STUDIES IN THE DEVELOPMENT OF THE OPOSSUM DIDELPHYS VIRGIANA L.

I. HISTORY OF THE EARLY CLEAVAGE

II. FORMATION OF THE BLASTOCYST

CARL GOTTFRIED HARTMAN

Contribution from the Zoological Laboratory of the University of Texas, No. 126

SIX TEXT FIGURES AND ELEVEN PLATES

CONTENTS

PART I. HISTORY OF THE EARLY CLEAVAGE

1. Introduction.....	2
a. Statement of the problem.....	2
b. Review of the literature.....	3
1) The work of Caldwell and Selenka.....	3
2) Cleavage and blastocyst formation in <i>Dasyurus</i> as described by Hill.....	6
c. Material and technique.....	9
1) Material.....	9
2) History of the animals.....	10
3) Securing of the eggs.....	12
4) Fixing and staining.....	14
2. Maturation and fertilization.....	15
a. The mature ovarian ovum.....	15
b. The tubal ovum.....	16
c. Appearance of young uterine eggs.....	19
d. The pronuclear stage.....	21
3. The first cleavage.....	22
a. The first cleavage spindle.....	22
b. The 2-celled stage.....	23
c. The elimination of yolk.....	23
4. The 4-celled stage.....	24
a. The orientation of the blastomeres.....	24
1) Shifting of the blastomeres.....	24
2) Consequences of shifting of blastomeres.....	27
b. Selenka's 4-celled stage.....	28
c. The distribution of yolk.....	28

5. The 8- to 16-celled stages.....	30
a. Third and fourth cleavages.....	30
1) General statement.....	30
2) Cleavage indeterminate.....	31
3) The 16-celled egg a blastocyst.....	33
b. The eliminated yolk.....	37
c. Absence of polar differentiation.....	38

PART II. THE FORMATION OF THE BLASTOCYST

6. The early unilaminar blastocyst.....	42
a. The just completed blastocyst.....	42
1) Formation of the blastocyst.....	42
2) First appearance of polarity.....	43
3) Inclusions within the cavity.....	44
b. Cells included within the blastocyst cavity.....	46
7. The formation of the entoderm.....	49
a. Entoderm formation as conjectured by Selenka.....	49
b. Entoderm formation in <i>Dasyurus</i>	50
c. Formation of entoderm in the opossum.....	52
d. Comparison of early and later blastocysts and growth of egg.....	56
8. General summary.....	58

INTRODUCTION

a. Statement of the problem

The desirability of increasing our knowledge of early marsupial development has frequently been emphasized by students of mammalian embryology. The almost yolk-free condition of the Eutherian egg, with its consequent holoblastic type of cleavage, is believed to have developed secondarily from a telolecithal and distinctly meroblastic ovum. This latter condition obtains in the monotreme egg, which may be considered transitional between the Sauropsida and the two higher classes of Mammalia. The large yolk-mass and the shell of the marsupial egg as well as other considerations make it highly probable that the Marsupials are intermediate between the Monotremes and the true Placentalia. It is reasonable, therefore, to expect the study of the marsupials to contribute to the solution of many problems in mammalian embryology which have arisen from the already very extensive study of Eutherian development.

Observations on the early development of marsupials have been very few. Up to 1910, when Hill published his excellent

account of the cleavage of the egg of *Dasyurus*, our knowledge of the early stages of marsupials rested largely on Selenka's ('87) work on the opossum. Because of the prominence of this author's famous 'Studien, in which the account was published, Caldwell's ('87) interpretation of the outer envelope of the marsupial egg as the shell membrane was completely overlooked until recalled by Hill. Inasmuch as his illuminating results cast doubt upon the validity of Selenka's interpretations, as well as the accuracy of his descriptions, which seemed to be based upon insufficient material, it seemed desirable to study the American marsupial in this locality where sufficient fresh and normal material could be secured. The problem was suggested to the writer by Dr. J. T. Patterson and the work was done under his direction. I am pleased gratefully to acknowledge his helpful suggestions throughout the progress of the work.

b. *Review of the literature on the early history of the marsupial egg*

1) *The work of Caldwell and Selenka.* In 1883-4 Caldwell collected a considerable amount of early marsupial and monotreme material in Australia. In 1884 he published several preliminary communications in which he recorded the discovery of the yolk mass of the monotreme egg. This discovery greatly increased Selenka's interest in marsupial embryology, in the study of which he had been for sometime engaged when Caldwell's paper appeared. Caldwell must also be credited with the discovery of the 'shell' of the marsupial egg; for he correctly homologized the "thin transparent membrane, 0.0015 mm. thick," of the marsupial, *Phascolarctus*, with the shell membrane of the monotreme egg. This interpretation Selenka completely ignored, for he mistook the shell membrane of the opossum for the corona radiata.

After several years of fruitless experiments in attempting to rear Australian and Brazilian species, Selenka imported a hundred or more individuals of the 'hardy' North American species, *Didelphys virginiana*, two females of which furnished the 16 eggs that formed the basis of his account (Selenka, '87) of cleavage and blastocyst formation.

He found that cleavage begins almost exactly 5 days after copulation (compare Hill and O'Donoghue's 'post-oestrus' period¹) and that birth takes place 7 days and 20 hours later. The author states that all of the 7 to 27 eggs or embryos found in any one animal are practically in the same state of development. No external indication of pregnancy, no changes even in the mammary glands, were to be noted until near the time of parturition.

According to Selenka, fertilization takes place in the upper part of the oviduct and cleavage begins in the uterus, the eggs scattering and remaining for three days floating in the uterine fluid. Maturation was not observed, although sections of ovaries were made and nine eggs were taken from the Fallopian tube.

Except for a few unessential details, Selenka correctly described the unsegmented egg of the opossum. His specimens were for the most part shrunken, as indicated by the shape of the ova and the 'perivitelline space' between the zona pellucida and the albumen layer, a condition I have not found in well-fixed normal eggs.

The first of Selenka's two specimens yielded one 2-celled and one 20-celled uterine egg and nine unfertilized eggs from the oviduct, all badly shrunken and of little value for study. It is interesting to note, however, that the 2-celled egg showed some elimination of yolk and that the 20-celled stage was practically a young blastocyst and contained an included ('entoderm') cell.

The second specimen furnished eggs in the following stages: one each in the 4-, 8-, 42- and 68-celled stages respectively; 2 young blastocysts containing some entoderm; and 8 advanced blastocysts ranging from 1.05 to 1.43 mm., the largest showing a layer of mesoderm in the embryonic area.

The blastomeres of the 4-celled stage were conical in form and of equal size and structure. They are radially arranged about a cleavage cavity. The 8-celled stage is made up of two superimposed rings of four cells each. From these two specimens Selenka concluded that the blastomeres of the 4-celled stage

¹ Hill and O'Donoghue ('13), p. 145.

maintain their radial arrangement, that is, they do not shift as is the case in the Eutherian egg. With these two stages before him the author could not avoid the conclusion that the third cleavage is horizontal, and in view of polar differentiation of his next, or 42-celled stage, he expresses the opinion that the third cleavage is qualitative, dividing the four blastomeres into an upper ring of ectodermal and a lower ring of entodermal cells.

The young 'gastrulae,' as Selenka calls the 42- and 68-cell stages, are unilaminar blastocysts in which a polarity is to be noted, in that the cells at one pole are larger and more yolk-laden than those of the other pole. The larger cells Selenka considered entodermal, i.e., lineal descendants of the lower ring of the 8-celled stage just described. In each case there was found within the blastocyst cavity a large included cell, which the author called the 'Urentodermzelle.' Hence the implication is that the entoderm is formed from the lower half of the blastocyst and from cells included within the cavity. Such an interpretation will be shown in this paper to be incorrect, as was already suggested by Hill in his study on the egg of *Dasyurus*.

The next blastocysts described by Selenka evidently suffered greatly in preparation and were drawn rather diagrammatically; hence I am unable to make any helpful comparisons between them and my own specimens. No light is thrown upon the manner of entoderm formation by these two specimens.

The other four blastocysts figured by Selenka are older, as indicated by the progress in the absorption of the albumen. The wall of the youngest of these is didermic for two-thirds of its surface area. In the oldest of the four, which is 1.45 mm. in diameter, the mesoderm and the primitive streak are already laid down. The six blastocysts intermediate between these two are fully formed didermic specimens and were faithfully described by Selenka. It is these blastocysts with the entoderm almost or quite completed of which Hill says: "Of Selenka's early material, I think it is these blastocysts alone which had any chance of giving origin to normal embryos."² It is my judgment that

² Hill ('10), p. 11.

the early blastocysts also represent normal stages of the opossum embryos. With so little material before him, some of which was indeed not entirely normal, it is no wonder that Selenka should have wrongly interpreted in every essential detail the cleavage of the opossum egg.

Mention should be made in passing of a short paper by Minot ('11) in which the late bilaminar blastocyst of the opossum is described in some detail. The article, however, adds little, if anything, to the work of Selenka, which it entirely ignores. Indeed, the author fails to recognize the shell-membrane, as did Selenka, and in his interpretation of Hill's ('10) work falls into other minor errors. The article by Dr. Minot will be discussed in Part III of these studies, to be published in a subsequent number of this Journal.

2) *Cleavage and blastocyst formation in Dasyurus as described by Hill '10*). Inasmuch as Professor Hill's thorough study of the Australian 'native cat' furnished a strong impulse for the study of the development of the opossum and since comparisons will be made in this paper between the two forms, it is deemed desirable to review in some detail the developmental processes as described by the British author.

The ovarian ovum of *Dasyurus*, aside from its much greater size and larger amount of yolk as compared with Eutherian ova, exhibits nothing unusual before the period of maturation. During the period of growth the ovum is more or less homogeneous throughout, or perhaps somewhat centrolecithal. But at the time of formation of the first polar body, which is given off in the ovary as in the Eutheria, the egg takes on a striking polarity through the accumulation of surplus yolk at the animal pole of the egg. The egg maintains this condition during its passage through the oviduct, in which it adds the albumen layer and the shell and where insemination takes place. The pronuclei and the first cleavage spindle lie in the granular formative cytoplasm, which occupies about two-thirds of the egg and which appears in longitudinal section as a broad crescent, the two horns of which partly envelop the yolk mass (text fig. 4A, see page 34).

The first cleavage plane is, of course, meridional and in this first division the cytoplasm of the two blastomeres round up in such a fashion as to cast off bodily the entire polar mass of yolk. The second cleavage plane which is also meridional, passes at right angles to the first, leaving the yolk body partly enclosed by the upper, more pointed apexes of the four blastomeres. These are, therefore, radially arranged and do not shift their position, as do the blastomeres of *Eutheria* in the corresponding stage. The third cleavages are again meridional, dividing the egg into eight equal blastomeres arranged in an open ring near or a little above the equator of the egg. Each cell again exhibits a well-marked polarity, both in greater deutoplasmic content of the lower pole as compared with the upper, and in the presence of the nucleus somewhat toward the upper pole.

In view of the subsequent history of the egg the author is fully justified in calling the upper the formative and the lower the non-formative pole of the egg; for the fourth cleavage is horizontal and divides the eight blastomeres quantitatively and qualitatively into an upper ring of eight, smaller, lighter-staining cells and a lower ring of larger, more darkly staining cells. All of these facts are fully evidenced by a set of superb photomicrographs. According to the author's interpretation, the upper ring of cells is destined to form the embryonic portion of the blastocyst; the lower, the non-formative portion.

For a time the rapidly multiplying cells of each ring occupy a more or less clearly horizontal position somewhat above the equator of the egg. They soon reach the periphery, however, that is, they become applied to the inner surface of the shell, and the formative cells proliferate toward one pole, the non-formative cells toward the opposite pole of the egg. The cells finally meet at the poles, and thus complete the unilaminar blastocyst. By this time the entire albuminous layer has disappeared, but considerable yolk still remains in the cavity. During cleavage the egg grows from about 0.35 mm. to about 0.45 mm. in diameter and the shell becomes somewhat thickened (text fig. 4 *G*).

Following this stage there is a period of growth during which the blastocyst maintains the unilaminar condition. The polar differentiation previously noted now disappears, for the cells of the entire blastocyst are found to have the same structural appearance both in surface view and in section (text fig. 4 *I*).

It is not until the blastocysts have attained a diameter of 3.5 mm. that the polarity again becomes evident structurally. At this time the cells of the formative ectoderm become differentiated, and a little later the entoderm formation begins. The upper formative and lower non-formative areas, of which the latter is usually the larger, are soon separated by a 'sutural line,' The formative cells on one side of the line are usually larger and more lightly staining; those below the line are smaller, more uniformly distributed and possess dark perinuclear zones.

The next stage is that of entoderm formation, which occurs in blastocysts a little more than 3.5 mm. in diameter. The entoderm arises by a proliferation of modified cells of the formative entoderm, the 'entodermal mother-cells.' These appear at various points in the formative region and seem both to migrate out of their position in the epithelial layer of the entoderm and then multiply, and to maintain their position and give off entodermal cells by proliferation. These cells as well as the daughter cells differ markedly in their darker staining qualities from the surrounding ectodermal cells. The young entodermal cells send out pseudopodia-like prolongations, which unite and form a fenestrated entodermal layer. This later closes up to form the primitive entoderm, which spreads until it lines the entire blastocyst cavity.

It should also be noted that the author homologizes the non-formative portion of the marsupial blastocyst with the trophoblast of the Eutheria, the embryo of the former, therefore, being without trophoblastic covering.

The work by Professor Hill here reviewed is the first connected account, supported by a sufficient number of stages, of the early development of a marsupial. It renders Selenka's views untenable and makes questionable some of the stages found by him.

Briefly stated, the present paper, together with one soon to follow, will show that the development of *Didelphys* differ from that of *Dasyurus*, first in its indeterminate type of cleavage, and second in the delay of structural evidences of polarity (aside from the clue offered by the position of the polar bodies) up to the time of entoderm formation. Agreement is found in the presence of large quantities of yolk in the egg and in the manner of entoderm formation. Other points of minor importance will be discussed in the body of the paper.

c. Material and technique

1) *Material.* The specimens of opossum eggs and blastocysts on which the present study (as well as two other articles shortly to appear) is based, represent collections made during three seasons, but especially during the winters of 1913-1914 and 1914-1915. During the first season, 1912-1913, the collections made resulted chiefly in establishing the breeding season of *Didelphys* for this locality. About 140 females were used, some of which were killed from time to time during the first season. The large number of specimens required to yield the desired stages is due, in part, to certain difficulties which were at first experienced in breeding the animals in captivity. It is my intention to say more concerning this matter in a subsequent publication on the physiology of reproduction of the opossum.

The present account deals with the unsegmented eggs, cleavage stages and blastocysts taken from twenty different animals. This number includes Female No. 112 which furnished only unfertilized and degenerating eggs; but the specimen is here added for a special purpose, as will appear below. 415 eggs were removed from the uterus or the oviduct of these animals, making an average of 22 eggs from each animal. The extremes in number were 1 and 45. No. 76 yielded 10 eggs from the oviduct on one side only and No. 144 yielded 11 from a single uterus; No. 117 discharged 43 eggs from the right ovary exactly one month after the left ovary had been removed.

Of these 415 eggs 230 were cut into serial sections five or six micra thick, mounted and stained. A few were mounted in balsam; some were lost in the handling and a few have been retained in 80 per cent alcohol. Perhaps one-sixth or one-seventh of the eggs taken from inseminated animals were clearly unfertilized and showed signs of degeneration.

Besides these, eggs were removed from 37 other females, so that about one thousand ova were handled in the progress of the work. In all of these last mentioned cases the eggs were unfortunately degenerate, not being fertilized, and were therefore usually not counted but discarded as worthless. Foetuses or pouch young were secured in several dozen cases.

Old degenerating eggs can easily be recognized. They are large and the shell is opaque and covered with a white incrustation. Later the shell collapses and the egg becomes a shapeless mass which sooner or later leaves the uterus. Young degenerate eggs in which little change can be noted in the shell or in the albumen may usually be recognized by the flattening of the ovum in the center. In side view the protoplasm (in which degeneration first becomes apparent) assumes the shape of a crescent, to be seen especially well in alcoholic specimens fixed in osmic acid mixtures.

A detailed account of each animal furnishing the eggs described in this study follows. Unless the time of capture is indicated it is understood that the animals were bred in captivity. The captives were kept in cages from a few weeks to two months or more. Nos. 40 to 58 were secured in 1914; Nos. 76 to 144 in 1915.

2) *History of animals.* No. 21. Copulation January 3; killed January 6; ovarian eggs only.

No. 40. Placed with male January 31; killed February 6; 11 eggs; unilaminar blastocysts with formation of entoderm; three eggs unfertilized.

No. 43. Placed with male January 28. Male removed February 2; killed February 7; 29 eggs: several late unilaminar blastocysts, mostly later bilaminar blastocysts, 0.8 mm. to 1.0 mm. in diameter; 6 large unfertilized eggs.

No. 46. Killed February 8; ovulation recent, as indicated by prominent and blood-shot stigmata on young corpora lutea; 21 small eggs;

3 in the 2-celled stage, 2 in the 4-celled stage, 2 in the 5-celled stage, 7 unfertilized, the remainder unaccounted for.

No. 50. Placed with male February 1; removed male February 2; replaced male February 5; killed February 10. 24 eggs: two 2-celled, two 4-celled, 10 blastocysts of about 50 cells, 2 unfertilized, 8 unaccounted for.

No. 52. With male February 4 to 6; killed February 10. 25 young (very small) eggs: one 4-celled stage; 3 in first cleavage; 14 unsegmented, 6 of which show pronuclei; the remainder unaccounted for.

No. 54. Killed February 13. About 25 small eggs: one 2-celled stage; one 4-celled stage; 2 in first cleavage; 11 undivided, of which 5 show pronuclei; 1 abnormal; 1 egg contained only shell and albumen, the place of ovum being taken by a mass of epithelial cells. Other eggs from this batch showed a number of epithelial cells from the oviduct. The remainder lost and unaccounted for.

No. 55. With male February 6; copulation probably February 7; killed February 13. 22 eggs: all advanced, bilaminar blastocysts 0.8-0.9 mm. in diameter, except 6 unfertilized eggs.

No. 56. Killed February 14. About 20 eggs removed from the Fallopian tubes, all devoid of albumen layer and shell; that is, just as passed from the ovaries.

No. 58. With male February 4; isolated February 8; killed February 15. Very young fresh corpora on ovary. Small, thin-shelled eggs, all unsegmented and unfertilized, but normal and just passed into the uterus.

No. 76. Caught between January 13 and 15; killed January 16. Both ovaries had discharged their follicles, but eggs were found in only one oviduct. These eggs, 10 in number, were almost exactly like those from No. 56.

No. 81. Caught January 18 and killed next day. About 20 eggs: all 4-celled with the exception of 3 or 4 unfertilized eggs.

No. 82. Caught January 18 and killed next day. About 25 eggs: almost fully formed bilaminar blastocysts with the exception of 6 unfertilized eggs.

No. 83. Caught January 18 and killed next day. About 9 eggs: 1 unsegmented; four 4-celled stages exactly like those of No. 81; 3 blastocysts of about 50 cells, like those of Nos. 50 and 88.

No. 85. Caught January 18 and killed the next day. About 25 eggs: one each in the following stages: 6, 7, 9, 10, 12, 14, 15, 17, and 18 cells; three of 8 cells; five of 16 cells and one abnormal egg.

No. 88. Caught about January 18 and killed January 20. Number of eggs not recorded, but 27 were sectioned and mounted, all young blastocysts of 30-55 cells, except 6 abnormal eggs.

No. 94. Killed January 21, several days after capture. A single advanced bilaminar blastocyst, like those of Nos. 43, 55, and 82 was taken.

No. 112. Secured in December. February 11, successful laparotomy performed, corroborating previous diagnosis of pseudopreg-

nancy; recovery perfect. March 2, all external indications of pseudopregnancy disappeared. March 14, approaching ovulation indicated. March 19, 2 p.m., left uterus removed and 20 eggs found. Animal was killed at 8 p.m. and 17 eggs recovered from the right uterus. Eggs varied in size and were in different stages of disintegration; but the case is here cited because one egg showed a '4-celled' stage, abnormal as I am convinced, but comparable in a way with the 4-celled stage of the opossum as figured by Selenka and with the normal 4-celled egg of *Dasyurus*.

No. 117. February 13, the female having shown external signs of ovulation, the left ovary and uterus were removed. 20 degenerate eggs were found. By March 2 all external signs of pseudopregnancy had disappeared. March 17, the sexual period being again indicated. animal was killed and 43 eggs, small in size, were removed from remaining uterus. The eggs included the following stages: twelve unsegmented (several of these showed signs of beginning disintegration); two 2-celled; two 4-celled (2 of these were apparently abnormal); one 5-celled; two 6-celled; two 8-celled (one of these had seven cells in mitosis); two 13-celled; one 14-celled; one 15-celled; three 16-celled.

No. 144. February 20, signs of pseudopregnancy. March 16, signs of second (or third?) sexual period approaching. March 20 killed. 11 eggs removed from one uterus only and over-fixed in Carnoy's fluid: one 12-celled stage, other eggs are young blastocysts like those of Nos. 50, 82, and 88, or somewhat more advanced.

3) *Securing the eggs.* On opening the body cavity of the female the ovary was first examined, from the condition of which the time that has elapsed since the discharge of the eggs may be roughly estimated. Prominent blood-shot stigmata indicate recent ovulation; a smooth surface with deep-seated dingy-yellow corpora shows that ovulation was not recent. If in the latter condition the uterus lacks fullness and turgidity, old degenerate eggs are likely to be found.

To secure the eggs the uterus is placed in a shallow vessel containing Ringer's solution at 40° C. and the muscular wall is slit longitudinally. The intrauterine pressure at once forces out the enormously swollen mucosa, which is richly supplied with blood vessels and convoluted uterine glands. The uterine mucosa is next gently pulled apart with two pairs of forceps, exposing the lumen of the organ, and in the folds of the mucosa the eggs may easily be seen with the aid of the binocular microscope, and picked out with a pipette. Old, encrusted eggs can easily be seen with the naked eye. Blastocysts of about 0.8 mm.

diameter are harder to see than the much smaller unsegmented eggs, for the former are perfectly hyaline and the latter possess a white core, the egg proper, surrounded by the transparent shell and albumen layer. After picking out all of the eggs which can be found by a search among the folds of the mucosa, the uterus is turned inside out and shaken in the dish of Ringer's solution. In this way usually several eggs are recovered which would otherwise be overlooked. A few cubic centimeters of fixing solution, such as Bouin's, added to the solution will facilitate the finding of eggs in the bottom of the dish. They can be located by means of the peculiar way in which they roll when agitated by a gentle stream from a pipette, for the eggs differ in this respect from tissue fragments which collect at the bottom of the dish.

Eggs were forced out of the Fallopian tubes by means of a stream of Ringer's solution sent through the tube with a fine-pointed glass pipette.

Aseptic operation was resorted to in about twenty cases in the hopes of securing two stages from the same animal. As opossums have a uterus duplex, they are well adapted for such experiments, for the removal of the uterus and the ovary of one side is a comparatively simple matter. The plan has the obvious advantage of enabling one to note the time of development with accuracy. If unfertilized eggs are found, moreover, the animal may be allowed to come into heat a second time, which happens about one month after the first. Recovery is prompt, and the wound usually heals perfectly.

Unfortunately, in these experiments unfertilized eggs were found in most cases at the time of the first operation. Thus from female No. 109, the left uterus was removed 6 days and 4 hours after copulation and 23 eggs recovered. Six hours later the other uterus was removed and 22 eggs recovered. Both lots, however, proved to be unfertilized and the eggs had begun to break up. Greater fortune, however, attended the study of female No. 114. This animal was operated on 5 days and 4 hours after copulation. The eggs had just been shed, as indicated by fresh stigmata on the ovary, but the eggs were not found, prob-

ably having been lost in the body cavity. Ninety-two hours later the animal was killed and embryos in the eleven somite stage were found in the remaining uterus. Female No. 117 was operated February 13, and 20 degenerate eggs were taken from the left uterus. On March 17, the remaining uterus furnished 3 eggs. The ovary was much enlarged and densely studded with large corpora lutea. The eggs were mostly fertilized. In the remaining cases, even where copulation was not observed, eggs were nearly always found, both after the first and the second oestrus periods, but were unfertilized and hence worthless.

Nos. 81 to 88 were killed in the field, where aseptic operation was impossible.

4) *Fixing and staining.* Four fixing fluids were most often used: Carnoy's, Bouin's, Fleming's and Hill's. Carnoy's fluid gave only fairly good results. Eggs fixed in Bouin's solution always proved usable: shrinkage does not occur, but the cell-elements are not so well fixed as with Hill's mixture. Fleming's solutions, both weak and strong, fix beautifully but cause shrinkage of the egg. The larger blastocysts invariably collapse in this fluid. I have found Hill's picro-nitric-aceto-osmic mixture to be a perfect fixing fluid for the opossum egg; it penetrates the tough shell of the egg rapidly and fixes the cell structures without causing shrinkage. The osmic acid stains the fat granules black in this as efficiently as does Fleming's solution. Hill's solution is made as follows: Mayer's picro-nitric acid, 96 cc., 1 per cent osmic acid 2 cc.; glacial acetic acid, 2 cc. Some specimens of every batch of eggs, except those of No. 144, were fixed in this last mentioned mixture.

It is important that the eggs be carried up into the higher percentages of alcohol by degrees to prevent collapse of the egg envelopes. Since the shell prevents penetration, this envelope may advantageously be removed with fine pointed needles. In the case of older blastocysts a hole may be punched in one side.

Toto preparations were stained in Delafield's haematoxylin and sections in iron-alum haematoxylin; the latter were counter-stained with orange G or eosin. A few sections of blastocysts were stained in safranin.

In order to determine with accuracy the arrangement of the blastomeres in 4- to 16- celled stages, wax models were made after the method of Born.

MATURATION AND FERTILIZATION

a. *The mature ovarian ovum*

A detailed account of the ovarian egg is not now attempted. It is deemed desirable, however, to determine two points: first, the time at which the first polar body is given off, and second, the distribution of yolk in the ovarian egg.

In the case of two females studied, ovulation had not yet taken place three days after copulation. The Graafian follicles were tensely filled with liquor folliculi and bulged out like great glassy beads from the surface of the ovary. The sectioned ovaries were found to contain ripe eggs (fig. 2), as was to be expected; but younger and much smaller follicles taken from other females also contained eggs which had given off the first polar body. Hence, the first maturation takes place some days before ovulation. More exact data cannot be given at this time.

The ripe ovarian egg is broadly elliptical in form and measures in section on the average 0.165 by 0.135 mm. It is surrounded by a well-defined zona pellucida, within which the flattened polar body is found. The polar body is given off at one pole of the elongated egg or at the equator, or it may indeed be seen at any other point on the margin of a section passing through the long axis of the egg. The germinal vesicle of the egg has not yet re-formed preparatory to the second maturation, for the chromosomes are very short rods arranged in the form of an open ring. They lie in the cytoplasm just beneath the surface of the egg and usually to one side of the polar body (fig. 2).

Aside from the position of the polar body there is no other structural evidence of polarity in the ripe ovarian egg of the opossum. Of the many eggs studied in sections none showed any massing of yolk toward one pole such as is strikingly the case in the egg of *Dasyurus*. This lack of polar concentration of yolk in the mature egg of the opossum is the first important

difference to be noted between the Australian and the American species. Further details of the ripe ovum will be given in the next section which deals with the freshly discharged eggs.

b. *The tubal ovum*

The eggs of the two lots (Nos. 56 and 76) removed from the oviduct agree in all essential respects. They had apparently just entered the oviduct, for only a mere trace of albumen had been laid down on the surface of the eggs. The eggs are normally shed simultaneously from a given ovary and both ovaries discharge their eggs almost if not quite at the same time. In the case of No. 76 the eggs from one ovary were a trifle late and were lost in the body cavity, for, though discharged, they were not to be found in the oviduct.

The eggs discharged in the second or third oestrus period of any given animal, however, show greater variation in size and age. In one case an egg was found in the oviduct, while its fellows had already been for some time in the uterus. The ovaries of animals in the second oestrus usually contain some large unruptured follicles along with fresh corporea lutea. Hence, it is apparent that the discharge of eggs in the second oestrus is not as regular as in the first. The opossum has only one sexual season a year and normally (i.e., in the wild state) only a single oestrus period.

The tubal eggs are usually elliptical, rarely oval or spherical in shape (figs. 1 and 3), as seen both in the fresh state and in most sections. They measure on the slide about 0.137 by 0.115 mm., but range from 0.135 by 0.12 to 0.098 by 0.086 mm. The eggs of batch No. 76 are a little the larger, measuring on the average 0.146 by 0.123 mm. against 0.127 by 0.106 mm. for those of batch No. 56.

The egg is surrounded by a rather well-defined homogeneous membrane, the zona pellucida (fig. 3). In several cases this is completely surrounded or only partly covered by a thin layer of albumen laid down in several laminae. The zona can be easily distinguished from the albumen on account of its dense, homogeneous structure. In some eggs the zona is not so well recog-

nized, the outline of the egg being diffuse and not bounded by a definite line. The zona is 0.004 to 0.0008 mm. in thickness.

Beneath the zona pellucida is a layer, very light in color and with very fine radial striations, clearly seen under the oil-immersion lens. In most eggs this has a uniform width of about 0.0012 mm. Whether or not this is in any way homologous to the zona radiata I am not prepared to say.

The cytoplasm is very definitely marked off from the lighter 'zona radiata' just mentioned. In fact, in some specimens the line of demarcation is so definite as to amount almost to a membrane.

As seen in sections the cytoplasm of the egg may be divided into three more or less distinct regions (figs. 1 and 3). The first of these is a narrow homogeneous band of finely granular protoplasm, devoid of yolk granules or yolk vacuoles. The band is darker than other portions of the cytoplasm, except occasional islands of granular structure surrounding yolk vacuoles. The center of the egg consists of a similar, homogeneous cytoplasm, granular throughout, with only here and there a fat vacuole. The central area extends from the center about half-way to the circumference. Between the marginal and central homogeneous portions of the cytoplasm is a broad band of highly vacuolated, reticular protoplasm, very clearly seen, especially in the eggs of lot No. 76. The structure of this ring seems to be the most delicate of the entire egg, for in two or three specimens the cytoplasm is somewhat broken down at this place. The vacuoles exist, even in eggs fixed with osmic acid mixtures (fig. 1). Sections of such eggs show also a ring of fat spherules, large and small, scattered around the margin of the central yolk free area. The fat spherules are numerous and may occupy a large portion of the vacuolated zone (fig. 1). In the region of the polar body the granular cytoplasm may come to the surface; in other words, there is at this point a break in the vacuolated zone.

The first polar body and the egg nucleus are to be seen in every specimen sectioned. Neither has undergone any material change since the egg left the ovary. The polar body is very small, containing a mass of chromatin and a minimum of cytoplasm.

In one case the chromosomes of the polar body were arranged in the equatorial plate of a division spindle. The position of the polar body is, in the majority of cases, though by no means invariably, at one pole of the elliptical egg, and the egg chromosomes lie in the cytoplasm just within the surface of the egg, either exactly beneath the polar body, or, as is more often the case, to one side of it. In a considerable proportion of the eggs, the polar body is found at the equator of the egg, less often at various levels between the pole and the equator, a variation found to obtain also in ripe ovarian eggs.

In a few of the eggs, particularly those of batch No. 56, one very large fat vacuole (or black fat spherule if stained with osmic acid) occurs at one pole of the egg, usually opposite the polar body (fig. 4). Many eggs also show a peculiar body about the size of a large fat spherule. It is homogeneous in structure devoid of all granulation, takes a pink or lavender stain with iron-haematoxylin and is surrounded by a light band, as if it had shrunk away from the surrounding cytoplasm. Its position in the different eggs is variable. Similar bodies have been noticed in other mammalian eggs.

Aside from the position of the polar body there is no evidence of polarity in the tubal egg of the opossum, such as has been described for ovarian and unsegmented uterine eggs of *Dasyurus*. In only one case was there a preponderance of yolk granules at one pole as compared with corresponding yolk-free granular cytoplasm at the opposite or 'animal' pole.

Insemination of the ovum no doubt takes place in the oviduct; for after the egg has received the albumen layer and the shell, the latter forms an impenetrable barrier to the entrance of the spermatozoa. Direct observations, however, of normal insemination in marsupials is lacking. In this connection Hill ('10) says of the eggs of *Dasyurus*: "Apparently the ova are shed almost simultaneously, and they must pass with considerable rapidity down the tubes to the uteri where cleavage begins, for I have only once found a tubal ovum, and that one had evidently been retarded for some reason, and was polyspermic."³ Selenka

³ Loc. cit., pp. 22-23.

('87) states: "Der Eintritt der Spermatozoen in den Perivitellinraum geschieht im oberen Theile des Oviducts, wie die Anwesenheit derselben in den auf dieser Strecke vorgefundens Eiern genügend beweist."⁴ But it will be observed that the actual entrance of the spermatozoon was not seen by Selenka, nor does he anywhere mention the pronuclei.

The ovum becomes surrounded with an albumen layer and a shell on its descent down the oviduct (fig. 5, 3). The albumen is laid down in successive delicate lamellae, between which numerous spermatozoa are seen in some of the eggs; or epithelial cells from the oviduct may become included in the albumen. One case was noted in which the spherical mass of epithelial cells instead of an ovum became surrounded by albumen and shell. In another case an ovum and a mass of cells were both included, and in a third case, two eggs, each separately provided with some albumen, were enclosed together in a shell.

c. Appearance of young uterine eggs

The eggs of female No. 58 are, to all appearances, the youngest found in the uterus. They are all unfertilized but otherwise are normal and show little or no signs of disintegration. Since the egg protoplasm very rapidly breaks up into fragments of various sizes, we may assume that the eggs of this lot had just arrived in the uterus. The shell membrane is, moreover, only 0.001 mm. in thickness and is so delicate as to shrink into many folds which lie, in the fixed specimens, snugly against the sphere of coagulated albumen. The shell grows in thickness in the uterus, hence the thickness of the shell is roughly a criterion as to the age of the eggs. But this is true only in very general terms, for there is no uniformity of thickness of shell among different batches of eggs in the same cleavage state (see Summary of this paper). The eggs of various individuals may, of course, pass through the Fallopian tube at different rates, hence the eggs passing the more slowly would be provided with thicker shells on entering the uterus.

⁴ Selenka ('87), p. 110.

The yolk in the eggs of No. 58 is distributed more evenly and in smaller granules than in tubal eggs described above, but the three regions may be made out. These eggs, if freed of albumen and shell, would be almost exact counterparts of the eggs just entering the Fallopian tube (Nos. 56 and 76). In the preparations the shrunken shells measure 0.25×0.22 mm. in diameter; the ovum proper, however, measures 0.125×0.092 mm.; about the same as the eggs of Nos. 56 and 76.

The polar body and egg chromosomes occupy the same relative positions as in the tubal ova. There is normally no 'perivitelline' space, as described and figured by Selenka. Sometimes, indeed, the zona pellucida cannot be distinguished, the protoplasm of the egg grading off, somewhat abruptly it is true, into the albumen layer, and in these cases no zona can be seen. Among some 230 preparations only rarely do I find any space between the egg and the albumen, hence I believe that the 'perivitelline space' of Selenka is an artefact due to shrinkage. In several eggs of batch No. 54 the protoplasm was slightly shrunken away from the zona, not the zona from the albumen. Yolk is eliminated in the 2- and the 4-celled stages, and lies between the blastomeres and the zona. After this the blastomeres become closely applied to the albumen layer, with the zona pellucida still apparent between the two in some cases.

When observed in the fresh state in the uterine liquid or in Ringer's solution the young eggs resemble markedly a half-cooked grain of tapioca or sago. The shell and albumen form a hyaline, almost transparent envelope with a white granular, rather opaque central core, which is the egg proper. The shell is turgid and well rounded out. As the fresh egg may measure through the shell 0.4 to 0.5 mm. (e.g., *D* text fig. 4, page 34) the albumen layer is seen to be much thicker than that of *Dasyurus* and the study of the small ovum in the center correspondingly more difficult (fig. 4). In the fixing fluid the albumen becomes so opaque that the egg cell can scarcely be seen, especially when the fixing fluid contains no osmic acid to differentiate the egg by the black stain of its yolk. On prolonged immersion of the egg in alcohol, the albumen clears up somewhat, so that the

ovum may again dimly be seen in the center. The concentric lamination of the albumen is apparent in the fresh state and in sections.

d. *The pronuclear stage*

The eggs of lots Nos. 54 and 52, among which specimens of the pronuclear stage are found, are apparently young eggs which have just entered the uterus. The shell of batch No. 54 is 0.001 mm. in thickness, that of No. 52, 0.0034 mm.

The pronuclei at first lie to one side of the egg and are surrounded by an almost yolk-free area of considerable size (fig. 4). To this extent the egg at this stage possesses an obvious polarity. Later, however, the yolk-free cytoplasm surrounding the pronuclei occupies approximately the center of the egg (fig. 6), and here the first cleavage spindle will form (figs. 7 and 8).

Egg No. 54 (2) d (fig. 4) was sectioned in such a manner that both pronuclei lie in the same section. The specimen appears normal in every way except for a third body situated between the pronuclei and in contact with one of them, as if extruded from it. This body has a structure similar to that of the pronuclei, and contains a little chromatin substance. Figure 6 is made from a section containing one pronucleus and the second pronucleus was inserted from its position in the sixth section beyond (sections 6 μ thick). In another case, No. 54 (2) a, the male pronucleus seems to be in the act of withdrawing from the surface as if the spermatozoon had just entered; for the pronucleus is top-shaped with the point at the extreme surface of the egg.

Neither the first nor the second polar body has been recognized with certainty in these eggs. This is perhaps due to the interference of yolk granules, not thoroughly bleached of osmic acid stain, and to the presence of numerous epithelial cells from the tuba, imbedded between the laminae of the albumen.

The eggs in the pronuclear stage do not exhibit any marked polarity aside from the temporary position of the pronuclei. Sometimes, it is true, the yolk is more or less concentrated in large granules toward the pole opposite the pronuclei; in other cases, as noted, the polar bodies are centrally located. An

obvious polarity is to be recognized in several eggs of batch No. 52 in which no chromatin is seen, but in other eggs of the same batch with similar absence of well-stained chromatic substance, the yolk is peripherally situated. Whether one study a given section through the center of the egg or the series of sections as a whole, no striking condition of polar differentiation into yolk mass and cytoplasm is encountered nor are minor polar differences in structure of the egg at all of uniform occurrence during the pronuclear stage.

From these facts, it is apparent that the pronuclei, at first eccentrically situated, soon migrate to the center, and the yolk maintains its peripheral distribution. This interpretation finds ample confirmation from a study of the next stage.

THE FIRST CLEAVAGE

a. *The first cleavage spindle*

The first cleavage spindle was observed in two specimens: No. 54 (4) b in metaphase (fig. 7) and No. 52 (3) a in early anaphase stage of division (fig. 8). The former shows a slight polarity in the distribution of yolk vacuoles, but the chromosomes are situated centrally. In the other specimen, there is no trace of polarity. The central area is perfectly yolk-free and is largely occupied by the spindle, only partly shown in figure 8. At the periphery there is a considerable number of fragments of yolk eliminated (not shown in the figure). At one side a new cell membrane appears to be forming just within the eliminated yolk-laden fragments.

The formation of the first cleavage spindle from the pronuclei was not observed, but sufficient material has been described to make it certain that the entire egg in the first cleavage divides approximately, if not exactly, into equal halves and that the yolk is not cast off in one large polar mass as in *Dasyurus*. That the yolk is given off at the entire periphery is shown by the stage just described and by the two-celled stage to be discussed in the next section.

b. The two-celled stage

Only three normal 2-celled stages, taken from two different females, Nos. 46 and 50, have been available. These specimens offer ample corroboration of the manner of cleavage and yolk elimination foreshadowed by the eggs of the preceding stage containing the first cleavage spindle.

The first cleavage plane divides the egg into approximately equal halves. While no quantitative difference is to be recognized, there seems to be a qualitative difference between the two blastomeres; for in one case the separation of one blastomere from its yolk has not yet been consummated (fig. 9). In other words, one blastomere is in advance of the other. This apparently insignificant difference may not be without meaning, for there is nothing in the subsequent history of the early cleavage out of harmony with the assumption that the daughter cells of one blastomere develop into the formative region, those of the other blastomere into the non-formative region of the blastocyst.

No polarity, either in the distribution of the yolk granules or in the position of the nuclei, can be recognized. In one section more yolk may appear at one pole of the cell; in the next section the other pole may contain the greater number of yolk vacuoles. This absence of polar differentiation is quite in harmony with the condition of the 4-celled stage presently to be described.

c. The elimination of yolk

The manner in which the yolk is given off is of importance. One cannot speak of 'extrusion' of yolk, for it is not cast out bodily as in *Dasyurus*; hence, the term 'elimination' is employed. The egg gets rid of surplus yolk by forming the new cell membranes of the two blastomeres at such a distance from the original surface of the egg as to leave a portion of the peripheral cytoplasm rich in yolk outside the blastomeres. This process is evident from a study of No. 50 (6) shown in figure 9. One blastomere has rid itself of yolk and has become rounded out. The other blastomere has not yet completed the cutting off of

the yolk, but the future surface of the blastomere is distinctly outlined by a marginal, yolk-free zone along which the membrane of the blastomere will form. In other words, one blastomere was caught in the act of eliminating its yolk. In the other specimen, No. 46 (1) shown in figure 10, both blastomeres have eliminated the yolk, but one has rounded off in advance of the other. After the cells have separated and become fully formed, the yolk remaining within them comes to have the same distribution in a submarginal region as in the unsegmented egg. This peripheral distribution is to be seen in blastomeres up to the 16-celled stage (fig. 23). The eliminated yolk and cytoplasmic substance, together with the layer of albumen, is later completely but slowly absorbed. Considerable yolk, in variable amounts, is retained even within the cells which form the walls of the younger blastocysts. But this yolk is also gradually absorbed until in late unilaminar blastocysts the fat granules are only occasionally observed.

It follows, then, from the absence of polar distribution of yolk in the opossum egg as compared with the egg of *Dasyurus*, that the yolk of the former is not given off as in the latter. In the opossum egg the yolk, being peripherally distributed, is given off peripherally; being concentrated in a dense mass at one pole in the egg of *Dasyurus*, the yolk is, in this case cast out at the pole when the two blastomeres form. The egg of the opossum is also much smaller (text fig. 4) and probably also proportionately less laden with yolk, for its egg measures on the average about one-half the diameter, that is, one-eighth the volume, of the egg of *Dasyurus*.

THE 4-CELLED STAGE

a. *The orientation of the blastomeres*

1) *Shifting of the blastomeres.* The orientation of the blastomeres in the 4-celled stage presents the chief point of divergence in the cleavage of *Didelphys* and *Dasyurus*, the former following the manner of *Eutheria*. The conclusions here presented are based upon abundant material secured from females just captured, as well as from cage animals.

It will be recalled that the four blastomeres of the corresponding stage in *Dasyurus* are radially arranged and that each cell shows distinctly an upper (formative) and a lower, yolk-laden (non-formative) pole. The four cells, furthermore, maintain the radial arrangement until the end of the third cleavages, which are also meridional. In the 8-celled stage the blastomeres are arranged in an open ring and each continues to exhibit a distinct polarity. The opossum egg, on the contrary, behaves at this time like the Eutherian egg, for the blastomeres shift and cleavage becomes indeterminate.

The second cleavage plane in the opossum is at right angles to the first, so that lines joining the centers of blastomeres having the same origin at first lie parallel. Several eggs show this parallel arrangement more or less distinctly, especially 52 (3) d, which has apparently just divided, for a membrane can be seen to have formed between each pair of blastomeres and the two cells of each pair are still in contact.

After division has been completed the cells assume the form of an almost perfect sphere. No exception to this has been seen in nineteen excellent preparations of this stage. The cells lie free in the semi-liquid egg content. They may or may not be in contact with each other and seldom touch the zona pellucida, which in some specimens, can still be seen closely appressed to the albumen layer. The four blastomeres do not show the slightest trace of polarity, being spherical in shape and having the nucleus usually in the center.

It is little wonder, then, that the blastomeres should shift their relative positions, and this is exactly what happens. The pairs of cells, at first parallel, shift until they lie at right angles, like the balls of a pair of crossed dumb-bells. The preparations show every gradation in the process of shifting. Drawings from wax models of these eggs clearly illustrate these points (fig. 1). The shifting of the eggs can also be seen by an inspection of figures 11 to 16, which represent three eggs, a separate drawing being made for each pair of blastomeres. The fragment of shell or the configuration of the albumen about the egg will enable the reader to note the angle taken by lines passing through

the centers of the two pairs of blastomeres of any one egg. Some of the eggs of this batch were studied in alcohol and, after staining with Delafield's, also in xylol. To facilitate the study of eggs in toto, the shells were removed, when the arrangement of the two blastomeres could be plainly made out. That the pairs of blastomeres of these eggs lie at different levels is shown by the fact that it is impossible to orient the eggs in such a way that a single section may pass through all four of the cells. Usually only two cells are found in any one section (figs. 11 to 16); some-

Fig. 1 Sketches made from wax models of 4- and 6-celled stages. *A*, No. 83 (2); *B*, No. 81 (6); *C*, No. 117 (4). In *B*, *ZP*, zona pellucida; *ALB*, albumen. The space between blastomeres and zona in *B* is occupied by yolk and coagulum ($\times 330$).

times a third cell may also be cut by the section (figs. 17 and 18).

In the 6-celled and 7-celled stages (text figs. 1, *C* and 2, *A*) the position of the pairs of daughter cells bears out the process described above for the 4-celled stages.

It is thus seen that if any polarity could be attributed to the undivided opossum egg on account of the original position of pronuclei at one side, or of other facts presented above, no stretch of the imagination could find a trace of polarity in the 4-celled stage. The blastomeres appear to be identical and shift their position as in the Eutheria.

2) *Consequences of shifting of the blastomeres.* The shifting of the blastomeres in the 4-celled stage results in an entirely different type of cleavage from that obtaining in *Dasyurus*, in which both qualitative and quantitative features are such as to enable the observer to trace out the fate of the blastomeres. The cell lineage thus determined is made more certain because the blastomeres of the 8-, 16- and 32-celled stages maintain a constant and definite relation to each other. In the opossum egg the absence of polar differentiation into large and small or light and dark blastomeres increases the difficulty of determining the cell lineage.

Fig. 2 Sketches from wax models: A, of 7-celled egg, No. 85 (7), and B, of 8-celled egg, No. 117 (10). Seven of the eight cells of B are in mitosis. Blastocyst formation is already anticipated in these eggs ($\times 330$).

In the 4-celled egg of the opossum in which the axes of the respective pairs of daughter cells are at right angles, one pair is clearly 'above' the other (text fig. 1, A and B). It is not improbable that the two pairs have different destinies. Certainly the blastocyst of the opossum agrees with that of *Dasyurus* in a polar differentiation with formative and non-formative regions. In *Dasyurus* the differentiation of the two kinds of cells comes in the fourth cleavage. In the opossum egg no differentiation is apparent in the cleavage stages, but there must needs be a potential difference in the cells of the two poles. If present in the 16-celled egg of the opossum, as in the 16-celled egg of *Dasyurus*, the polar differences already exist in the 8-celled, the 4-celled and the 2-celled egg. Hence, also, the opossum egg

possesses a potential, though concealed, polar differentiation into formative and non-formative cells. This I believe is the true interpretation. The subject will be pursued further below.

b. *Selenka's four-celled stage*

The normal 4-celled egg of the opossum as here described is totally at variance with the single 4-celled specimen seen by Selenka⁵ as well as with the normal stage in *Dasyurus*. But I am fully convinced that Selenka's only 4-celled specimen was abnormal and in the process of disintegration. I have several times seen such eggs consisting of four radially arranged 'blastomeres' or egg fragments. A striking case is an egg of batch No. 112, in which the four cells seem an almost perfect replica of Selenka's specimen. At their upper ends the blastomeres fit together by their inner angular surfaces but their lower ends are spread apart. The cells are crowded to one pole of the egg, touching the zona with their upper ends, a condition to a certain extent true of the egg figured by Selenka. This egg was studied in alcohol and xylol in toto and was then perfectly oriented and imbedded in paraffin, and sectioned. The previous diagnosis of abnormality was sustained by a study of the sections. The nuclei of the cells were very abnormally placed and other signs of degeneration were apparent. All other eggs of this batch No. 112 were in various stages of disruption. I am perfectly convinced that Selenka's 4-celled stage is abnormal and does not represent the normal condition in the opossum, notwithstanding that it is comparable with the 4-celled egg of *Dasyurus*.

c. *The distribution of yolk*

The blastomeres of the 4-celled eggs of the entire collection are in the same stage of advancement; they have fully formed nuclei and no case of mitosis occurs among them. It would seem, therefore, that the blastomeres of the 2-celled stage divide practically at the same time. In all cases the relation of cells to yolk is the same.

⁵ Loc. cit., p. 113; figures 4 and 5, plate 17.

The distribution of eliminated yolk is as would be expected, *a priori*, if one accept the interpretation of the 2-celled stage advanced above. If there were any doubt as to these views on the expulsion of the yolk in the 2-celled stage on account of the meager available material on which the conclusions were based, the explanation presented is fully justified by a study of the abundant material secured at the end of the second cleavage. The four blastomeres in all cases are almost completely surrounded by precipitated material, the 'yolk.' Rarely do the blastomeres touch each other and still more rarely are they in contact with the albumen layer or with the zona, in case the latter still persists.

The structure of the 'yolk' in the eggs of specimens Nos. 81, 83, and 54 is peculiar to these eggs (figs. 11 to 18) and was not met in any others, not even in the blastocysts furnished by No. 83, in addition to the 4-celled stages. It is a more or less homogeneous mixture of discharged cytoplasm, fat (yolk) and coagulum (protein of the egg sap)—all of these mixed together and precipitated by the fixing fluids into flocculent masses (fig. 18). There is often a more or less even distribution of small yolk granules (fig. 17) or fat vacuoles, but they are not as prominent as in other eggs. In other stages the eliminated material is usually massed in lumps, in which the black yolk globules or clear vacuoles are plainly seen; and in many eggs the coagulum appears as a thin precipitate separate from the other substances. Of the 4-celled eggs only egg No. 52 (3) d shows the usual appearance of the yolk; perhaps because of a different treatment of the preparation.

In less than half of the specimens at this stage one polar body was seen; in a few both polar bodies were found. The zona pellucida was observed in only two or three eggs. The blastomeres themselves have no cell membranes, excepting again No. 52 (3) d. The cells are usually well marked but there is no sharp, clear-cut line or membrane (figs. 17 and 18).

The blastomeres of the different eggs vary considerably in size, measuring from 0.043 to 0.056 mm. But this variation is not due to differences in size of eggs, that is, of volume enclosed

by the zona, for a large egg may contain small blastomeres. It is due rather to differences in quantity of yolk and cytoplasm eliminated in the previous stages; the more yolk thrown off the smaller the blastomeres. The end result is, however, the same, for the yolk is eventually absorbed and used as nutriment, whether it remains within the blastomeres or not. The yolk remaining within the blastomeres is arranged peripherally, as in previous stages, and indeed in the 8- and 16-celled ova as well.

The peculiarities of the first two cleavage stages of the opossum egg and their interpretation as presented above are fully confirmed by the succeeding cleavage stages now to be discussed.

THE 8- TO 16-CELLED STAGES

a. *Third and fourth cleavages*

1) *General statement.* Eggs of five to eighteen cells were furnished by specimens Nos. 85 and 117; these two batches included five 8-celled and eight 16-celled eggs. Every other number of blastomeres from five to eighteen inclusive was represented in different specimens. The eggs of No. 85 are to be considered perfectly normal, for the female furnishing them was fresh from the field and the eggs are of normal size (figs. 19–24). Yet the eggs of batch No. 117 appear normal in every respect except as to size (*A* and *B* text fig. 2). The reduction in size is to be expected in view of the fact that a single ovary of female No. 117 discharged 43 or more eggs at the second oestrus period, having one month previously discharged about twenty. The measurements of several eggs of both batches (measured from sections) are given in the following table:

TABLE I

		<i>mm.</i>			<i>mm.</i>
85 (7)	7-celled	0.124 x 0.095	117 (4)	6-celled	0.103 x 0.101
85 (18b)	12-celled	0.11 x 0.102	117 (10)c	10-celled	0.087 x 0.083
85 (3)	9-celled	0.13 x 0.11	117 (10)b	8-celled	0.093 x 0.084
85 (11)	16-celled	0.13 x 0.117	117 (11)a	16-celled	0.087 x 0.085

A study of this abundant material makes it certain that the third and fourth cleavages take place in the opossum ovum in a manner quite different from that shown by Hill to hold for the egg of *Dasyurus*. In that form the fourth cleavage is for the first time horizontal and divides the single ring of eight conical cells into an upper ring of small, relatively yolk-free cells and a lower ring of larger, yolk-rich and more darkly staining cells. The former are said to be destined to produce the formative portions of the blastocyst. Thus a single section of the 16-celled egg may pass through all of the eight blastomeres of a given ring (text fig. 4, *E*). This description does not hold in the slightest degree for the opossum egg in the corresponding stage.

The blastomeres of the 4-celled opossum egg having shifted about at right angles as described above, one would expect the planes of the third cleavage also to pass irregularly and at various angles through the four blastomeres. This is the case. The shifting of the blastomeres continues as cleavage progresses. The cells migrate more and more to the periphery and spread about irregularly into the form of a hollow sphere. When the 16-celled stage has been reached, or even before, all semblance of orderliness seems to have vanished.

In another respect the opossum egg of sixteen cells differs from the corresponding stage of *Dasyurus* (compare *E* and *F*, text fig. 4). In the latter species the albumen layer is entirely absorbed and the progeny of the sixteen blastomeres, as these multiply, come at once to lie against the shell. In the 16-celled stage of the opossum, however, there has been as yet no perceptible absorption of albumen, the layer of which is enormous as compared with that in the egg of *Dasyurus*. The albumen of the opossum egg is not exhausted until the bilaminar blastocyst is some 1.4 mm. in diameter and the formation of the mesoderm and the primitive streak is initiated (Selenka).

2) *Cleavage indeterminate*. After the opossum egg has passed the 4-celled stage the planes of cleavage of any two dividing cells are never parallel; that is, the axes of the division spindles in any given stage point different directions. Already in the 6- or 7-celled egg (text figs. 1 and 2), the continued shifting may

be noted from a study of wax models of such eggs. In models of eggs from the 6- to the 16-celled stages the blastomeres are grouped into pairs of common origin. Thus a 6-celled egg has two large cells and two pairs of small cells, that is, $2 + (2 \times 2) = 6$. Likewise, a 10-celled egg has six large and four small cells, or $6 + (2 \times 2) = 10$. If lines be drawn connecting the centers of the cells of each pair, scarcely any two lines are parallel (text fig. 3).

One need not rely upon wax models, however, to note that the cleavage planes are no longer parallel. Several eggs were fixed at the time two or more of their blastomeres were in mitosis and

Fig. 3 Sketches of wax models during fourth cleavage. A, 10-celled egg No. 85 (18) a; cells 1 and 6 and 3 and 4 are pairs of cells of common origin; cells 9 and 10 are in mitosis. B, 12-celled egg No. 85 (18) b; the four pairs of smaller daughter cells are easily recognized in the figure; cells 5 and 6 are in mitosis (fig. 21) ($\times 330$).

from the direction of the spindles the angle of the cleavage planes may be determined. This is strikingly true of three eggs containing 8, 10 and 12 cells respectively (text figs. 2 B, and 3 A and B). In the 8-celled egg (text fig. 2 B) two cells are in the telophase stage of division, the nuclei of the daughter cells undergoing reconstruction; five cells are in metaphase or beginning anaphase with the chromosome-loops clearly seen at or near the equator of the cells; one cell is in the resting condition. The directions of the spindles obey no apparent rule except that these are tangential to the surface of the egg, as the mitotic figures show.

The 10- and 12-celled stages are also instructive in this connection. In the 10-celled egg (text fig. 3 A) two blastomeres have just divided and two others show mitotic figures (late anaphase). Blastomeres 3 and 4 and 1 and 6 make two pairs respectively; 9 and 10 are in mitosis, with their spindles oriented at right angles to each other. Cell 7 is elongated like cell 10, as if soon to divide. All of these divisions are tangential to the surface, but besides this no order in arrangement of cleavage spindles can be noted. The same is true of the 12-celled egg (text fig. 3 B). Eight small blastomeres are irregularly massed to one side or 'pole' of the egg and are definitely arranged in pairs. Four larger cells, also irregularly placed, are found at the other side. Two of the larger cells are in mitosis (fig. 21) and the axes of their spindles are almost at right angles and nearly if not quite tangential to the surface.

The cases just described leave no doubt, therefore, of the fundamental differences between the fourth cleavage of *Dasyurus* and of the opossum. In the latter the 16-celled stage is established by an indeterminate cleavage of the cells, the division planes passing through the cells in no apparent order except that the spindles are tangentially arranged with respect to the more or less imperfect sphere made by the blastomeres.

The indeterminate type of cleavage as just described recalls a similar process obtaining in the Eutheria. The similarity between the early cleavages of the opossum and the higher mammals is further illustrated by the disparity in the time that cleavage of the various blastomeres takes place; for eggs of every number of cells, from five to eighteen, were found along with the 8- and 16-celled eggs. These occurred, however, in larger numbers than other cleavage stages.

3) *The 16-celled egg practically a blastocyst.* The blastomeres of the 16-celled stage are arranged about a cleavage cavity of considerable size (figs. 22 to 24). They are irregularly scattered in the form of a sphere with gaps of various sizes between most of the blastomeres, for but few of the cells are in contact with each other. They are in contact rather with the albumen layer, against which they tend to flatten out. In *Dasyurus*, on the con-

trary, the two superimposed rings maintain a position more or less parallel with the equator, so that a given section through the egg in the plane of the equator would pass through all of the eight cells of either ring (text fig. 4 *E*). A section through a 16-celled egg of the opossum (figs. 22-24) never passes through the centers of more than four or five cells. Such a section of the 16-celled opossum egg is not unlike a section through the 16-celled stage of a Eutherian mammal (fig. 22).⁶ The early arrangement of the cells of the opossum egg into a hollow sphere makes possible the early formation of the completed blastocyst. The blastomeres in the eggs of *Dasyurus*, which are arranged in rings near the equator, must proliferate toward their respective poles. The blastocyst is completed when the cells have met and closed the gap at either pole. In the opossum egg the blastocyst is completed when the scattered gaps seen in the wall of the 16-celled eggs have been filled by a further division and a flattening of the cells. This process may be completed in this way in eggs having as few as thirty cells.

The blastocyst formation, however, is anticipated in the 8-celled stage or indeed even before this time. For in 5-, 6- and

Fig. 4 Diagrams to show comparative size and structure of the eggs of *Dasyurus* and *Didelphys* ($\times 75$). *A*, *C*, *E*, *G*, *I* and *K*, eggs of *Dasyurus* (all except *K* after Hill); *B*, *D*, *F*, *H*, *J* and *L*, eggs of *Didelphys* (original). *ALB*, albumen; *EMB.ECT*, embryonic ectoderm; *TR.ECT*, trophoblastic ectoderm; *SM*, Shell membrane; *Y*, yolk; *ZP*, zona pellucida.

A and *B*, pronuclear stage; the ovum of the opossum is seen to be much the smaller; the yolk mass (*Y*) is seen at one pole of the egg of *Dasyurus*. By comparing *C*, a fresh 2-celled egg of *Dasyurus*, with *D*, a fresh 4-celled egg of *Didelphys*, one can readily note the relative size of entire egg and of ovum proper and the relative thickness of albumen and shell membrane of the two species. Note extruded yolk in *C* and *D*. Compare also *E* and *F*. *E* represents a section taken through an entire 'ring' of the 16-celled *Dasyurus* egg; no such ringed arrangement occurs in the opossum egg of 16 cells (*F*). *G* (surface view) and *H* (section) are blastocysts of equal size, but in the former the cells lie against the shell membrane, whereas in the latter the blastocyst itself is only a little larger than the unsegmented ovum. *J*, opossum blastocyst at time of entoderm formation; *K*, arc of *Dasyurus* blastocyst in corresponding stage, after long period of growth, which does not occur in *Didelphys*. *I*, section of blastocyst of *Dasyurus* early in the period of growth, the period of 'no differentiation' (Cf. 1 with *G* and *J*). *L*, section of complete bilaminar blastocyst of *Didelphys*.

⁶ Cf., for example, Assheton ('98), figure 6, plate 15.

7-celled stages the tendency of the cells to migrate toward the periphery and to become closely appressed to the albumen layer is very apparent. One 12-celled egg is an almost fully-formed blastocyst (text fig. 5 A); the cells are very flat and closely applied to the albumen, precisely as in eggs of more than double the number of cells. On the other hand, in a number of 8- to 16-celled eggs, not all of the blastomeres touch the albumen layer and in these cases they maintain their spherical shape (fig. 21). Attention should be called at this point to a 16-celled egg, 85 (12), of which a model was made. This egg is unique for specimens of this batch in that it contains an included cell some distance removed from the peripheral cells making up the wall of the 'blastocyst' and so situated as to make it improbable that it could ever have attained a place in the wall of the blastocyst. This egg will be referred to again.

b. *The eliminated yolk*

The processes in the development of the opossum egg up to the 16-celled stage described above fully answer the question propounded by Selenka as to the manner in which the yolk reaches the cavity of the blastocyst. To quote:

Auf welche Weise die in Figure 8, 10 and 11 mit *i* bezeichneten Dotterballen in die Furchungshöhle gelangen, weiss ich nicht zu sagen; in den ersten beiden Stadien der Furchung fehlen sie ganz, ebenso auch

Fig. 5 Sections through early unilaminar blastocysts ($\times 250$). *i*, gap in wall of blastocyst.

A, early attempt at blastocyst formation in 12-celled egg No. 144 (1) b. B, 31-celled egg, No. 18 (18), with only half of blastocyst formed normally. C, No. 88 (1), the only blastocyst here figured which is entirely devoid of included cells. D, No. 50 (4), 50-celled blastocyst with almost uniform wall; this specimen has several included cells, not shown in this section. E, No. 88 (2), shows polar differentiation in its greater thickness of cells at one pole as compared with cells at the opposite pole (Cf. G, O and others). F, No. 88 (14), showing excessive proliferation from cells in wall of blastocyst (Cf. I and L). G and N, two sections of No. 88 (4). H and I, two sections of No. 88 (7). J and M, two sections from opposite poles of No. 88 (6); note large included blastomere in J. K and L, two sections of No. 88 (16); note large cell in wall of K, and included cells in L. O, more advanced blastocyst, No. 144 (1) a; transition between the series shown in this figure and those shown in figure 6.

in den älteren Gastrulastadien; man wird daher zu der Annahme gedrängt, dass diese Dotterballen von den Furchungszellen ausgestossen, bald aber wieder resobirt werden.⁷

Selenka was thus of the opinion that the yolk is given off in the 8-celled stage and not earlier.

It has been shown above that in the 4-celled stage of the opossum egg the yolk practically surrounds the blastomeres. In the next division the blastomeres exhibit a centrifugal tendency and sooner or later come to lie closely appressed to the albumen layer. Hence the eliminated yolk comes to occupy a central position until absorbed. By what physiological activities the blastomeres are forced against the albumen I am not prepared to say. The fact is they do pass peripherally and occupy a point of vantage where they may best absorb the nutriment from the albumen and the included yolk as well.

The distribution of yolk remaining within the blastomeres in eggs from five to sixteen cells occurs as in preceding stages, namely in a peripheral belt (figs. 19 and 23). The relative quantity of yolk within and without the cells is again a variable one. Where the blastomeres touch the albumen layer there is often less yolk or even none at all, the yolk granules or vacuoles being concentrated at the surface toward the cleavage cavity (fig. 23). Preparation of eggs fixed in Bouin's fluid show large vacuoles at the rounded free side of the cell. Sometimes these vacuoles coalesce into irregular spaces as if the cells broke down at this point under the action of fixatives, which recalls the behavior of the delicate deutoplasmic portion of the unsegmented egg of *Dasyurus*.⁸

c. *Absence of polar differentiation*

Although there is a shifting of blastomeres in the egg of the opossum after the second cleavage, the daughter cells of either of the first two blastomeres are, no doubt, to be found in one or the other hemisphere of the 16-celled stage (text figs. 1 to 3). In models of certain eggs containing 6, 7, 8, 10, 12, and 16 cells

⁷ Loc. cit., p. 115.

⁸ Hill, p. 12.

respectively, the blastomeres are very clearly arranged in pairs, the two daughter cells of any given division remaining close together for a time, their common origin being judged by the juxtaposition. The blastomeres of any given stage do not shift about sufficiently so as to destroy the orientation here implied, namely the identical lineage of all of the cells in either hemisphere of the egg.

This being true, a thorough search was made for structural evidences of polarity: differences in size, staining qualities and yolk content of cells. None were discovered. The cells in section usually appear of uniform size, except, of course, in eggs in which certain blastomeres have divided in advance of others (text figs. 1 to 3). The differences that may occasionally be noted in 8- or 16-celled eggs are sporadic and accidental and are not to be correlated with polar differentiation, as both sections and models of these eggs show. Neither were the differences in staining qualities or yolk content of the cells at all regular in any given egg.

Especially favorable specimens for testing the assumption of polar differentiation among the constituent cells are three eggs of 6, 10 and 12 cells respectively, already referred to in another connection. The 6-celled egg (fig. 1 C) has four small cells at one 'pole' and two large cells at the other [$2 + (2 \times 2) = 6$]. The 10-celled egg (text fig. 3 A) has two pairs of small cells and two larger cells in one rather clearly marked 'upper' ring and four large cells in the 'lower' ring [$6 + (2 \times 2) = 10$]. The 12-celled egg (text fig. 3 B) is similar to the 10-celled egg just described but has four cells divided into pairs [$4 + (4 \times 2) = 12$]. The smaller cells are concentrated at one pole. From models made of these eggs this orientation is clearly seen.

Fortunately, in these three eggs the sections were taken vertically, that is through both the divided cells at one pole and the undivided cells at the other. Here, then, if anywhere, are specimens exhibiting a polarity, slight though this be, in the retardation of cells division at one pole of the egg. If a comparison with the egg of *Dasyurus* in this regard be valid, the more rapidly dividing cells are formative, the others non-formative.

A careful study and painstaking comparison of the cells at one 'pole' with those of the opposite pole led to no discovery whatever of qualitative differentiation among the cells. Though the differences in the rate of division of the cells may have some significance they are certainly not correlated with structural differences.

That the 16-celled stage of the opossum, in common with the corresponding stage in *Dasyurus*, possesses a polarity, seems highly probable. But, unlike the egg of *Dasyurus*, the polarity of the opossum egg is only potentially present and is not reflected in structural differences. Polar differentiation begins in the blastocyst of about 40 cells, that is, soon after the fifth cleavage, when the blastocyst is just completed. As the polarity of the egg becomes manifested so soon, the assumption that polarity is also present, though concealed, in the 16-celled stage, is not at all unreasonable. If, therefore, the egg of the opossum in the 16-celled stage possess a potential polarity, even though this be not reflected morphologically, such polarity must needs be established before the 8-celled stage is reached—indeed, upon the completion of the first cleavage. For, having arrived at the 8-celled stage, the blastomeres are so shifted and arranged around the surface of a sphere that the 'fourth cleavage plane' could not possibly divide "the blastomeres of the 8-celled stage to form two superimposed rings," as in *Dasyurus*. Again, supposing that the fourth cleavage does divide each of the eight scattered blastomeres qualitatively into a formative and non-formative cells, the formative blastomeres resulting from the division of the four cells at the lower pole would have to migrate to the upper pole and the non-formative halves of the divided blastomeres in the upper portion of the egg would have to migrate to the lower pole—a process not to be seriously considered in the face of the facts. Again, the qualitative differences among the upper and the lower cells must be present as early as the 2-celled stage, if present in the 16-celled stage, for the study of eggs of 4, 6, 7, 8, 10 and 12 cells shows clearly, as noted above, that the cells of either pole of the 16-celled egg are lineal descendants of one pair of blastomeres of the 4-celled egg. The

whole matter may be summarized by the statement that cleavage proceeds in the opossum from the 1-celled to the 16-celled stage superficially somewhat as in the Eutheria and without evident polar differentiation, but that potentially the cells at the poles differ, those of one hemisphere being destined to become the formative, those of the other the non-formative region of the early blastocyst.

PART II. THE FORMATION OF THE BLASTOCYST

THE EARLY UNILAMINAR BLASTOCYST

a. *The just completed blastocyst*

1) *Formation of the blastocyst.* In the preceding section it was seen that the formation of the blastocyst was plainly foreshadowed in the 8- to 16-celled stages. Early in cleavage the cells migrate toward the albumen layer, to which they soon become closely appressed. The stage at which the cells arrive and begin to flatten out beneath the zona varies in different eggs. The process may begin in the 5-, 6- or 7-celled stage (fig. 19) or it may be delayed beyond the 16-celled stage. In one 31-celled egg (text fig. 5 *B*), for example, the blastomeres of only one hemisphere have flattened out and completed this half of the blastocyst; the cells of the other half are rounded and still have gaps between them. In most cases of 15- to 18-celled eggs, however, the blastomeres have definitely established a contact with the albumen and this relation they do not change until, in the large bilaminar blastocysts, the albumen is all absorbed. Hence in blastocysts of the opossum there is no 'perivitelline space' nor are the cells at all normally rounded off peripherally in the manner figured by Selenka. The early blastocysts described by him are no doubt damaged specimens in which the cells have shrunken away from the albumen layer.

Since, therefore, the blastomeres of the 16-celled egg are already arranged in a hollow sphere, the blastocyst is completed at a much earlier stage than that of *Dasyurus*. Of nearly four dozen specimens of young blastocysts, each approximating 50 cells, all have completed walls except five (text fig. 5 *N*). The blastocyst wall is indeed complete in some specimens containing from 30 to 40 cells.

In *Dasyurus*, on the contrary, blastocysts of 89, 93, 121, 128 and 130 cells are still incomplete, the last being on the point of closing at the poles. The difference between the two forms is due to differences existing between the respective 16-celled stages. In the Australian species the two rings of cells, equatorially placed, must fill the space from the equator to the poles by sheer force of crowding, as they multiply and spread. As they proliferate, the cells are held in the form of a sphere by the enveloping shell. In the opossum egg the cells are already distributed over the sphere in the 16-celled stage; hence with one more division and a little flattening of the cells the blastocyst wall is completed. At this stage the diameter of the eggs of *Didelphys* and *Dasyurus* is about the same, 0.45 mm. But this measures practically the diameter of the *Dasyurine* blastocyst, the cells of which are flattened out under the shell of the egg. In the opossum the thick albumen layer, hardly yet attacked by the metabolic activity of the cells, intervenes between the small blastocyst (0.12-0.15 mm. in diameter) and the shell membrane (*G* and *H*, text fig. 4).

2) *First appearance of polarity.* Selenka describes and figures two early unilaminar blastocysts of 42 and 68 cells respectively. Both exhibit an obvious polarity in that they consist of large, yolk-laden cells at the lower pole, and these taper off gradually to small, somewhat elongated, flatter and smaller cells at the upper pole. An inspection of figure 5 discloses undoubted evidence of polarity in the specimens of my collection. A polar differentiation is then apparent for the first time in the history of the egg. So far as I have been able to discover, however, the difference between the cells at the two poles of the blastocysts is one of thickness only, there being no difference in yolk content or staining qualities of the cells.

The differences in thickness of cells is sometimes considerable (text fig. 5 *E-H*), sometimes hardly noticeable (text fig. 5 *D*) and in some cases the wall appears practically uniform throughout (text fig. 5 *C*). The shape of the section, whether circular or elliptical, is not correlated with appearance of polarity. Orientation was not feasible in imbedding the specimens, since the

thickness of the albumen layer made it impossible to distinguish the thick from the thin-walled portion of the blastocyst. Hence, some eggs were sectioned equatorially, others meridionally, and still others obliquely. Measurements of cells through the series, however, indicated that in some of the blastocysts with about 50 cells a polar differentiation had not yet taken place. It must be assumed, therefore, that the greater flattening of the cells at one pole as compared with those at the other is delayed in some cases. In general however, the polar differentiation may be said first to manifest itself at about the 50-cell stage or almost immediately upon the completion of the blastocyst wall.

Occasionally one or more large cells occur at one pole of the egg (text fig. 5 K). Figure 25 is a blastocyst consisting of 52 cells. The large cell in the section is one of four similar cells in juxtaposition at this pole. Perhaps these represent a somewhat retarded generation of blastomeres. The case brings to mind such a cell in an advanced blastocyst already in process of forming the entoderm (figs. 37 and 38). Occasionally these larger cells situated in the wall are darker and have their long axes radially arranged (text fig. 5 K). Sometimes they are found in process of division.

In the opossum egg there is no polarity due to the presence of a yolk mass at the 'formative' pole, as in *Dasyurus*, nor to the presence of included cells at the 'entodermal' poles, as *Selenka* would have it. In a few cases, however, where there is a great attenuation of the cells at one pole of these early blastocysts, such thinning away may be due to the presence of large numbers of included cells. The matter of included cells will be discussed below.

3) *Inclusion within the blastocyst cavity.* There may always be distinguished within the blastocyst cavity such inclusions as were mentioned in connection with the 16-celled stage (figs. 25 and 26). Masses of coagulum (precipitated proteins of the blastocystic fluid) are seen in one part or the other of the cavity. Yolk granules are scattered in varying quantity throughout the cavity and are nearly always surrounded with cytoplasmic fragments. Most of these are the result of yolk elimination from

the 2-celled egg, as previously described. Their position within the cavity of the blastocyst is due to the migration of the cells to the albumen layer some time after the 4-celled stage is passed.

Besides these inclusions, the blastocyst cavity usually contains entire cells as well as cell fragments bearing chromatin matter (text fig. 5). Selenka also described and figured an included cell in his 42 and 68-celled stages and mentions an included cell in his much damaged 20-celled egg. Such cells are found in the cavities of thirty-nine out of forty-four blastocysts in my possession.

The cellular material mentioned in the preceding section comes to lie in the cavity of the blastocyst in two ways: first, by failure of cells in about the 16-celled stage to reach the periphery and second by proliferation from the blastocyst wall. The first method seems highly probable, *a priori*, and is strongly suggested by the presence of an included cell in the 16-celled egg, specimen 85 (12), cited above. The model made of this egg shows that the included cell had little chance of finding its place in the blastocyst wall. The included cells of the 50-celled stages are, moreover, in some cases fragmentary and show signs of disintegration.

The second method suggested is apparent from an inspection of certain blastocysts shown in text figure 5 (*F*, *I*, *L*). There are four types of cells in these blastocysts: 1) the ordinary flat to cubical cells definitely located in the blastocyst wall; 2) one or more much larger cells, often columnar in shape, also situated in the blastocyst wall and frequently darkly stained; these cells sometimes jut out far into the cleavage cavity (text fig. 5 *K*); 3) included cells connected with the cells of the blastocyst, clearly cases of proliferations from the cells of class 2; these proliferated cells in several cases make a complete bridge across a sector of the blastocyst (text fig. 5 *F*); 4) ordinary included cells floating in the cavity singly or connected with other cells as if just divided. Since the majority of these early blastocysts are absolutely normal, the included cells doubtless have no special significance. A study of blastocysts in the next stage indicates that the included cells do not take part in the formation

of the entoderm, as suggested by Selenka. To suppose that the cells in various parts of the blastocyst cavity migrate to the formative pole of the egg, attach and proliferate as definitive entoderm of the blastocyst is asking too much of them; nor does this presumption accord with facts observed in studying the later stages.

It will be recalled that Selenka ('87) observed included cells in the 20-celled egg and in the two young blastocysts studied by him. Inasmuch as Hill ('10) criticised these stages as abnormal, and as the matter is one of importance, it will now be discussed in detail.

b. Cells included in the blastocyst cavity

Of the included cells above mentioned Hill says:

Whilst the 42- and 68-celled blastocysts described by Selenka may be regarded as normal so far as the occurrence of polar openings and the constitution of their wall are concerned, I hold them to be abnormal in respect of the presence in each of a single large yolk-laden cell, regarded by Selenka as entodermal in significance. It is well to point out that Selenka was not able actually to determine the fate of this cell; he merely presumed that it took part in the formation of the definitive entoderm. No such cell occurs in normal blastocysts of *Dasyurus* at any stage of development, and in my opinion Selenka's 'urentoderm-zelle' is no other than a retarded and displaced blastomere. . . . I am strengthened in this interpretation by the occurrence in an abnormal blastocyst of *Dasyurus* of just such a large cell as that observed by Selenka. . . . This cell corresponds in its size and cytoplasmic characters with a non-formative blastomere of about the 16-celled stage, and I regard it simply as a blastomere which has failed to undergo normal division.⁹

In two other incomplete blastocysts of less than 0.4 mm. diameter Hill found abnormal included cells; but in later blastocysts 0.5 to 0.8 mm. in diameter, taken from three different females, he often finds included cells or fragments thereof. To quote again:

In blastocysts of this stage of growth a variable number of small spherical cells or cell-fragments are frequently met with in the blastocyst cavity, usually lying in contact with the inner aspect of the cellular wall. In some blastocysts such structures are absent, in others

⁹ Loc. cit., p. 42.

one or two may be present, in yet others numbers of them may occur. They may be definitely nucleated, but this is exceptional; more usually they contain one or more deeply staining granules (of chromatin?), or are devoid of such. They are of no morphological importance, and I think there can be no doubt that they represent cells or fragments of cells which have been separated off from the cellular wall during the process of active growth. They are of common occurrence in later blastocysts, and it is possible the so-called 'yolk-balls' observed by Selenka in *Didelphys* are of the same nature.¹⁰

It is thus seen that Hill does not consider his later blastocysts abnormal, no doubt because of the large proportion of specimens containing included cells.

Again, in later full-grown bilaminar blastocysts Hill finds islands of deep-staining cells, both in the formative and the non-formative areas, in contact with the blastocyst wall. These cells have large nuclei and may be actively proliferating or, on the other hand, they may be in process of degeneration. He considers them "of no morphological importance and (they) are destined sooner or later to degenerate." I have not seen cells like these last described, but Selenka mentions similar ectodermal growths in opossum vesicles containing embryos with three somites.

It is not amiss at this point to recall that cell fragments have been reported in normal cleavage stages of Eutherian embryos, e.g. by Assheton ('98) in the sheep. In the 8- to 17-celled stages of the sheep ovum the segmentation cavity "always contains spherical or otherwise shaped masses of substance in every way similar to the cytoplasm of the segments." In three specimens chromatin spots were recognized, in others chromatin-like granules. Of these Assheton says: "I cannot give any explanation of the fragments of cells seen within the segmentation cavity, nor of the origin of the nucleus-like body."¹¹

These cases of included cells or cell fragments within the cleavage cavity as described by Selenka, Hill and Assheton for *Didelphys*, *Dasyurus* and *Ovis* respectively, are cited as probably illustrating cases of such inclusions in normal embryos.

¹⁰ Loc. cit., p. 46.

¹¹ Loc. cit., p. 212.

In the forty-four preparations of young opossum blastocysts in my possession, taken from four different females, only five do not show clear cases of included cells with nuclei and in three of these cases I am in doubt as to whether or not chromatin fragments are present. If, therefore, cell-inclusion constitute an abnormality, my forty-four blastocysts are only 10 per cent or less normal. This I am certain is not the case.

There is, of course, every gradation in the volume of included material: lipoid, cytoplasmic and chromatic. There may be only a few fat grains and lumps of cytoplasm (fig. 25 and text fig. 5 *D*) or a single perfect cell in addition to these amorphous masses (fig. 26). Other eggs have two, three or a dozen small cells, or, as many or more large cells which may finally almost completely fill the segmentation cavity (fig. 5). One egg, indeed, is practically a solid morula, the cavity being all but obliterated by cell masses. But the included cells are fragmenting and the whole structure has the appearance of degeneration. This and other abnormal eggs are listed as such and are not among the forty-four on which the description of this stage is based. All of these forty-four I am convinced are normal and they are among the best fixed and stained preparations in my possession.

For three reasons, therefore, the conclusion is fully justified that blastocysts containing a limited number of cells within their cavities are perfectly normal, but that these cells have no morphological significance: 1) They are of undoubted occurrence in thirty-nine out of forty-four blastocysts of approximately the 50-celled stage, which are normal and the walls of which do not differ essentially from blastocysts apparently free of included nuclear material. The three cases of similar inclusions of cells observed by Selenka should also be noted in this connection. 2) Included cells without morphological significance have been observed in cleavage stages in other mammals. 3) Later stages of *Didelphys* and *Dasyurus*, apparently normal in every respect, show fragments of cells not entirely digested or absorbed.

To recapitulate, the blastocyst of the opossum, formed in the 16-celled stage, is completed soon after the fifth cleavage or at about the 40-celled stage. At this time or a little later a polar

differentiation may be made out for the first time in the development of the egg. This difference in the cells of the two poles is merely quantitative, so far as may be observed from a study of the specimens at this stage alone. The only evidence of polarity at this stage lies in the thinning of the cells at one pole of the egg. The question as to which of the poles is formative and which non-formative will be discussed in the next section.

THE FORMATION OF THE ENTODERM

a. Entoderm formation as conjectured by Selenka

The method of entoderm formation in the opossum naturally interested Selenka, but he lacked the requisite stages to determine the matter. It has been shown above that the included cell in Selenka's 42- and 68-celled stages, celled by him 'Urentodermzelle,' has no morphological significance. His surmise, moreover, that the 8-celled stage is made up of four ectodermal and four entodermal cells is a mere conjecture, based solely on analogy. Hence it follows also that the early blastocyst is not divided into a lower thick-walled 'entodermal' half and an upper 'ectodermal' half, as he suggested.

Selenka's later blastocysts (his two 'young gastrulae') already contain entodermal (?) cells massed at one pole of the egg. From these masses proliferation of flat cells are seen advancing along the blastocyst wall. Little or no differences exist in the cells of the ectodermal wall, the polarity of the egg being indicated merely by the position of the entodermal cells. The ectodermal cells are represented by the author rather ideally as a reconstruction, so that I am at a loss to make accurate comparison with my material. In my collection I find several eggs of batch No. 144 (fig. 27) which show a massing of cells at one pole, though not in the numbers represented by Selenka's young gastrulae. On the whole I believe these two specimens of Selenka to be badly shrunken. Hence, also, his attempt to figure out the axis of the gastrula from the shape of one of his specimens must be characterized as futile, for the differences in the several diameters of the blastocyst were one of chance merely. The blastocysts secured by me are mainly spherical;

where they deviate from this shape in the preparations they have suffered collapse to that extent. In the older of Selenka's two gastrulae the enormous 'Perivitellinraum' between the blastocyst and its albumen layer is to be regarded with suspicion. I have never seen a blastocyst, the cells of which were not in close contact with the albumen layer. Judging from the quantity of albumen remaining in Selenka's figure 1, Tafel 18 (although it is a rough criterion at best) I regard his older gastrula there figured to be a shrunken specimen of a stage just preceding my own No. 40 (4), shown in outline in text figure 6 *D* and in detail in figure 32; and this in turn is a stage just preceding Selenka's almost complete bilaminar blastocyst which he shows in figure 3, Tafel 18.

It is evident then, that Selenka did not, on account of a lack of the requisite stages, hit upon the true method of entoderm formation in the opossum.

b. *Entoderm formation in Dasyurus*

In Hill's account of the history of the blastocyst of *Dasyurus* he gives us a new method of entoderm formation in mammals, and his interpretation of the facts as he observed them in *Dasyurus* finds confirmation in similar conditions independently discovered by Patterson ('10) in the embryo of the *Tatusia*.

In *Dasyurus* there appears in the still unilaminar wall of the blastocyst, when this has attained a diameter of nearly 4.0 mm., a number of modified ectodermal cells, the 'entoderm mother cells.' These produce the entodermal cells by a process of proliferation and migration on to the inner surface of the blastocyst wall. They appear at the formative pole of the blastocyst and at an early stage are united into a fenestrated tissue by means of pseudopodial prolongations. Eventually they form the entire inner lining of the blastocyst.

Hill's conclusions are borne out by young blastocysts of other Australian forms. In all cases the germ layer formation differs essentially from that obtaining in *Eutheria* in the absence of the morula stage. That the same facts are substantially true in the development of the opossum I now propose to show.

Fig. 6 Sections through the embryonic area of blastocysts at the stage of entoderm formation ($\times 90$). A, No. 144 (1 d); B, No. 40 (2); C, No. 40 (3); D, No. 40 (4); E, No. 40 (1); F, No. 43 (9); G, No. 43 (7); H, No. 43 (8). ALB, albumen; CG, coagulum; EMB A, embryonic area; TR, extra-embryonic area; SM, shell membrane. The albumen is seen to be absorbed more rapidly over the embryonic area. In A and E, the shell membrane is misshapen and a large space is left between it and the albumen layer.

c. Formation of entoderm in the opossum

The conclusions which follow are based upon four blastocysts of No. 40 and three of No. 43 and corroborative evidence is furnished by a study of the somewhat transitional stages of eggs in batch No. 144. From a study of these blastocysts the conclusion is inevitable that entoderm formation in the opossum proceeds essentially as in *Dasyurus*, *Macropus* and *Parameles* as described by Hill ('10), namely by a proliferation from specialized cells of the unilaminar blastocyst.

An inspection of text figure 6 and figures 27 to 38 will show that at this stage the formative or 'embryonic' area of the blastocyst is sharply marked off from the extra embryonic region. The cells of the former vary from cubical to somewhat elongated, those of the latter are greatly attenuated. In cross-section through the center of the embryonic disc this is seen to occupy from one-fifth to one-third of the circumference of the section, but on account of the great thinness of the non-formative cells, the volume of this region is greater than that of the non-formative.

At different points in the embryonic portion of the blastocyst wall certain cells appear which stain more darkly than cells surrounding them. These show a tendency to push out from their place in the wall in quite the same manner as described by Hill for similar cells in sections of blastocysts of *Dasyurus*. Inasmuch, furthermore, as these cells are seen frequently to divide and give off dark-staining entodermal cells on to the inner surface of the embryonic disc, we may conclude that we have here the counterparts of Hill's 'entoderm mother cells.'

This differentiation of entoderm mother cells into primitive entoderm does not proceed as fast as the multiplication of all of the cells in the embryonic disc. For scarcely a section can be taken through this region without cutting through one or more cells in mitosis. As a result, the area becomes at first densely studded with nuclei (fig. 30) and later the cells may be crowded in a flattened mass three cells deep (fig. 36). Sections of such blastocysts are almost exact copies of the corresponding stage figured by Hill ('10) of blastocysts of *Macropus*. The

innermost cells in the last mentioned stage are clearly primitive entoderm. From the margins of the discs entodermal cells may be seen migrating out beneath the wall of the blastocyst. There can be no doubt from this brief statement, that entoderm formation proceeds in the opossum very similarly to the method obtaining in the Australian marsupials. Further details will now be mentioned.

The youngest blastocysts to give any indication of cell proliferation as just described are those of batch No. 144 (fig. 27). The blastocysts contain somewhat over 100 cells. Very little albumen has been absorbed and the cells of the non-formative region have not become much flattened; hence, these specimens are only a little advanced over the 50- to 60-celled blastocysts described in the preceding section. In figure 27, which has formative cells on the reader's left, a large cell is dividing and other cells proliferating from the wall at other points. These latter appear in the figure as mere included cells, but a study of the series of sections shows them to be attached to the wall. The specimens, though poorly fixed, are introduced here because they are clearly somewhat advanced in the direction of the older blastocysts now to be described. These latter are well fixed and for the most part excellently stained preparations.

Specimen No. 43 (9) shows the fewest entodermal cells. Though somewhat crushed at right angles to the edge of the microtome blade (text fig. 6 *P*) it is otherwise well preserved and the cellular elements are perfectly stained. A portion of the blastocyst wall reproduced in figure 28 shows an 'entodermal mother cell' (ENT^1) in the wall and also a primitive entodermal cell (ENT), both stained darker than the other cells of the blastocyst. The former cell shows a tendency to pull from its place in the wall in precisely the manner illustrated by Hill in his figure 75, Plate 8. In surface view this cell (ENT^1 fig. 28) would appear underneath the adjoining cell of the blastocyst wall.

More cell elements are seen in specimen 43 (8), figures 29 and 30. The primitive entoderm cells (ENT in the figures) are mostly in close contact with the ectodermal cells of the wall.

At ENT^1 an entodermal mother cell has apparently just divided. If we conceive cell ENT^1 of figure 28 as dividing across its long axis (that is obliquely to the surface of the blastocyst), we should have a condition exactly like cell ENT^1 , figure 29. At M figures 29 and 30, a large cell is in mitosis. Its staining qualities, however, do not as yet indicate its entodermal nature.

Another large cell is in mitosis in a section of No. 40 (2), figure 31. The spindle of this cell (ENT^2) is oblique to the surface of the blastocyst. At other points (O) there are elongated cells and nuclei with their long axes oblique to the surface. The cell figured at ENT^1 is a just divided primitive entoderm cell: and others are shown at ENT .

A section through the embryonic area of No. 40 (4), illustrated in figure 32, shows at ENT^1 a large darkly-staining cell, which one might interpret as an entodermal mother cell that has left its position in the wall of the blastocyst and has migrated to the inner surface. The section also shows several primitive entodermal cells, clearly darker staining than the overlying cells. Blastocyst No. 40 (1) contains several interesting cases of primitive entoderm cells (figs. 33 and 34) which give every indication of a tendency to migrate from the original position within the wall, so as to underlie the superficial cells. The cell marked ENT^1 , figure 34, is an especially striking case in point. Yolk granules (Y , figs. 29, 32 and 33) are of frequent occurrence in these blastocysts, chiefly in the formative region, where, indeed, the only cells thick enough to hold a yolk granule are situated.

A blastocyst containing a large retarded blastomere is shown in figures 37 and 38. The cell is situated in the blastocyst wall within the formative region, which is rather limited in superficial extent. The large cell is almost covered with other cells proliferating from it. The presence of large nuclei in the formative region of other blastomeres indicates that retardation in division of cells is not uncommon.

The cells of several of the blastocysts just mentioned, show a marked tendency toward rapid multiplication in the formative region, so that this area becomes densely studded with nuclei

(figs. 29 and 32). All of the cells multiply faster than the entodermal cells differentiate and as a result the cells become massed and the embryonic area tends to thicken. The process is continued until the embryonic disc is about three cells deep (figs. 35 and 36). The innermost of these cells are clearly entodermal. From the margin of the disc, entodermal cells may be seen to migrate out in an effort to complete the lining of the blastocyst.

If one compare sections through the embryonic area of the opossum blastocysts which have just been described with sections through corresponding stages of *Macropus* and *Parameles*, as figured by Hill, the similarity is very striking. In all cases the area tends to be much thicker than that of *Dasyurus*. This agreement of the opossum with *Macropus* and *Parameles* is correlated with the agreement in size of blastocysts as compared with the blastocysts of *Dasyurus* at the time of entoderm formation; for this process in *Dasyurus* is preceded by a period of extensive growth. The bearing of this on the subject under discussion will appear presently.

Surface views of this stage of the opossum blastocyst were not studied, since the technical difficulties of making surface preparations are almost insurmountable on account of the small size of the opossum blastocyst and the interference of the superimposed albumen. The corresponding stage in *Dasyurus* has a diameter of 4.0 mm. (eight to ten times that of the opossum blastocyst) and the albumen has long since disappeared (*K* and *J*, text fig. 4). Inasmuch as surface views were not available, the entoderm mother cells were not studied in toto, hence it was not possible to determine whether or not these cells possess pseudopodial prolongations and are capable of amoeboid movements. Several cells seen in section, however, as pointed out in detail above, strikingly resemble those shown by Hill in drawings of sections through the embryonic area and interpreted by him as primitive entoderm cells. These facts as they occur in the opossum are presented as observed, and the interpretations of the conclusions suggested are those which a careful study of the material seems fully to justify.

d. *Comparison of early and later blastocysts and growth of the egg*

A comparison of figures *H* and *J* (text fig. 4) will show the reader at a glance the changes which the blastocyst, the albumen and the shell have undergone from the 50-celled stage to the time that the entoderm formation is initiated.

The entire egg has grown comparatively little in size—from a diameter of 0.40 mm. on the average to about 0.50 by 0.60 mm. in the largest specimen (text fig. 4 *J*). This increase is due largely to the turgidity of the entire structure, the liquid within the ovum pressing against the plastic wall, which gradually responds. In the fresh state and in alcohol the blastocyst eggs in both stages are practically spherical. The blastocysts shown in figure 6 differ more in the preparations than they did in the fresh state, on account of differences in amount of shrinkage. Measurements of the thickness of shell membranes resulted as follows: No. 40, 0.0035 mm.; No. 43, 0.0027 mm.; No. 144, 0.0023 mm. Practically no change in thickness of shell has taken place since the 16-celled stage.

The blastocysts themselves vary more in diameter than do the shell membranes, chiefly because of the differences in quantity of albumen still left unabsorbed. It will be noticed that absorption of albumen proceeds most rapidly in the region of the blastodisc, less at the opposite pole. This is as one would expect from the very apparent difference in protoplasmic mass as well as from differences in metabolic activity of the cells at the two poles. The absorption of albumen continues in this proportion; so that in bilaminar blastocysts, when the formative region comes to lie against the shell, the non-formative region may still have a greater or less thickness of albumen separating it from the shell (text fig. 4 *L*). The ratio of activity of the formative cells (and the consequent thickness of the embryonic area) to quantity of albumen absorbed holds true only in general terms. Thus *B*, text figure 6, represents an egg with thickened embryonic disc whereas considerable albumen is left in the egg; figure *F*, on the contrary, shows little albumen as well as few cell elements in the formative area.

Included yolk and coagulum are still seen in several of the specimens, and large yolk granules, such as observed regularly in the earlier stages, still occur within the blastomeres (figs. 29, 32 and 33). Inasmuch as the thickened area of these blastocysts is very clearly the formative region, the question arises: which portion, the thicker or the thinner half, of the earlier (50-celled) blastocysts is formative and which the non-formative portion? Polar differentiation in the opossum, it will be recalled, first manifests itself by a more rapid thinning at one pole of the blastocyst of 50 or more cells. It will be recalled also, that Hill, interpreting Selenka's two earliest blastocysts in the light of the cleavage process in *Dasyurus*, considers the larger, more yolk-laden, cells of the lower pole as non-formative; the other as formative. I venture the assertion that the thicker-walled pole is the formative region (compare text figs. 5 and 6). The thinning of half or more of the blastocyst is simply the accompaniment of the differentiation of the embryonic disc. Blastocysts of No. 144, although shrunken, are especially instructive in this connection, since several of them are clearly a little more advanced than those described in the last section. From figure 27 and text figure 6 A, the change here indicated may well be seen. One region of the blastocyst (to the right in the figures) is more attenuated and larger in extent than the other. The differentiation has gone further than in any blastocyst shown in figure 5, and figure 27 is only a little more advanced. The whole series is so nearly complete as to justify the conclusion that, once begun, the differentiation of the formative region is progressive.

From the specimens described in this section, therefore, there can be no doubt whatever that, in common with other marsupials, the opossum lacks the morula stage in its development. It is furthermore clear that cell proliferations do occur from the blastocyst wall in the region of the blastodisc and that these proliferations result in the formation of the definitive entoderm.

GENERAL SUMMARY

1) Maturation of the opossum egg begins in the ovary, where, as in other mammals, the first polar body is given off (fig. 2, pages 15-16).

2) An average of twenty-two eggs are shed at one ovulation, but the number may rise to forty-five. This overproduction of eggs is accompanied by a high percentage of mortality at each stage in the development of the opossum, for unfertilized or otherwise abnormal eggs are found in each lot and dead fetuses are of frequent occurrence. On removal of the ovary and uterus from one side at the time of the first ovulation, the remaining ovary may compensatorily double the normal production of eggs at the second ovulation (pages 10-15).

3) Measurements of a dozen full and unshrunk ovarian ova gave an average of 0.165×0.135 mm. Unsegmented tubal and uterine eggs in the sectioned and stained preparations measure less, namely from 0.146×0.123 mm. to 0.095×0.080 mm., or an average from six lots of 0.111×0.107 mm. Hence the eggs of the opossum are on the average larger than most mammalian eggs. They are much smaller than the eggs of *Dasyurus*, which measure 0.27×0.24 mm. (fig. 4 and its legend).

4) The ripe egg of the opossum exhibits no evidence of polarity except that afforded by the position of the polar body and chromosomes of the egg nucleus. No polar concentration of deutoplasmic substance is ever encountered in the unsegmented opossum egg. The yolk granules or yolk spherules are for the most part situated in a peripheral or submarginal region, just beneath a thin yolk-free layer of granular cytoplasm (figs. 1, 3, 6, 8; pages 15-18).

5) Insemination occurs in the oviduct, where doubtless the second polar body is given off; neither process was, however, actually observed. After insemination the egg receives, through the secretive activity of the gland cells lining the oviduct, the secondary egg envelopes:—albumen and shell membrane. The albumen is homologous with the albumen covering of other Amniota; the shell membrane is homologous with the shell of Monotremata (figs. 5 and 6, pages 18-20).

6) When the eggs pass from the oviduct the shell membrane is thin and delicate, measuring 0.0012 mm. in thickness. It grows thicker rapidly, soon attaining a maximum which remains practically constant until the didermic blastocyst is fully formed. There is no progressive growth in thickness of shell membrane as in *Dasyurus*. That different batches of eggs, even in the same stage of advancement, vary considerably in this respect may be seen from the following table (figs. 4 and 5, page 19).

TABLE 2

NORMAL STAGE OF EACH LOT OF EGGS	NO. OF LOT	AVERAGE THICKNESS OF SHELL
Unsegmented.....	{ 54 & 58	0.0012
	{ 52	0.0034
	{ 46	0.0055
4-celled.....	81	0.0022
8-18-celled.....	{ 85	0.0033
	{ 117	0.0030
Just completed blastocyst.....	{ 50	0.0034
	{ 88	0.0045
Early bilaminar blastocyst.....	{ 144	0.0023
	{ 40	0.0035
	{ 43	0.0027
Completed bilaminar blastocyst.....	{ 55	0.0033
	{ 82	0.0048

7) Uterine eggs in early cleavage measure from 0.4 to 0.5 mm. through the shell membrane, or an average of about 0.45 mm. Hence the albumen layer is on the average 0.15 mm. in thickness. This thickness is maintained until the fully formed blastocyst begins to grow at the expense of the albumen. The egg grows very little in diameter of shell, being only 0.6 mm. in diameter at the time entoderm formation sets in or 0.7 to 0.9 mm. at the completion of the didermic blastocyst, when nearly all the albumen has been digested (fig. 4, pages 20 and 56).

8) The pronuclei are fully formed when the egg enters the uterus. At first they occupy a position within a yolk-free area at one pole, but soon migrate to the center, where the first cleavage spindle takes its position (figs. 4, 6 to 8, pages 21-22).

9) The first cleavage plane divides the egg into approximately equal halves. At this time the elimination of yolk takes place. This occurs, not by the extrusion of a deutoplasmic mass at one pole, but by the elimination from the entire periphery. The two blastomeres form a new membrane just within the yolk-laden submarginal layer, leaving masses of cytoplasm rich in yolk between the blastomeres and the zona pellucida. In this condition the eliminated material is later found in the cleavage cavity of every stage until absorbed. The two blastomeres exhibit no evidence of polarity. The yolk remaining within the blastomeres in the 2-celled, as well as in later stages, occupies a submarginal zone as in the unsegmented egg (figs. 7-10, pages 22-24).

10) The second cleavage plane is again meridional and at right angles to the first. The four resulting blastomeres are spherical and shift their position until the pairs come to lie at right angles to each other. From this time on the cleavage is indeterminate, as in the *Eutheria* (text fig. 1 and figs. 11-18, pages 24-28).

11) As cleavage proceeds the cells migrate to the periphery and become closely appressed against the zona pellucida or, if this has disintegrated, against the albumen layer. As a result, at the end of the fourth cleavage (and sometimes before this) the cells are arranged in the form of a sphere enclosing a considerable cleavage cavity, in which the eliminated yolk and coagulum are to be found. The cells of the 8- and 16-celled stages are practically alike in every particular (text figs. 2 and 3, figs. 19-24).

12) A study of an unbroken series from the 2-celled to the 18-celled stages, together with a fairly complete series up to the time of entoderm formation, makes it highly probable that the cells of each hemisphere are lineal descendants of one or the other of the two blastomeres of the 2-celled stage. The cells of the two hemispheres are destined to form the embryonic and the non-embryonic regions, respectively, of the blastocyst. This interpretation is supported by a full series of wax models of the cleavage stages. Hence, also, the unsegmented opossum ovum possesses a potential though concealed polarity and con-

sists of a formative and a non-formative portion. This interpretation homologises the opossum egg with that of *Dasyurus*, in which polar differentiation is structurally evident throughout early cleavage (text figs. 1, 3, 4 and 6, pages 39–40).

13) Since the blastocyst of the opossum is already anticipated in the 16-celled stage by the arrangement of the cells into a hollow sphere, the blastocyst is completed at an early stage, that is, in eggs of about 40 cells. By this time the ovum has grown very little, the albumen layer being as thick as in previous stages. The corresponding stage of *Dasyurus* contains about 125 thin cells which lie immediately against the shell membrane. About this time polar differentiation is manifested for the first time in the history of the opossum egg as a thinning of the cells at the pole destined to be the extra-embryonic area of the definitive vesicle. Included cells are almost always present in the early blastocysts (figs. 4, 5, 6, 26, 27, pages 42 and 49).

14) The morula stage is absent in the opossum egg. The entoderm is formed by the proliferation of specialized 'entoderm mother cells' which appear only in the embryonic area. The entoderm mother cells may or may not migrate from their position in the wall of the blastocyst, but in either case they give off by division primitive entodermal cells which spread out at first immediately beneath the embryonic ectoderm and later beyond this area, until they completely line the entire vesicle. Entoderm formation sets in when about half the albumen is absorbed and the blastocyst is 0.6 mm. or less in diameter. Hence there is no such great period of growth as obtains in *Dasyurus*. The didermic blastocyst of the opossum is completed when the egg is only about double the original size as measured through the shell membrane (text fig. 4, figs. 28–38, pages 49–57).

LITERATURE

- ASSHETON, R. 1898 The segmentation of the ovum of the sheep, with observations on the hypothesis of a hypoblastic origin of the trophoblast. Quar. Journ. Micr. Sci., vol. 41.
- CALDWELL, W. H. 1887 The embryology of Monotremata and Marsupalia, Part I, Phil. Trans. Roy. Soc., vol. 178 B.
- HILL, J. P. 1910 The early development of the Marsupalia, with special reference to the native cat (*Dasyurus viverrinus*). Quar. Journ. Micr. Sci., vol. 56.
- HILL AND O'DONOGHUE 1913 The reproductive cycle in the Marsupial *Dasyurus viverrinus*. Quar. Journ. Micr. Sci., vol. 59.
- MINOT, CHARLES R. 1911 Note on the blastodermic vesicle of the opossum. Anat. Rec., vol. 5, no. 6.
- PATTERSON, J. T. 1913 Polyembryonic development in *Tatusia novemcincta*. Journ. of Morph., vol. 24.
- SELENKA, E. 1887 Studien in der Entwicklungsgeschichte der Theire, Band 4, Das Opossum, (*Didelphys virginiana*). Wiesbaden.

PLATE I

EXPLANATION OF FIGURES

1 Tubal ovum, No. 76 (3). The distribution of yolk spherules is not quite typical, an unusual number of them having invaded the central, generally yolk-free zone ($\times 600$).

2 Ovarian egg, No. 21 (20), showing discus proligerus (*DP*), polar body (*PB*), and chromosomes of the egg nucleus (*CH*). The zona pellucida is best seen at the lower margin of the egg. The section is taken somewhat to one side of the center ($\times 430$).

GENERAL ABBREVIATIONS

<i>CG</i> , coagulum	<i>TR.ECT</i> , trophoblastic ectoderm
<i>CH</i> , chromosomes of egg nucleus	<i>Y</i> , yolk
<i>EMB.A.</i> , embryonic area	<i>ZP</i> , zona pellucida
<i>PB</i> , polar body	<i>ZR</i> , zona radiata
<i>SM</i> , shell membrane	

Note: The figures in these plates were drawn by Miss Aimée Vanneman, technician in the School of Zoology, the University of Texas. The outlines for the drawings were made with the aid of a Bausch and Lomb drawing apparatus.

PLATE 2

EXPLANATION OF FIGURES

3 Tubal ovum, No. 56 (10). The yolk-free peripheral and central zones and the yolk-laden submarginal zone are well shown ($\times 607$).

4 Uterine ovum, with pronuclei, No. 54 (2). The specimen is somewhat abnormal in the possession of the third body (*A*) adjacent to one of the pronuclei ($\times 790$).

PLATE 3

EXPLANATION OF FIGURES

5 Unfertilized uterine egg, No. 58 (2), just after it entered the uterus. On account of its thinness, the shell membrane has shrunk considerably. The large yolk spherule is seen at one pole. The egg is slightly shrunken away from the zona at one side ($\times 525$).

PLATE 4

EXPLANATION OF FIGURES

6 Uterine ovum, No. 52 (7), with pronuclei near the center. The pronuclei are not in the same section in the series, but are here represented together, the drawing having been made by superimposing the two sections containing the pronuclei ($\times 790$).

7 Fertilized uterine ovum, No. 54 (4), with chromosomes of first cleavage spindle. An unusual preponderance of yolk vacuoles is to be noticed in the lower hemisphere of the egg ($\times 790$).

6

7

PLATE 5

EXPLANATION OF FIGURES

8 Portion of first cleavage spindle in uterine egg No. 52 (3) ($\times 790$).

9 2-celled stage showing elimination of yolk; egg No. 50 (6). The cell to the left has separated from the eliminated material and rounded off. The cell to the right has not yet separated, but the line of separation is indicated by the dark granular zone (*CM*) ($\times 405$).

10 2-celled egg, No. 46 (3). The yolk has been eliminated; the cell to the left has not yet rounded off. The yolk granules are again assuming a submarginal arrangement in the blastomeres ($\times 405$).

PLATE 6

EXPLANATION OF FIGURES

11 and 12 Two sections of 4-celled egg No. 81 (5), showing relative position of the two pairs of blastomeres. The configuration of the shell membrane (*SM*) and the mass of albumen (*ALB*) will enable the reader to determine the axes of the pairs of blastomeres. It will be noted that these are nearly parallel ($\times 125$).

13 and 14 Two sections of 4-celled egg No. 81 (11). The blastomeres have shifted more than those of figures 11 and 12 (text figure 1 *B*). The cells are relatively small ($\times 125$).

15 and 16 Two sections of 4-celled egg No. 81 (5), showing the two pairs of blastomeres almost at right angles to each other. Compare text figure 1 *A* ($\times 125$).

PLATE 7

EXPLANATION OF FIGURES

17 Section of a 4-celled egg No. 81 (2), showing characteristic appearance of yolk in this batch of eggs. In some of these eggs the blastomeres are without clear-cut cell membranes ($\times 390$).

18 Section through three blastomeres of the 4-celled egg No. 83 (7). The blastomeres of the 4-celled stages are in all cases so oriented that no section contains more than three cells ($\times 390$).

19 Section through a 7-celled egg, No. 85 (7). The tendency of the cells to flatten out against the zona is already manifested at this stage. The peripheral arrangement of yolk remaining within the blastomeres is well shown ($\times 390$).

20 Section through 9-celled egg, No. 85 (3). The blastomeres are in contact with the albumen layer. The cells are large and little yolk is seen in the cleavage cavity. The vacuolization in the inner margins of cells is due to breaking down of deutoplasmic content of cells in this region. The yolk has not all as yet been crowded into the cleavage cavity ($\times 390$).

21 Section through the four blastomeres of the 12-celled egg No. 85 (18) b. The blastomeres are numbered to correspond to the figures employed in text figure 3 B. Cells 5 and 6 are in mitosis. Unlike the two preceding specimens, the cells of this egg are not yet in intimate contact with the zona ($\times 390$).

22 Section through the 16-celled egg No. 85 (2). The centrifugal migration of the cells and their flattening out against the zona are well shown. The yolk and coagulum occupy the cleavage cavity ($\times 390$).

17

18

19

20

21

22

PLATE 8

EXPLANATION OF FIGURES

23 and 24 Sections through the 16-celled eggs, Nos. 85 (11) b and 85 (11) a, respectively, to show the relative distribution of yolk granules within the blastomeres and in the cleavage cavity. As in figures 19-22 the blastocyst formation is anticipated. The blastocyst will be completed when the gaps (i, i) have been filled by the continued flattening and multiplication of the cells ($\times 390$).

25 Section of 52-celled completed blastocyst, No. 50 (3) a, showing included deutoplasm and coagulum in cavity. The large cell in upper portion of figure is one of four retarded blastomeres situated at this pole ($\times 563$).

PLATE 9

EXPLANATION OF FIGURES

26 Section of blastocyst No. 50 (3) b, to show distribution of yolk granules. The yolk granules in the cleavage cavity are to be found chiefly within the cytoplasmic fragments and the included nucleated cell (*C*). Much yolk is still within the blastomeres ($\times 540$).

27 Section of blastocyst No. 144 (1) d, somewhat more advanced than those shown in figures 25 and 26 (*A*, text figure 6). The specimen is somewhat shrunken. The non-formative area (to the reader's right) has become considerably attenuated. A study of the series from which this section is taken shows that the apparently included cells are attached to cells situated in the wall of the blastocyst ($\times 375$).

26

27

PLATE 10

EXPLANATION OF FIGURES

(Magnification of all of the figures $\times 375$.)

28 Portion of formative region of blastocyst No. 43 (9). *ENT*,¹ entodermal mother cell in the blastocyst wall; *ENT'*, primitive entodermal cell. Both cells are much darker than the adjacent ectodermal cells. See *F*, text figure 6, for outline sketch of entire section of blastocyst.

29 and 30 Adjoining sections through the embryonic area of blastocyst No. 43 (8), shown in outline in *H*, text figure 6. *ENT*,¹ primitive entodermal cell dividing from an entodermal mother cell; *M*, large cell in mitosis. This cell contains a large fat spherule shown in section in figure 30.

31 Section through embryonic area of specimen No. 40 (2). (Cf. outline sketch *B*, text figure 6. *ENT*,¹ entoderm cell being given off from entodermal mother cell situated in the wall of blastocyst; *ENT*,² large cell dividing obliquely to surface; *O*, elongate cells and nuclei, with axes oblique to surface of blastocyst.

32 Section through embryonic area of *D*, text figure 6, specimen No. 40 (4). *ENT*,¹ entodermal mother cell which has migrated from its position in the wall of the blastocyst.

33 and 34 Two sections of blastocyst No. 40 (1), shown in outline in *E*, text figure 6. *ENT*,¹ primitive entoderm cell just divided from entodermal mother cell and pushed out underneath the superficial ectodermal cell.

35 Section through middle of the embryonic area of blastocyst No. 43 (7), shown also in *G*, text figure 6. The area has thickened on account of rapid cell division at this point, nearly every section of the series containing one or more cells in mitosis.

PLATE 11

EXPLANATION OF FIGURES

36 The tenth of the series of 40 sections through the same egg of which figure 35 represents the 21st section. The thickening of the embryonic area is better shown here. Three cells are in mitosis. At *ENT*¹ the entodermal cells are seen to migrate from the margin of the embryonic area.

37 and 38 Sections of blastocyst No. 40 (3) shown also in *C*, text figure 6. The embryonic region (*EMB.A*) is thick, but limited in superficial area. *C*, large cell, a retarded blastomere; this cell comes to the surface in a number of sections of the series. *TR.ECT*, trophoblastic ectoderm.