrated that all blastomeres from four- and eight-cell ova have the inherent ability to develop into trophoblastic cells.

Dalcq[6] and Mulnard,[7,8] utilizing cytochemical and cytological techniques, concluded that there did exist a "bilateral symmetrical" organization in the uncleaved ovum. The findings of Dalcq[6] gave way for the interpretations that Tarkowski[3] and Seidel[1,2] proposed from the results of experiments mentioned previously.

Mulnard's conclusions[7,8] were based on experimental results of cytochemical studies specifically designed for the detection of acid phosphatase activity associated with cells of the inner cell mass.

Our investigation was conducted in an effort to determine the extent of blastomere lability when isolated as various components of two-, four-, and eight-cell ova. The interpretations drawn from these experiments are in accord with views of Tarkowski and Wroblewska[5] and Mintz[4]; that is, the blastomeres of the eight-cell ovum and possibly even later stages have the capability of becoming either trophoblast or inner cell mass, depending on their location in the cell aggregate at the time of blastulation. The formation of "trophoblastic vesicles" from single blastomeres of four- and eight-cell ova is a result of an inadequate number of cells at time of cavitation.

## II. MATERIALS AND METHODS

### A. ANIMALS

This work was performed with $C_{57}$BL/6CUM and random-bred Swiss albino mice. All animals were at least 6 weeks old when used.

### B. TECHNIQUES

Ovulation was induced by intraperitoneal (IP) injection of 5 international units (IU) of pregnant mares' serum (PMS) followed 40 to 48 hours later by an IP injection of human chorionic gonadotropin (HCG). The techniques of recovery, manipulation, and culture of intact ova are as described in a previous communication[9] with the exception that 0.6 ml of 4% methylcellulose was added to each 10-ml portion of culture medium. The ova were recovered at appropriate time intervals after HCG injection so as to assure the presence of the desired cell stages (Table 1).

TABLE 1. PROPORTIONS OF TWO-, FOUR-, AND EIGHT-CELL OVA RECOVERED AT VARIOUS TIME INTERVALS AFTER LUTEINIZING HORMONE INJECTION

| Hours from Injection of Luteinizing Hormone to Recovery | Percentage of Each Cell Stage Recovered[a] | | |
|---|---|---|---|
| | 2-Cell | 4-Cell | 8-Cell |
| 48 | 99.5 | | |
| 49 | 76 | 24 | |
| 50 | 58 | 42 | |
| 51 | 70 | 30 | |
| 52 | 28 | 72 | |
| 53 | 7 | 93 | |
| 54 | 8 | 92 | |
| 55 | 15 | 85 | |
| 56 | 1.5 | 98.5 | |
| 57 | 1 | 99 | |
| 58 | 1 | 99 | |
| 59 | | 96 | 4 |
| 60 | | 68 | 32 |
| 61 | | 36 | 64 |
| 62 | | 5 | 95 |

a. Data taken from 1,497 ova recovered from 272 mice.

After recovery, the ova were deposited in a 0.5% solution of Streptomyces griseus protease (Pronase®, CalBiochem Co.) at room temperature to remove the zona pellucidae.[10] The Pronase was prepared in Hanks balanced salt solution without bicarbonate. Dissolution of pellucid membranes was complete in 3 to 5 minutes. After removal of the zona pellucidae, the ova were washed several times in culture medium to remove any trace of Pronase. After sufficient washing, the ova were repeatedly pipetted through a micropipette (inside diameter approx. 50μ), which facilitated blastomere separation. Blastomeres of two-cell ova separated quite readily; in most cases, four-cell ova separated into two two-cell components and, with additional pipetting, separated into four single blastomeres. The eight-cell ova rarely separated into two four-cell components; generally, they separated irregularly, one or two cells at a time. Pipetting was continued until the desired number of cells remained (four), and the cells that were "shelled off" were reassembled in aggregates containing four blastomeres or explanted as four separate cultures, each containing a single blastomere. Versene (EDTA) was initially employed to facilitate blastomere separation; however, this practice was discontinued when toxic effects were observed.

The component parts of two-, four-, and eight-cell ova that were cultured and the number of blastomeres in these parts are given in Table 2.

TABLE 2. EXPLANTATION PROTOCOL

| No. of Cells in Intact Ovum | No. of Cells in Fraction Explanted | | |
|---|---|---|---|
| | 1/2 | 1/4 | 1/8 |
| 2 | 1 | - | - |
| 4 | 2 | 1 | - |
| 8 | 4 | - | 1 |

In these experiments, no attempt was made to identify explanted blastomeres with their original intact ovum. Instead, blastomeres were randomly selected from a common pool in a microdrop that contained cells of the same cleavage stage. However, in one group of experiments, the two halves from the same intact ovum were explanted together in the same microdrop, along with a control; care was taken to prevent contact which might result in recombination or fusion into the original intact form. In all experiments, one or more intact ova, of the same developmental stage as that from which the blastomeres came, were deposited in each microdrop to serve as controls and for photographic demonstration.

## III. RESULTS AND DISCUSSION

A total of 717 halves from two-, four-, and eight-cell ova (Fig. 1-3) were cultured; their development efficiency is recorded in Table 3. Overall, 73.2% of these component parts developed into distinct morphological entities as morulae or blastocysts (Fig. 4-6). The percentage of components developing was noticeably higher for those isolated from the later cleavage stages. The percentages of components developing to blastocyst when isolated from two-, four-, and eight-cell ova were 41, 58, and 66%, respectively (Table 3).

TABLE 3. PERCENTAGES OF MORULA- AND BLASTOCYST-LIKE STRUCTURES DEVELOPING FROM "ONE HALF" OF TWO-, FOUR-, AND EIGHT-CELL MOUSE OVA

| Cell Stage at Time of Isolation | Total No. Blastomeres Explanted | Morula | Blastocyst | Total % |
|---|---|---|---|---|
| 2 | 427 | 102 (24%) | 176 (41%) | 65.1 |
| 4 | 199 | 51 (26%) | 117 (58%) | 84.4 |
| 8 | 91 | 19 (20%) | 60 (66%) | 85.7 |
| Total | 717 | 172 | 353 | 73.2 |

Some difficulty was encountered in consistently demonstrating development of single blastomeres from both four- and eight-cell ova. In one series of experiments, seven of eight blastomeres from an eight-cell ovum developed into the characteristic "trophoblastic vesicles" as described by Tarkowski and Wroblewska.[5] These structures contained no visible inner cell mass (Fig. 7).

In the intact ovum, four cleavage divisions can be observed quite readily. Immediately after the third division, there seems to be a fusion of blastomeres that prevents further separation and also makes the number of subsequent intermitotic divisions difficult to ascertain. However, by careful examination, one can estimate with reasonable accuracy the total number of cells and, in turn, determine the number of mitotic divisions prior to blastulation. We gave particular attention to the number of cleavage divisions each component ("one half" of two-, four-, or eight-cell ova; 1/4 of four-cell ova; and 1/8 of 8-cell ova) underwent. This phenomenon is illustrated in Figure 8.

When one blastomere is isolated from a two-cell ovum and grown in vitro for 72 hours, it undergoes five mitotic divisions. Blastomeres from four-cell ova and four from eight-cell ova undergo four and three mitotic divisions, respectively. The total number of cells in each of the components is exactly equal at the time of cavitation, which occurs between the fifth and sixth mitotic division.

Those structures developing from single blastomeres of four- and eight-cell ova underwent four and three mitotic divisions, respectively. In this case, the single blastomere from the four-cell ovum contained no more than 16 cells at the end of the in vitro culture period; those blastomeres from eight-cell ova contained a maximum of eight cells. Irrespective of the number of cells, after the fifth mitotic division (counting from the intact ovum) cavitation proceeded. Blastomeres developing as an aggregate in the intact ovum or separated into component parts appear to have an inherent ability to cavitate after a predetermined number of intermitotic divisions.

Structures that developed from a single blastomeres from eight-cell ova never contained embryonic inner cell mass. These findings seem to substantiate the interpretations proposed by Tarkowski and Wroblewska.[5] Any particular cell can become inner cell mass or trophoblast, depending on whether it lies inside the cell mass or on the periphery of the mass. In the case of the "trophoblastic vesicles" formed from single blastomeres of eight-cell ova, a maximum of eight cells are formed from three mitotic divisions after isolation from the intact eight-cell ovum. When cavitation occurs, this presents a difficult situation from the standpoint of geometry alone. Upon secretion of the blastocoelic fluid, resulting in the formation of the blastocoele, the number of cells is insufficient to form a cell-lined cavity and still "cut off" or "trap" inner cells that would constitute the embryonic inner cell mass.

In separating eight-cell ova into two four-cell halves, blastomeres were particularly difficult to remove other than by "shelling off" one, two, or three blastomeres at a time until only four blastomeres remained. Those blastomeres "shelled off" frequently showed obvious damage upon reaggregation, and, in most cases, deteriorated or became necrotic shortly after explanation. This gave rise to the possibility that only ventral components were being explanted, which would account for the preponderance and increased incidence of "blastocyst-like" structures developing from the "half eggs" of four- and eight-cell ova. However, experiments consisting of explanting pairs of blastomeres from four-cell ova, in which blastocysts developed from each half with visible inner cell mass, indicated this was not the case. This also seems to indicate that no regional determination has occurred at the four-cell stage. Attempts to achieve comparable results from each four-cell component of eight-cell ova were not as successful. However, a limited number of four-blastomere pairs from eight-cell ova did develop into smaller, but quite normal-appearing, twin blastocysts (Fig. 5).

The mechanism of monozygotic twinning is poorly understood in the rodent and other mammals. Transfer experiments are being conducted to determine if identical twins can arise from the fission of two-, four-, and eight-cell ova after development to blastocyst in vitro.

The importance of the ovum culture technique for experimental twinning studies has been indicated.[5] Tarkowski and Wroblewska[6] mentioned that naked blastomeres transferred to the oviducts of mice did not survive and could not be recovered for some unknown reason. One of us[11] transferred 336 separated blastomeres from 168 two-cell rat ova into the oviducts of 51 recipient animals and observed no implantation sites on females autopsied or living young from females allowed to go to term. Two-cell ova deprived of the zona pellucidae likewise failed to develop, whereas 75% of intact two-cell controls resulted in the birth of living young. In this case either the importance of zona pellucidae or developmental failure attributable to Pronase damage is indicated.

Living young produced from each half of an eight-cell ovum would prove conclusively that the blastomeres of the eight-cell ovum are completely equipotent and that regional determination occurs at later stages of development. The fact that Tarkowski[3] and Seidel[1,2] demonstrated that single blastomeres of the two-cell ovum could give rise to living young, and interpretations drawn from the results of this study, do not rule out the possible existence of another mechanism of twinning. On several occasions, Spears has noted a subsequent separation into two quite normal-appearing structures after the fusion of early preimplantation ova (two-, four-cell). The work of Mintz[4] and Tarkowski[18] with chimeric embryos suggests the possibility that complete integration of genotypes could result, with the subsequent fission into two embryos which would be indistinguishable because of this complete genetic integration prior to differentation. Experiments are being planned to test this hypothesis.

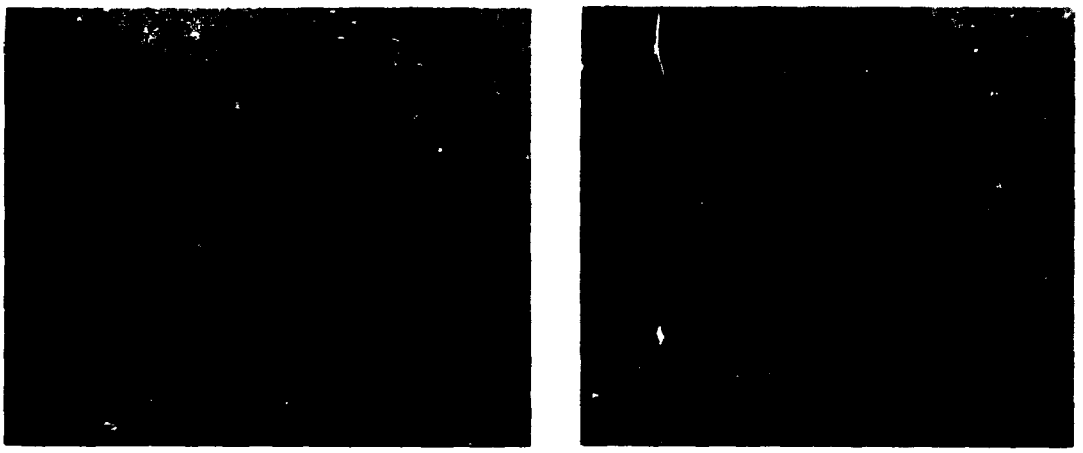

FIGURE 1. Twin Blastomeres from a Two-Cell Ovum with Intact Control. A, at the time of explantation; B, after 28 hours in vitro. 200X

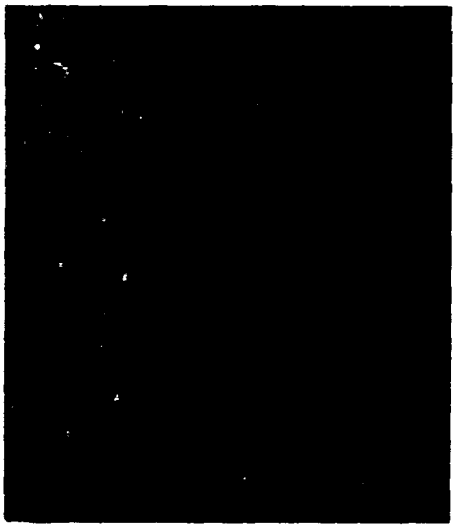

FIGURE 2. Twin Components from a Four-Cell Ovum with Control at the Time of Explantation. 200X

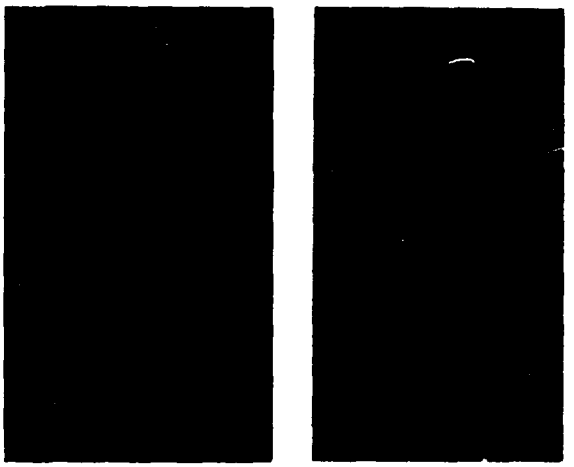

FIGURE 3. Twin Components from an Eight-Cell Ovum with Control at the Time of Explantation. 200X

FIGURE 4. Twin Morula Typical of Those Developed from One-Half of Two-, Four-, or Eight-Cell Ova. This particular pair developed from halves of a four-cell ovum after 30 hours in vitro. Control is in the center. 200X

FIGURE 5. Twin Blastocysts Developed from Halves of an Eight-Cell Ovum after 30 Hours in vitro. Two control ova are present. 200X

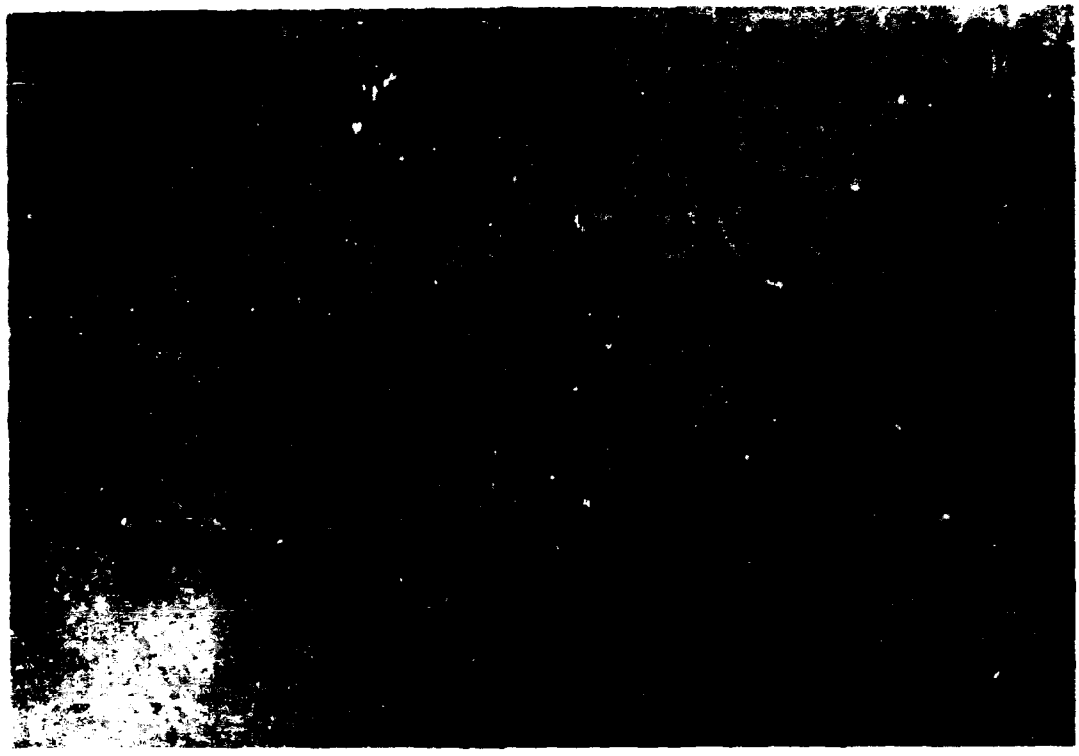

FIGURE 6. Twin Blastocysts Developed from Structures (Morula) Shown in Figure 4. Photographed the following day after 45 hours in vitro. Control embryo is beginning to hatch. 200X

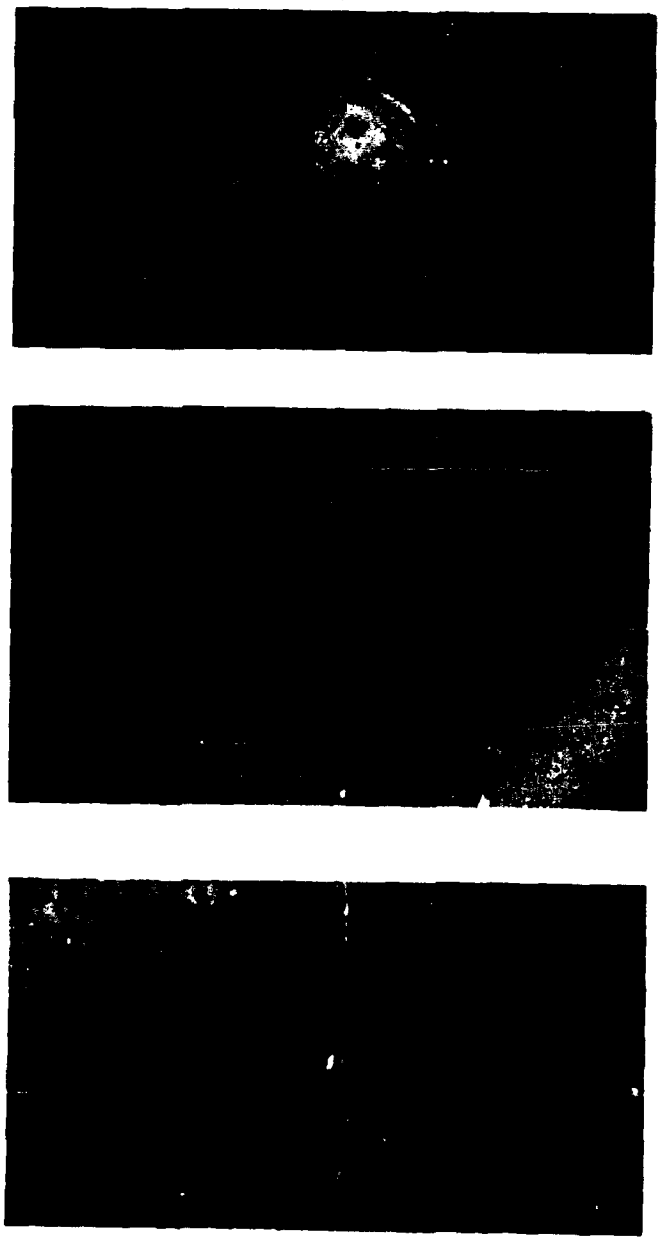

FIGURE 7. Trophoblastic Vesicles Developed from One Blastomere of an Eight-Cell Ovum. 200X

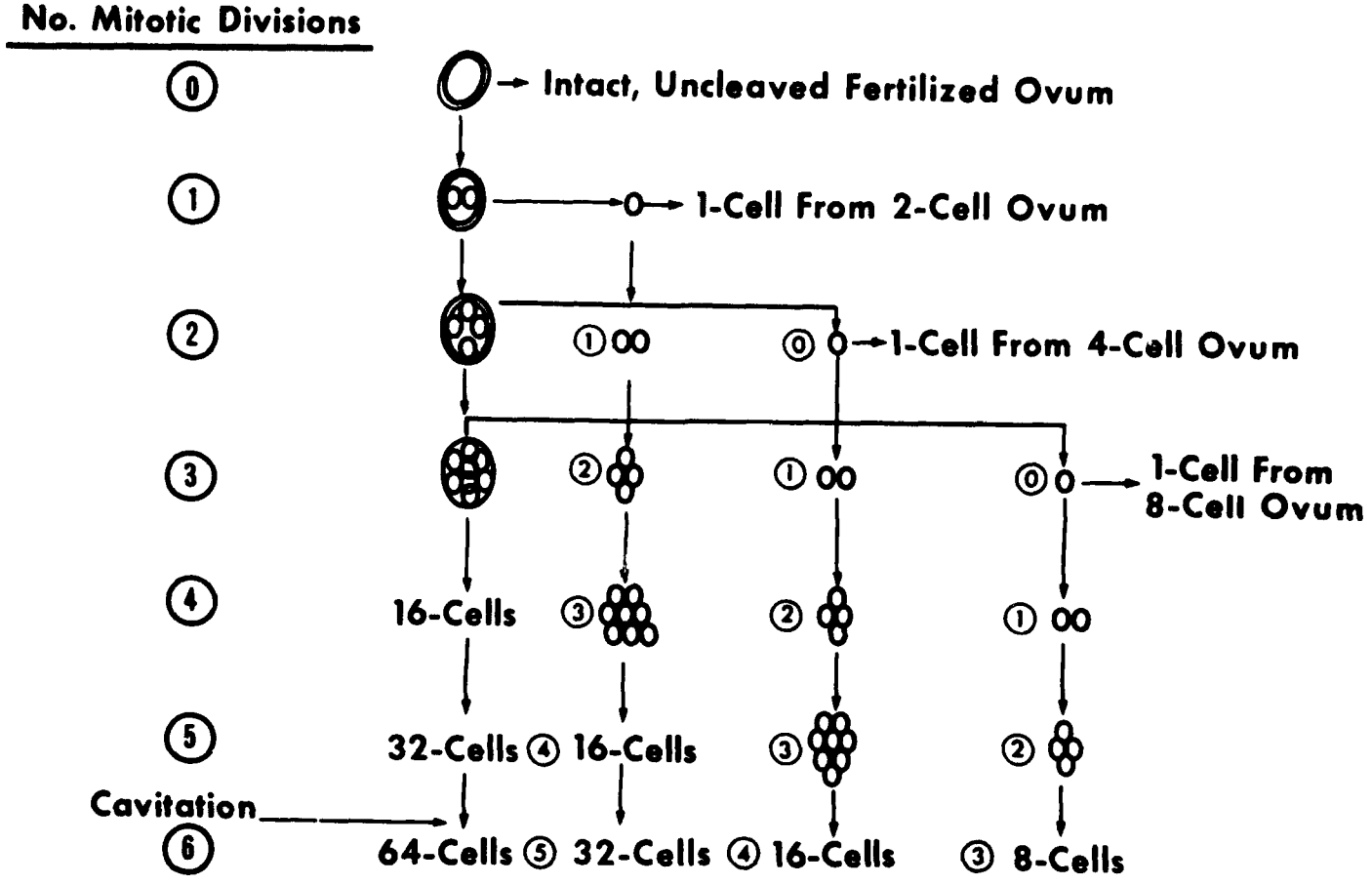


FIGURE 8. The Inherent Ability of Blastomeres to Cavitate after Five Mitotic Divisions, Regardless of the Total Number of Cells Present or the Cell Stage from which Single Blastomere was Isolated.

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13. ABSTRACT

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