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THE FORMATION AND STRUCTURE OF THE ZONA PELLUCIDA IN THE OVARIAN EGGS OF TURTLES

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TWELVE FIGURES

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INTRODUCTION

In the active research upon eggs of all groups of animals, which has been in progress for nearly fifty years, the zona pellucida, a cuticular membrane, formed around the egg in the course of growth at some stage preceding maturation, has not failed to be an object of interest to investigators and to share with other portions of the egg the most painstaking examination. According to Waldeyer ('01, '02, '03, p. 287) there occurs at least one membrane in all vertebrate eggs whereas among invertebrates some eggs remain naked. The majority of vertebrate eggs which have been subjected to careful study show this membrane to be the zona pellucida. It consists typically of two concentric layers, one of which exhibiting characteristic radiating striations perpendicular to the egg surface is termed the zona radiata.

That in the same species this membrane varies to a considerable degree in thickness is shown by figures given below where in the turtle's egg its thickness ranges from 1μ in initial stages to 17μ. The variation of this dimension in the eggs of different groups of vertebrates can be demonstrated by comparing these figures with those given by Prenant, Bouin, and Maillard (Traité d'Histologie, p. 1097) who quote Nagel's measurements, 1.2μ to 1.5μ in the mouse and 2.0μ to 2.5μ in man. The zona pellucida must be distinguished from the yolk or egg membrane, a very thin membrane observed in some vertebrate eggs surrounding the yolk before maturation is completed (Van Beneden, '80, Fischer '05, p. 595).

In respect to thoroughness and extent of investigation upon the structure of the zona pellucida, mammalian eggs naturally stand first. Less complete and comprehensive study has been given to it in the lower vertebrate groups.

HISTORICAL SKETCH

Authors who have given detailed contributions upon the mammalian zona pellucida may be grouped into three classes: first, those who regard this membrane as originating from the egg cytoplasm; second, those who maintain that it is derived from processes of the egg epithelium or from an exoplastic or intercellular substance of the cells of this epithelium; third, those who frankly state its origin to be uncertain. The first class includes Van Beneden ('80), Kölliker ('98) and Sobotta ('02). In the normal Graafian follicles of the bat (Rhinolophus ferrum equinum, Schreb.), Van Beneden observed egg cells in such close contact that the zona pellucida of one touched the zona pellucida of its neighbor to the exclusion of the epithelial layers. He, therefore, concluded that the zona pellucida developed from the surface of the egg cytoplasm in the absence of the egg epithelium. Kölliker stated that egg cells not yet surrounded by epithelium but already arranged in islets or nests were inclosed in a distinct membrane which he designated as the first anlage of the zona pellucida.

Flemming ('82), Retzius ('89), Paladino ('90), Von Ebner ('00), and Fischer ('05) affirm that the zona pellucida is formed of cytoplasmic prolongations of the epithelial cells. Flemming describes fibers which "may be protoplasmic connections of the egg cell with its neighboring cells; in the spaces between these bridges the intermediary mass of the zona, gradually becoming firmer, may be laid down." Retzius states that in the rabbit cylindrical cells send out branched processes which gradually interlace so that a thick network originates around the egg. A consolidation occurs on the inner belt of this network forming the zona pellucida. In the completely developed zona pellucida the outer zone is also consolidated and between the inner zone and the surface of the egg radiating striations can be recognized. These represent granular filaments, which bore through the substance of the zona and are attached to the egg surface by small conical basee. In the opinion of Paladino, bridges exist in the rabbit between the epithelial cells as a whole, as well as between these and the egg cell. In ripe eggs a fiber net exists between the epithelium and the outer surface of the egg, the inner meshes of which contain finely granular substance. Paladino gave a rather bizarre interpretation of this granular substance, regarding it as nutrient material derived from the breaking down of the epithelial cells formerly existing in these areas. A true zona pellucida is evolved from this substance which becomes hyalin in character and strongly refractive. Von Ebner substantiates in general the statements of Flemming and Retzius. The first anlage of the zona shows a network closely attached to the egg surface; this gradually moves back toward the epithelium leaving radiating filaments in connection with the egg surface to give place to the "secondary zona substance." According to Fischer the zona pellucida arises from unbranched cytoplasmic prolongations of the epithelial cells interwoven and pressed together. Compression occurs to such an extent on the inner portion of the zona as to eliminate the individual outlines and thus form a homogeneous substance. In the completely developed zona pellucida he distinguishes three layers, spongy, radiating and homogeneous, of which the last named is the oldest and firmest.

Regaud and Dubreuil ('08, p. 152) deny any protoplasmic connections between the egg and the cells of the egg epithelium.

In a fully developed ovarian follicle (rabbit) the zona is formed of three concentric layers. The first is a very thin internal layer applied to the surface of the egg but substantially independent of it; to this layer, which is not homogeneous but fenestrated in the manner of a grating (or grill?), we have given the name fenestrated epiovular membrane. The second is an external layer in connection with the prolongations of the cells of the corna radiata: it is formed by a thick felt of filaments running in all directions, the felted layer. The third or middle layer, the zona pellucida properly called comprises two substances, radiating filaments irregularly extending from the felted layer to the periovular membrane and an amorphous or granular substance (following the action of the fixative), laid down in abundance in the spaces between the radiating filaments which it bathes. . . . The felted filaments, the radiating filaments and the epiovular membrane which have been interpreted up to the present time as anastomosing elements are not protoplasmic but an exoplastic production of the follicular cells about the egg.

The investigations of Rubaschkin and Waldeyer have left them in doubt as to the exact origin of the homogeneous substance (so-called by them) of the zona pellucida. According to Waldeyer ('01, '02, '03) the zona pellucida is composed of a fiber felt and a homogeneous substance across which protoplasmic connections from the epithelium to the ooplasm make their way. The homogeneous substance is perhaps a product of the ooplasm and the mammalian zona pellucida, derived in part from the epithelium, in part from the ooplasm. Rubaschkin ('05, p. 519) describes as the zona pellucida in guinea pigs, a thick homogeneous layer directly surrounding the egg or yolk membrane. Central processes from the epithelial cells penetrate this zona substance where they lose their protoplasmic appearance. These processes do not form intercellular bridges because they are prevented from actual contact with the ooplasm by the presence of the egg membrane. They do not end with enlargements or knobs as Retzius figured them to do. A number of eggs, however, show a thick layer of coarse fibers, the processes of the epithelial cells which wind about the zona substance but do not penetrate it at any point. This layer corresponds to the perizonal fiber net of Retzius. Waldeyer is inclined to regard the zona pellucida, contrary to his earlier opinion, as a product of the ooplasm, while Rubaschkin favors the view that it is derived from the epithelium.

Of those who express an opinion on the zona pellucida of the egg in vertebrate groups below the mammals there may be cited Lams on the European smelt (Osmerus eperlanus), Munson on the turtle (Clemmys marmorata), Waldeyer on selachians amphibia, reptiles and birds, and Mlle. Loyez on reptiles in general. In Osmerus eperlanus, Lams ('03, '04) describes the zona pellucida which he calls a chorion, thick and radially striated. The striated appearance is due to innumerable canalicules running perpendicular to the surface of the egg. Also, in the cytoplasm of the egg directly beneath the yolk membrane, he sees granular striations which "do not properly, in all probability, belong to the egg cell but correspond to prolongations of the follicular cells which have traversed the canalicules of the chorion and the yolk membrane and become continuous with the cytoplasm of the egg." Munson ('04, p. 331) states that in Clemmys marmorata there occurs an egg membrane which is composed of two layers, the outer homogeneous and the inner striated. In selachians, amphibians, reptiles and birds Waldeyer ('01, '02, '03, p. 293) shows that a zona pellucida consisting of an outer homogeneous and an inner striated layer can be seen well only in developing eggs, that it atrophies in mature eggs leaving only a very thin egg membrane. The striated appearance is due to radial canals. According to Mlle. Loyez ('05, '06, p. 147) three membranes arise in eggs of reptiles. The vitelline membrane which originates directly from the primitive membrane of the oocyte is at first very thin. As it increases in thickness it becomes finally striated and then granular. The heavily striated zona radiata forms on its inner surface early in the course of development. A very transitory third membrane is differentiated from the internal surface of the zona radiata. After its disappearance the inner surface of the zona radiata becomes less and less distinct and finally the striations come to appear in the superficial layer of the egg. Mlle. Loyez' vitelline membrane and zona radiata together make up the zona pellucida, without doubt and her third transitory membrane is the yolk membrane.

This short résumé proves that most authors agree upon the existence of protoplasmic bridges connecting the epithelial cells with the egg cytoplasm. But when they mention the homogeneous cuticular substance few give satisfactory descriptions and illustrations of the origin of this or of its structure in later phases of development. Only Regaud and Dubreuil go into the subject in detail; they lay emphasis upon the different stages of its development from the exoplastic fibers formed between the epithelial cells. The object of the present paper is to show that this membrane in the species studied consists neither of real cytoplasmic structures nor of real exoplastic structures but of intercellular substance and of cytoplasmic prolongations of the epithelial cells combined in a definite manner. The intercellular substance is represented by a series of walls ramified and anastomosed in such a way as to create cylinders or canals of which the transverse section appears as a reticular network. Extending down through these cylinders cytoplasmic filaments from the epithelial cells make their way to the yolk substance.

MATERIAL AND METHODS (INCLUDING POSSIBLE SOURCES OF ERROR)

Twenty-one series have been prepared from the ovarian eggs of the following turtles: Clemmys guttatus (Schneider), Graptemys geographicus (Lesueur), Emydoidea blandingi (Holbr.), Aromochelys odoratus (Latr.) and Chrysemys picta (Hermann) in various stages of growth. The identification of these species is so simple that I shall not stay to discuss the particular features by which they were identified. The animals were killed as soon as possible after their arrival in the laboratory to reduce errors in observation, the result of any prolonged starvation due to improper feeding, a condition which has marked influence upon the general ovarian structure as shown recently by Walsh (Loeb '17). The time which elapsed between the capture of the turtles and their arrival in the laboratory and the conditions under which they were kept prior to their arrival are not known. The majority of the ovaries examined had the appearance of being perfectly normal. In a few of them, however, at least one egg which must already have attained a diameter of 2 to 3 mm. showed processes of degeneration well under way. In several of these pathological eggs I found an object which Dr. Van der Stricht and Dr. Todd identified as a parasite. To the influence of this parasite the pathological condition of the egg was probably due but no one to my knowledge has so far made a study of this subject.

All the eggs examined were very much less than the size of the deposited egg. No essential differences are apparent in the structure of the zona pellucida of the various species, hence the stages described below have been chosen as representative of all the material.

The following methods of technique were employed. Fixation by the fluids of Hermann, Flemming or Benda, followed by staining with iron haematoxylin and Congo red or with safranin and picric acid. Fixation by Bouin's mixture or trichloracetic acid followed by staining either with iron haematoxylin and Congo red or with Mallory's connective tissue stain. The sections are cut four or five micra thick. All investigators have studied their material in cross section but, judging from their text and illustrations few have seen the importance of examining tangential and oblique sections. Fischer mentions that he could see the fiber work of the spongy layer very beautifully in tangential sections. In tangential sections Lams is able to interpret the structure of the chorion of Osmerus eperlanus. Dr. Van der Stricht called my attention to the significance of this method of study.

Because of shrinkage in paraffin and because of flattening from the action of fixatives and from the pressure of the knife in cutting the circumference of the egg almost always becomes ovoid: this necessitates the taking of averages from measurements of the long and short axes. Because also of the method of measuring with the camera lucida, the figures given for the diameters of the egg, taken through the zona pellucida, are only approximate. The figures given for the thickness of the zona are more exact, having been obtained from prints of microphotographs by computing the magnification.

The microphotographs, all of which were taken at a magnification of 750 diameters, represent the structure of the zona pellucida in eggs ranging in diameter from 0.65 mm. to 2.6 mm. and from younger stages in which the zona pellucida measures only 1μ up to a stage where it is 17μ in thickness. I do not know if this last measurement may approximate the maximum thickness of the zona since I have no measurements from larger eggs. Mlle. Loyez states that in reptiles the zona is very thin upon completion of development. Since it is extremely difficult in microphotography to focus upon an entire field unless that field is perfectly flat in all its parts some portions of the figures are not sharply defined. The endeavor has always been to focus upon the most important part of the section.

OBSERVATIONS

The epithelium

When the oocyte has reached the size two or three times, at a rough estimate, that of the oogonium from which it originated it is surrounded by a flattened epithelium which remains of one layer throughout the course of development. With the gradual growth of the oocyte the epithelial cells take on a definite prismatic shape and increase in height in the axis perpendicular to the surface of the egg until this axis may become as long as the transverse. The transverse axis appears the longer, however, in the majority of cases especially in the later stages herein described. Upon cross sections through the epithelial layer of oocytes less than 1 mm. in diameter the nuclei of the epithelial cells are seen to be rather widely spaced (fig. 2, *ep.*) while in older stages, because of reduction in size of the nuclei and in content of the cytoplasm, the arrangement is more compact (figs. 3, 7, 9, 10, 11, *ep.*). Occasional mitoses prove that to accommodate the increasing volume of the egg the epithelium extends itself by divisions of its constituent cells. In eggs much larger than those figured very numerous mitoses occur. The epithelial cells are sharply marked off from one another by intercellular channels filled with intercellular substance. Unfortunately this does not show clearly in the photographs. Some preparations fixed in Bouin and stained by Mallory's connective tissue method, show this substance very clearly colored by aniline blue. The intercellular substance early undergoes a change of constitution and becomes transformed, at the level of the surface of the cells, into the special cement known as the terminal bars (Schäfer '12, p. 86, Stöhr '98, p. 68). It is well known that sections cut perpendicular to the plane of the surface of the epithelial cells show in well fixed and stained preparations a continuous dark line representing the lateral surfaces of the terminal bars sometimes thickened noticeably at points marking the limits of two adjacent cells. In other portions of the sections this line may not be seen but cross sections of the bars appear as dark round spots. The former picture is represented in the turtle's eggs in figure 2, *t.b.* The lateral surfaces of the terminal bars of adjacent cells form a rather thick distinct boundary line between themselves and the oocyte thus marking the beginning of the zona pelucida.

Cytoplasmic bridges of various sorts connect the cells with one another (Fischer '05, Paladino '90). Filamentous and thin or short and coarse, they traverse the intercellular spaces and retain their identity for considerable distances within the cell cytoplasm where they finally mingle with the denser portions encircling the large nuclei (fig. 1 *l.b.*, *s.b.*). A dense opaque mass, the attraction sphere, is closely attached to each nucleus usually either on that face which is nearest the surface of the cell or at one side (figs. 2, 4, *a.s.*). Often such clearness is obtained through successful fixation or through the thinness of the section as to determine the character of the sphere. It is composed of three elements, a small granule (or sometimes two) the central corpuscle in the center or slightly to the side of an oval or circular clear field, the medullary layer marked off from the mass by a distinctly larger, more opaque zone, the cortical layer (Van Beneden). Loosely interwoven filaments extend out from the dense attraction sphere to the clear exoplasm at the periphery of the cells thus forming a delicate network.

Zona pellucida

Solely for purposes of clearness the developmental history of the zona pellucida may be presented in three successive stages.

The first stage covers those phases of formation in which the zona pellucida, on cross section, is but a thin one layered cuticle while on oblique and tangential sections the beginnings of a reticular network are found.

The second stage includes that period during which the zona pellucida becomes divided into two concentric layers, the inner thin and radially striated, the outer, denser with striations more or less obscured.

The third stage is co-extensive with the period of growth during which both layers just mentioned become very much thicker.

Stage 1. The terminal bars, as viewed on a cross section, divide the epithelial cells from the oocyte by an apparently continuous line which at first is thin and uniform but later becomes thicker until it is a cuticle of double contour and of rather uneven outline especially on its deep surface where it lies in connection with the epithelial cells. On this front the junctions of the intercellular substance, separating the lateral surfaces of the epithelial cells, make with the bars triangular thickenings. The change in the terminal bars initiates the development of the zona pellucida. From the time when the cuticle reaches an average thickness of 1μ it may be termed the zona pellucida (fig. 2, *i.z.p.*). Filaments of the cytoplasmic network extending from the attraction spheres (*a.s.*) seem to attach themselves directly to the deeper limit of this cuticle (fig. 2) the actual structure of which is not demonstrable on cross sections. Oblique and tangential sections, however, make it clear that the zona pellucida is of complicated organization even at this early stage. It is perhaps well to explain at once that in an oblique or tangential section of an egg one may see two, three or more irregular rows of epithelial cells, the number depending upon the size of the egg and therefore upon the curve of the epithelial layer. These represent cross sections of the epithelial cells at various heights. These portions in the section furthest away from the yolk show the bases of the cells;

then appear successively the clear cytoplasm and perhaps the basal segments of the nuclei; next various segments through the nuclei; and nearer the yolk, sections through the central spheres and terminal bars and therefore through the incipient zona pellucida. These tangential sections (figs. 3, 4, 5) prove that the cuticle is composed of large polygonal fields (*p.f.*) marked off from one another by a system of dark lines, the terminal bars (*t.b.*). These large polygonal fields are not homogeneous but inclose smaller fields of similar outline formed by a fine pale network, the meshes of which are a little thicker and darker at some points and in close connection with the terminal bars, thus giving the impression of extensions of the bars over the surface of the epithelial cells. The meshes of this fine reticulum seem exactly to overlie the deeper cytoplasmic network (fig. 4 *c.n*) of the cell which arises from the interwoven filaments extending from the central spheres. The zona pellucida then takes its origin as a veil-like formation consisting of a mosaic of terminal bars and polygonal fields within which may be recognized the small, pale areas, future canals of the adult membrane separated by pale and dark filaments giving origin to the future fundamental substance of the adult membrane.

In older oocytes several changes take place. Those portions of the network, in which the meshes are a little thicker and are stained in the same way as the terminal bars, have become much more numerous (figs. 5, 6).

It may render the description clearer at this point to distinguish the network of darkly stained meshes which follows the pattern of the original terminal bars around the large polygonal fields, calling this the primary network (*p.n.*) from that which follows the outlines of the original cytoplasmic reticulum, using for this the term secondary network (*s.n.*) Dr. Van der Stricht observes a similar distinction in structures of the membrana tectoria. The meshes of the primary network appear to send out short extensions to the secondary network and to soften their sharp angles so that these assume circular or oval shapes rather than clear cut polygons (figs. 5, 6, *p.n.*). So far I have been unable to assure myself definitely of a longitudinal splitting of these meshes and of the development of cuticular bridges connecting the parts as has been shown to take place in the membrana olfactoria (Van der Stricht) although certain figures do suggest such an interpretation. A superficial and older portion of the veil of the zona pellucida shows the beginnings of the adult structure, regular small round spaces inclosing dark granules, the cross sections of prolongations of the epithelial cells (figs 5, *pr.*).

Stage 2. In more advanced phases of growth the nuclei of the epithelial cells are crowded nearer to one another and lie closely on the zona pellucida. A cross section (fig. 7) shows that the zona pellucida has become thicker and is divided concentrically into two layers, the outer of which (*o.l.*) is more or less homogeneous and very dark in the figure whereas the inner (*i.l.*) is less opaque and distinctly striated in a direction perpendicular to the surface of the egg. This layer is separated from the yolk substance by a sharp boundary, the nature of which together with the two layers of the zona must be investigated in tangential sections. The real importance of the study of tangential sections is well demonstrated here for the extremely intricate structure of the zona pellucida, of which one could obtain no true conception from cross sections, is revealed with remarkable clearness. In many preparations, as portrayed in figure 8, *o.l.* the outer denser layer appears separated into three concentric belts, a middle clearer stratum (*s'.*) between two bordering darker thicker strata (*s.s''.*). In other preparations stained either more deeply or very slightly this concentric division into belts is not seen. A completely satisfactory explanation for this phenomenon cannot be given. There is a possibility that it may be due to accidental causes, for instance uneven penetration of the fixative or other media used though its explanation is more probably to be found in differences in constitution between the older and the more recently formed parts of the zona pellucida.

Far from being homogeneous the outer layer consists of clear spaces, the cross sections of a system of canals (*c.*) within which are seen filaments, the cytoplasmic prolongations (*pr.*) from epithelial cells. The canals are separated by a meshwork much thicker and larger than in earlier stages, representing the cuticular part of the zona pellucida (*f.s.*) already observed in the first stage. Immediately beneath the epithelium in the zona (*s.*) a series of polygonal or circular fields occupies an area corresponding to that originally marked off by the primary network. On the whole one receives the impression that merging occurs between the primary and the secondary networks so that distinction between them is no longer possible. The three elements of which the outer layer is composed also make up the clear inner striated layer though in the latter region the network of the fundamental substance of the zona stains far less deeply and appears to be of a much less dense character. The striations (fig. 7, *f.s.*) are undoubtedly produced by filaments connecting the epithelial cells with the yolk and by walls of the tubes of the fundamental substance of the zona which these filaments traverse. Since tubes, canals and filaments occur in the outer layer it seems at first remarkable that the striations in it are not obvious in cross sections. In favorable and largely decolorized preparations, the outer layer does appear striated but in more darkly stained preparations the fundamental substance obscures the prolongations because of its great affinity for the stain. The striation in the inner layer is quite evident in cross sections because its fundamental substance takes up very little stain. The inner layer is evidently the older part of the zona and must have been originally identical in substance with the outer layer, the later differentiation resulting from a change in properties of the older fundamental substance causing it to become less dense and to have less affinity for stains. For the site of active proliferation of the fundamental substance is the surface of the epithelial layer which moves back as the epithelial cells withdraw in the centrifugal growth of the egg. It is a still more significant fact, I believe, that living eggs show striations in the outer layer also: at least I have lately observed this appearance in preparations of more advanced stages of the living eggs of Aromochelys odoratus, the eggs of which differ in no essential manner from those of the species previously mentioned in the general structure of the zona pellucida. In eggs of A. odoratus approximately 1.5 to 2 mm. in diameter examined in normal saline the striations of the outer layer seemed continuous with those of the inner layer yet the line of demarcation between the layers was in no way obliterated. The difference in the nature of the layers apparently is one simply of refraction since there is no distinct structure dividing them nor indeed any distinguishable boundary line. The presence of an egg or yolk membrane which might have been represented by a sharp line of demarcation between the striated layer and the oocyte in figure 7 cannot be confirmed in tangential sections. The boundary line (fig. 7) seems to be produced by thickenings of the ends of cell prolongations at the points where they reach the yolk. No trace of an egg membrane can be discovered in the living oocytes of A. odoratus.

Stage 3. The inner layer of the zona pellucida grows in thickness comparatively slowly whereas the outer, increasing more rapidly, becomes two or three times as thick as the former (figs. 9 and 10). The area of proliferation often stains very deeply (fig. 9, *a.p.*) so that a densely colored belt borders the surface of the outer layer remote from the yolk. At certain points in the outer layer (fig. 9) are seen cross sections of the canals (*c.*) with their contents (*pr.*) at other points a real striation, the result of rows of granular filaments in continuity with identical rows of filaments in the striated layer. This confirms the observation made on living eggs in which was noted the presence of striation in the outer layer. When the area of proliferation has chanced to stain less deeply one can see that the filaments are actually prolongations extending down from the scanty cytoplasm surrounding the epithelial nuclei into the substance of the zona (figs. 10, 11, *pr.*) There are no indications that these filaments branch as Retzius has reported in the case of the rabbit oocyte but the small conical or knob-like bases described by him appear (figs. 9, 10, *k.e.*) as enlargements of the prolongations. Among the granular filaments within the striated layer there appear more homogeneous elements in continuity with the meshes of the fundamental substance of the outer layer (figs. 9, 10, 12, *f.s.*). In this stage the constituents of the zona are shown to be the same as in stage 2: a system of clear openings, cross sections of cylinders (*c.*) with their contents the prolongations (*pr.*) of the epithelial cells and the mesh work of the cuticular fundamental substance (*f.s.*, fig. 12, *o.l.*, *i.l.*). In the inner layer the meshes of the fundamental substance stand out more clearly than in figure 8 since they are more deeply stained. Here the tubes and filaments have increased greatly in length and the fundamental substance in amount. In the series of stages showing these elements in various phases of development it can be noticed that whereas in numerous openings the prolongations are very well seen in other openings no contents are perceptible. The absence of prolongations from some spaces may be due first to imperfect fixation and staining, secondly to the real lack of systems of cavities corresponding to and overlying the intercellular spaces and consequently the primitive terminal bars in the first stages of development. A very thin discontinuous line between the knob-like enlargements of the ends of the granular filaments and the yolk substance in figure 9 may represent a real egg membrane. This appearance is very rare and further investigation with more refined methods is required to explain it. In tangential sections one sees nothing convincing of the presence of an egg membrane. It may be as Van Beneden asserts regarding the eggs of the rabbit that it can never be isolated in ovarian eggs until a short time before impregnation. In that case it could not be seen in turtles, eggs as small as these which are at present being investigated.

SUMMARY

1. The epithelium surrounding the ovarian egg in all turtles herein reported is represented by one layer of prismatic cells between the sides of which short and long bridges extend. The intercellular spaces at the surface of these cells are closed by a special cement, the terminal bars. The cell is formed by a nucleus and by cytoplasm consisting of an attraction sphere composed of a central corpuscle, a medullary and a cortical layer. These spheres form a dense endoplasm around the nucleus from which filaments extend to a clear layer near the periphery, the exoplasm in a delicate network.

2. The zona pellucida varies in thickness from 1μ to 17μ according to the stage of development of the egg. Beginning with a stage where it is on an average 3μ thick two different layers appear, the outer denser and thicker and the inner narrower, clearer and striated. In the course of development the outer layer differentiates, grows and extends to a greater degree than the inner.

3. The zona pellucida during its growth is always formed by two or three different elements:

a. The fundamental homogeneous substance filling up the spaces between

b. A system of numerous canals or tubules which inclose

c. Filaments or prolongations of the epithelial cells which are connected with the surface of the yolk. The fundamental substance of the zona pellucida is more abundant and dense in the outer layer than in the inner.

4. The fundamental substance of the zona pellucida is developed as a cuticular element by the terminal bars or primary network, that is by a definite special intercellular cement possessing the property of extension over the free surface of the epithelial cells and forming connections there with the delicate secondary network apparently produced directly by the superficial cytoplasm of the epithelial cells. The secondary network seems able to give rise at its surface to a cement similar to that resulting from the activity of the terminal bars. This superficial cuticular network gradually becomes thicker and by the development of fresh cuticular material builds up the entire fundamental substance of the zona pellucida. The prolongations of the epithelial cells, at first short, traverse the zona pellucida and become longer as this increases in thickness. Enclosed in canals, the prolongations reach the surface of the yolk to end in knob-like enlargements.

5. The structure of the zona pellucida just described presents a condition most favorable for the conveyance of nutritive material from the epithelial area in contact with the maternal capillaries to the actively growing and extending yolk.

In conclusion I wish to acknowledge my indebtedness for constant advice and criticism to Dr. Van der Stricht under whose direction this work has been carried out and to Dr. Todd who obtained and identified the material.

BIBLIOGRAPHY

Cattaneo, Donato 1913-14 Richerche sulla struttura dell'ovario dei mammiferi. Arch. Ital. di Anat. e di Embriol., vol. 12, pp. 1–34.

Fischer, A. 1905 Zur Kenntnis der Struktur des Oolemmas der Säugetiereizellen. Anat. Hefte, Bd. 29, pp. 557–589.

Flemming, W. 1882 Zellsubstanz, Kern, und Zellteilung. Leipzig, p. 35.

Koelliker, A. von 1898 Über die Entwicklung der Graafschen Follikel und Eier. Sitzungsber. d. physiol. med. Ges. Wurzburg, pp. 1–7.

Lams, H. 1903–04 Contribution à l'étude de la genése du vitellus dans l'ovule des Téléstéens. Arch. d'Anat. micr., T. 6, pp. 633–652.

Loeb, L. 1917 Factors in the growth and sterility of the mammalian ovary. Science, N. S., vol. 45, pp. 591–592.

Loyez, M. 1905–06 Recherches sur le développement ovarien des oeufs méroblastiques à vitellus nutritif abondant. Arch. d'Anat. micr., T. 8, pp. 69–237.

Munson, John P. 1904 Researches on the oogenesis of the tortoise, Clemmys marmorata. Am. Jour. Anat., vol. 3, pp. 311–347.

Paladino, G. 1890 I ponti intercellulari tra l'uovo ovarico e le cellule follicolari, e la formazione della zona pellucida. Anat. Anz., Bd. 5, pp. 254–259.

Regaud et Dubreuil 1908 Sur les productions exoplastiques des cellules folliculeuses de l'ovaire chez la lapine. Verh. d. Anat. Ges. Berlin, pp. 152–156.

Retzius, G. 1889 Die Interzellularbrücken des Eierstockeies und der Follikelzellen sowie über die Entwickelung der Zona pellucida. Verh. d. Anat. Ges. Berlin, pp. 10–11.

Rubaschkin, W 1905 Über die Reifungs- und Befruchtungsprozesse des Meerschweineneies. Anat. Hefte, Bd. 29, pp. 509–548.

Schäfer, E. A. 1912 Text-book of Microscopic Anatomy. London, p. 86.

Sobotta, J. 1902 Atlas und Grundriss der Histologie, p. 89.

Stöhr, P. 1898 Text-book of Histology. 2nd Amer. from 7th German ed., Philadelphia, p. 68.

Van Beneden, E. 1880 Contributions à la connaissance de l'ovaire des mammifères. Arch. de Biol., T. 1, pp. 475–551.

Van der Stricht, O. 1909 Le Neuro-epithélium olfactif et sa Membrane Lim'tante Interne. Mém. cour. de l'Acad. roy. de Méd. de la Belgique, T. 20, f. 2.

Waldeyer, W. 1906 Die Geschlechtszellen. Handbuch der vergl. u. exper. Entwickelungslehre der Wirbeltiere. Hertwig, pp. 287–293.

PLATE 1

EXPLANATION OF FIGURES

For abbreviations see page 256

Fig. 1 Tangential section of the epithelium of an egg 0.75 mm. in diameter from the ovary of Chrysemys picta. Benda. Safranin and picric acid. 5μ. Note the long filamentous (*l.b.*) and short thick (*s.b.*) cytoplasmic bridges connecting the adjacent cells. The intercellular substance is not stained.

Fig. 2 Transverse section of an egg 0.69 mm. in diameter from the ovary of Chrysemys picta. Bouin. Mallory's stain. 4μ. The epithelial cells one of which shows an attraction sphere (*a.s.*) very well are widely spaced. The terminal bars (*t.b.*) form the anlage of the zona pellucida (*i.z.p.*) which is 1μ in thickness.

Fig. 3 Oblique section of the egg represented in figure 2. The zona pellucida (*z.p.*) is seen to develop from a system of large polygonal fields (*p.f.*) marked off by the terminal bars (*t.b.*).

Fig. 4 Tangential section of the egg represented in figure 2. A number of epithelial cells are cut through their bases, others at various heights through the nucleus, a third group through the attraction sphere, a fourth through the cytoplasmic network and terminal bars at their surfaces. Central corpuscles can be seen in some of the spheres. The polygonal fields (*p.f.*) are sharply outlined by the terminal bars (*t.b.*).

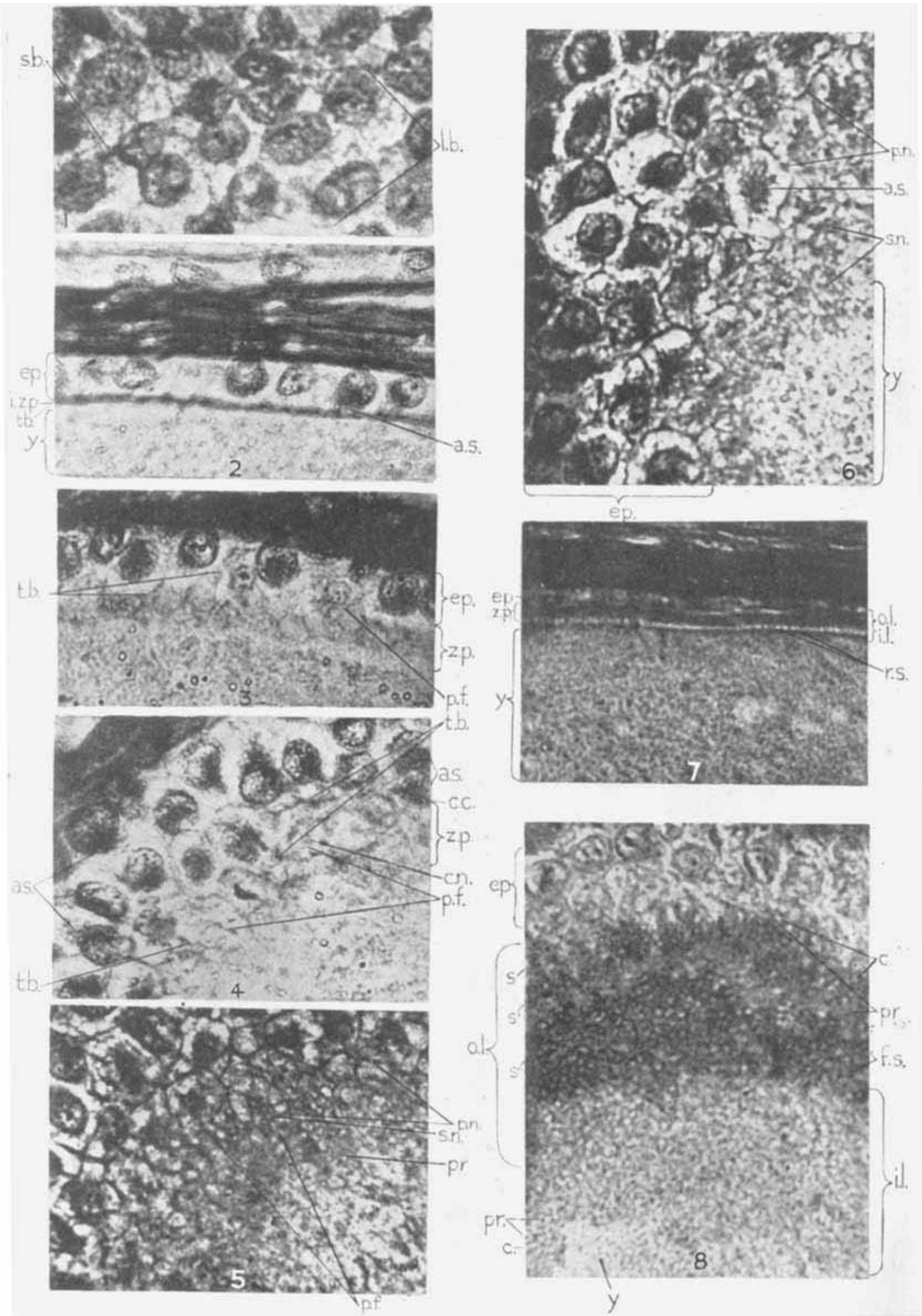
Fig. 5 Tangential section of an egg 0.99 mm. in diameter from the ovary of Chrysemys picta. Bouin. Heidenhain's haematoxylin, Congo red. 4μ. The primary network (*p.n.*) of the zona pellucida follows the outlines of the original terminal bars and the secondary (*s.n.*) the outlines of the superficial cytoplasmic network of the epithelial cells.

Fig. 6 Tangential section of an egg 0.74 mm. in diameter from the ovary of Chrysemys picta. Bouin. Heidenhain's haematoxylin, Congo red. 4μ. The details are similar to those of figures 4 and 5.

Fig. 7 Transverse section of an egg 1.1 mm. in diameter from the ovary of Graptemys geographicus. Trichloracetic acid. Heidenhain's haematoxylin, Congo red. 5μ. The zona pellucida has divided concentrically into two layers, the inner of which shows radiating striations very clearly. It measures 3.6μ in thickness.

Fig. 8 Tangential section of the egg represented in figure 7. Same fixation and stain. 5μ. Both layers of the zona pellucida *i.l.* and *o.l.* are seen to be formed by three elements:

1. A system of canals (*c.*) separated by
2. Meshes of the fundamental substance (*f.s.*) which enclose
3. Prolongations of the epithelial cells (*pr.*)


sb.
l.b.
1
ep
i.zp
tb
y
a.s.
2
p.n.
a.s.
s.n.
y
6
ep.
t.b.
ep.
zp.
p.f.
3
ep
zp
o.l.
i.l.
r.s.
y
7
t.b.
a.s.
c.c.
zp.
c.n.
p.f.
as.
t.b.
4
ep
c.
pr.
f.s.
s
s
s
o.l.
i.l.
pr.
c.
y
8
p.n.
s.n.
pr.
p.f.
5

ABBREVIATIONS

a.p., area of proliferation
a.s., attraction sphere
c., canals
c.c., central corpuscle
c.n., cytoplasmic network
ep., epithelium
f.s., fundamental substance
i.l., inner layer
i.z.p., incipient zona pellucida
k.e., knob-like enlargements
l.b., long bridges
o.l., outer layer
p.f., polygonal fields
p.n., primary network
pr., prolongations
r.s., radiating striations
s., outer stratum
s', middle stratum
s'', inner stratum
s.b., short bridges
s.n., secondary network
t.b., terminal bars
y., yolk
z.p., zona pellucida

The figures are not reduced in reproduction. They are microphotographs taken at a magnification of 750 diameters. Leitz microscope. Obj. 7. Oc. 1.

PLATE 2

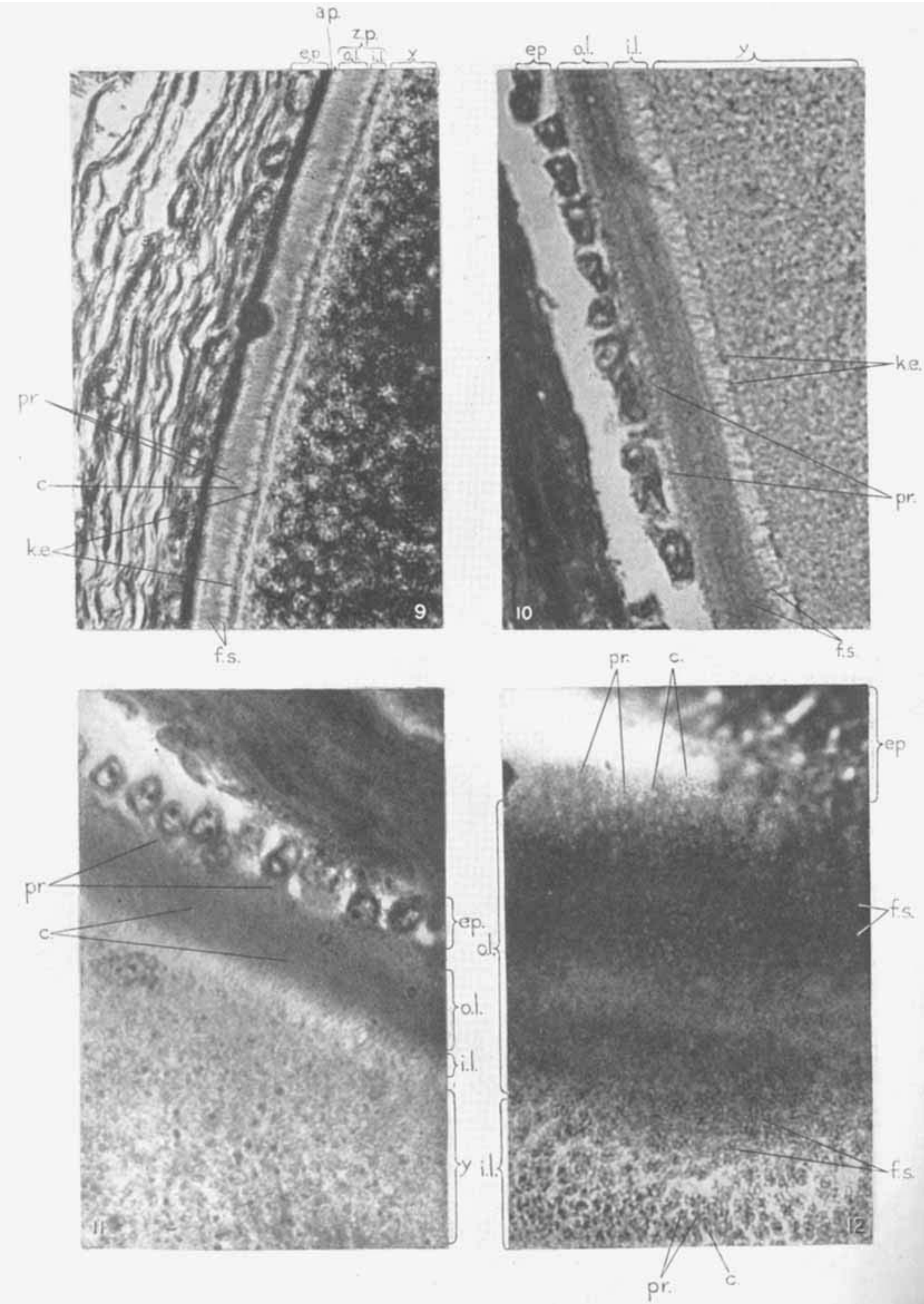
EXPLANATION OF FIGURES

Fig. 9 Transverse section of an egg 1.42 mm. in diameter from the ovary of Clemmys guttatus. Benda. Safranin and picric acid. 5μ. The outer layer (*o.l.*) of the zona has thickened to a greater extent than the inner (*i.l.*). The prolongations show knob-like enlargements at their tips (*k.e.*)t The area of proliferation (*a.p.*) is deeply stained. Zone measurement 12μ.

Fig. 10 Transverse section of an egg 2.6 mm. in diameter from the ovary of Chrysemys picta. Bouin. Heidenhain's haematoxylin. Congo red. 4μ. The prolongations (*pr.*) are clearly seen extending from the scant cytoplasm of the epithelial cells down into the outer layer. The zona measures 17μ in thickness.

Fig. 11 Oblique section of the egg represented in figure 10. Same fixation and staining. 4μ. Note the canals and the prolongations of the epithelial cells.

Fig. 12 Tangential section of the egg represented in figures 10 and 11. Bouin. Mallory's stain. 4μ. With figures 10 and 11 this shows the great increase in thickness in both layers of the zona (cf. with figure 8).


a.p.
z.p.
ep
o.l.
i.l.
y
pr
c
k.e
f.s.
9
ep
o.l.
i.l.
y
k.e.
pr.
f.s
10
pr
c
ep.
o.l.
i.l.
y i.l.
11
pr.
c.
ep
o.l.
f.s.
f.s.
12
pr.
c.