Secretion of Embryonic Envelopes and Embryonic Molting Cycles in *Hemioniscus balani* Buchholz, Isopoda Epicaridea

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ABSTRACT The fertilized egg of *Hemioniscus balani,* a viviparous isopod, is encased in two envelopes, I and II, the structure of which is described.

In the course of its development, the embryo secretes five successive sacs called embryonic envelopes. Both their structure and the means of secretion by the embryo are analyzed through thin sections. The presence of these successive secretion cycles together with exuviations involving these embryonic envelopes lead us to believe that during embryonic development in *Hemioniscus balani,* embryonic molting cycles exist.

The appearance and shedding of the envelopes of the fertilized egg and embryo allow determination of ten embryonic stages which are briefly described.

During its development, the *Hemioniscus balani* embryo undergoes considerable growth. It is around 380 μ by 180 μ at hatching. This size represents 100–150 times the volume of the egg whose diameter is merely 50 μ (Teissier, '29).

This relatively large increase requires special adaptation of the protective envelopes to allow them to increase their size to encompass the embryo during embryogenesis. The enlargement might be explained in several hypothetical ways. The envelopes may be permanent and stretchable or they may be transitory so that the increasing volume would inevitably lead to their shedding in the course of growth. They may even demonstrate several of these traits.

Some of these envelopes are observed at an early stage around the fertilized eggs, while others appear during much later stages of embryonic development. These are called the embryonic envelopes.

In Crustacea, the fertilized egg usually is supposed to have two envelopes. They have been called the "chorion" and "vitelline membrane," in Isopoda (McMurrich, 1895; Nair, '56; Stromberg, '65, '67, '72), or the external and internal membrane in some other Isopoda (Ellis, '61; Holdich, '68; Davis, '68), in Ostracoda (Tétart, '70), in Copepoda (Davis, '59), in some Mysidacea (Davis, '66) and in Decapoda (Davis, '64, '65).

Sometimes only one envelope surrounds the fertilized egg of Isopoda (Van Beneden, 1869; Forsman, '44; Kjennerud, '50; Jensen, '55; Naylor, '55; Davis, '64). Finally, in some Decapoda, Cheung ('66) noted two envelopes which were separated by a thick, dense layer.

Considering the Epicaridea, one of which is the subject of the present study, only observations on *Bopyroïdes hippolytes, Hemiarthrus abdominalis* and *Pseudione crenulata* (Stromberg, '71) have yielded precise information on the number of egg envelopes in the Bopyridae. In these species, a protective chorion is present when the egg is deposited in the marsupium. A vitelline membrane appears next to the surface of the egg right after fertilization.

Former studies have shown 1, 2 and even 3 egg envelopes in various Epicaridea (Rathke, 1837; Hesse, 1861; Cornalia and Panceri, 1861; Fraisse, 1877–78; Hiraiwa, '36). This lack of agreement in both the naming and numbering of the fertilized egg envelopes in Crustacea is probably due to the fact that neither their ultrastructure (aside from both the study on the egg envelopes of Ostracoda and the particular

case of Anostraca) nor the origin of their secretion has been precisely defined.

Embryonic envelopes have also been seen in some Crustacea like Leptostraca (Manton, '34), Mysidacea (Manton, '28), Ostracoda (Tétart, '70), Amphipoda (LeRoux, '33; Ginet, '60; Turquin, '67; Graf, '72) and Isopoda (Forsman, '44; Naylor, '55; Jensen, '55; Ellis, '61; Holdich, '68; Stromberg, '67, '71, '72).

Terms designating these envelopes, as well as their quantity, vary according to the example studied. Furthermore, certain authors having observed shedding of the embryonic envelopes during growth conclude that embryonic molts exist in those particular Crustacea.

In the specific case of *Hemioniscus balani*, Caullery and Mesnil ('01) pointed out the presence of a glassy transparent shell around the embryo from the earliest stages. Without really examining this hypothesis, they suggested that this shell could be replaced several times, thus producing a series of embryonic molts.

These studies furnish no evidence whatsoever concerning how the embryonic envelopes are formed, nor of their origin. Only an ultrastructural approach could resolve this question and, indeed, clearly establish the moment during embryonic development when these envelopes appear.

In the present research on *Hemioniscus balani*, the embryonic stages are considered first. Stage delimitation corresponds to the appearance and shedding of protective envelopes seen during embryonic growth. Thus, the criteria for determining stages are adapted to this particular investigation, and do not present complete embryological study.

Next, the structure of the fertilized egg envelopes is described through observation of thin sections. Finally, the structure and secretion of the embryonic envelopes appearing during growth are analyzed.

The evidence given for successive cycles of embryonic envelope secretion by the embryo itself, together with evidence of veritable ecdysis affecting one or two of these envelopes simultaneously, prove for the first time in Crustacea that embryonic molting cycles are present.

The example of *Hemioniscus balani* is then placed in the context of other examples of embryonic molting which, though little analyzed, are already known in some Crustacea.

MATERIAL AND METHODS

Samples of gravid female *Hemioniscus balani* in stages F 4 and F 5 of the female state, having undergone four or five exuviations of their posterior region (Goudeau, '72a,b), were collected for this study from the Green Island Channel near Roscoff, where they feed on the barnacle, *Elminius modestus*.

(1) *Preparation of specimens for electron microscope observation*

The osmotic pressure of the liquid surrounding the embryo within the female's internal incubating pocket was measured with a Ramsay micro-osmometer ('55).

Embryos at different stages were fixed with 1.4% glutaraldehyde in 0.4 M sodium cacodylate buffer at ph 7.3 with 1 ‰ calcium chloride added. The osmotic pressure of the liquid fixation was adjusted by adding sucrose in order to obtain in each case light hypertonicity compared to the liquid of the incubating pocket. At laboratory temperature, fixation lasted 20–30 minutes, for the early embryonic stages and 45–60 minutes for later stages.

Specimens then were postfixed in 1% osmium tetroxide with the same buffer for 45 minutes. After extremely gradual dehydration, some embryos were embedded in ERL 4206 whose low viscosity seems to increase tissue penetration (Spurr, '69), and others in Epon 812 in order to be able to apply the enzymatic digestion technique with pronase (Monneron and Bernhard, '66). Sagittal and parasagittal sections made by the Reichert microtome were stained with uranyl acetate in alcohol solution and counterstained with lead citrate (Reynolds, '63). In addition, sections relative to some embryonic stages were collected on gold grids for detecting polysaccharides by the Thiéry method ('67). After 30 minutes of 1% periodic acid oxidation, the sections were treated with 0.2% thiocarbohydrazide (TCH) in 20% acetic acid solution for 90 minutes or 24 hours, then placed in a solution of 1% silver proteinate for 30 minutes without light. Other preparations similarly collected on gold

grids were tested with pronase (Monneron and Bernhard, '66). Following oxidation with 10% periodic acid solution for 30 minutes, the sections were treated with 0.1% pronase solution in 0.01 M Tris -HCL (Tris = Tris (hydroxymethyl) aminomethane) buffer at ph 7.4, during 1 or 24 hours at 40°C. The sections were finally stained with uranyl acetate and lead citrate.

The various series of sections were examined on a Hitachi (types HU 11A and HU 11B) and on a Philips EM 300.

(2) *Measurement of embryonic stage dimensions*

The different embryonic stages were drawn with camera lucida. Outlines thus obtained allowed estimation of each stage's dimensions, which showed in later stages especially a certain variability.

RESULTS

A. *Definition of embryonic stages and determination of egg envelopes and embryonic envelopes* (figs. 1, 2)

The fertilized egg, slightly oblong in shape, measures about 65 μ by 50 μ. It is not entirely without yolk and shows on thin sections small yolk globules each enveloped in a membrane, sparsely distributed throughout the cytoplasm. The small size and number, and the cytoplasmic dispersion, of yolk globules exclude the possibility of a distinct layer of clear periplasm without yolk as described in the eggs of certain Bopyridae (Stromberg, '71).

When observed with the electron microscope, the egg in its first cleavage appears surrounded by two envelopes, the inner and outer, called egg envelopes I and II respectively. Even though these envelopes of the dividing egg seemingly correspond to the chorion and vitelline membrane described in some Bopyridae (Stromberg, '71), *a priori* employment of these terms seems arbitrary. Only further study of the origin of the envelopes and the way they are formed will permit their accurate identification.

(1) *Definition of stage A* (fig. 1)

The segmentation of the egg is holoblastic and equal (Buchholz, 1866; Caullery and Mesnil, '01). The early cleavages form a pear-shaped morula described by Buchholz (1866) and Caullery and Mesnil ('01).

The dimensions of the morula are approximately 75 μ by 55 μ and differ little from those of the fertilized egg, suggesting that growth has not yet begun. The morula, corresponding to stage A in this study's nomenclature, is always encased in envelopes I and II of the fertilized egg.

(2) *Definition of stage B* (fig. 1)

While segmentation continues, the size of the blastomeres slightly diminishes. In the period where about 20 blastomeres are formed, the beginning of a segmentation cavity appears. This stage corresponds to the "preblastula" defined in *Chrysaora hysocella*, a viviparous hydrozoan (Teissier, '29). In *Chrysaora* as in *Hemioniscus balani*, the decrease in size of the blastomeres during the next few cleavages indicates that segmentation is not complete and thus indicates an intermediate stage. This preblastula, or stage B, has a rounded shape, whereas the blastomeres which bear no marked difference are radially oriented. Stage B, which is 85 μ by 65 μ, does not undergo appreciable growth. It always remains enclosed by envelopes I and II of the fertilized egg.

(3) *Definition of stage C* (fig. 1)

On completion of segmentation, a blastula is formed by a regular layer of blastomeres which have lost completely their yolk and which encircle a spacious blastocoel. This coeloblastula is approximately 120 μ by 110 μ, thus showing considerable size increase from stage B.

One of the poles of the coeloblastula soon develops a group of tall, narrow blastomeres which represent a zone of intense cell proliferation signifying the formation of a blastodisc. Its elaboration, described by Caullery and Mesnil, seems almost identical in *Hemioniscus balani* and in Bopyridae (Stromberg, '71).

This stage, called C, possesses a well specified blastodisc outlining the embryo's future ventral side. Examination of thin sections shows that stage C has three different envelopes, one inside another. The outermost of these, egg envelope II, is in-

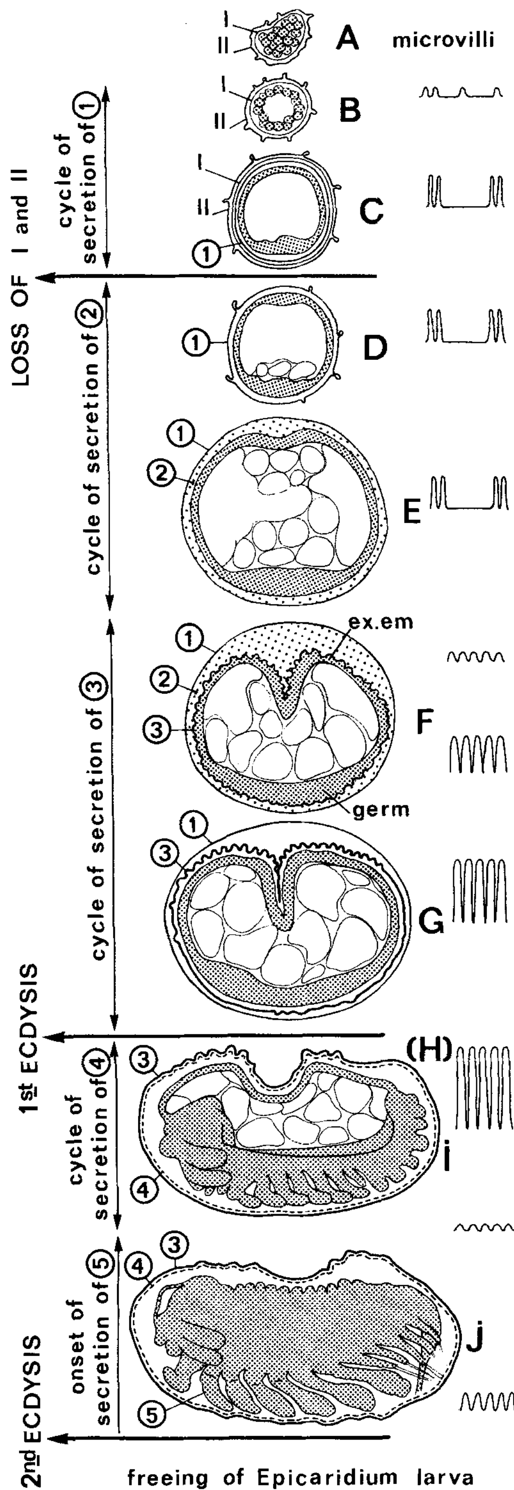

Figure 1

ternally lined by egg envelope I and then by another protective lining called embryonic envelope①, since it is secreted by the blastomeres themselves. The secretion of①, which starts at the end of stage B, is entirely finished with stage C.

(4) *Definition of stage D* (fig.1)

Hemioniscus balani like some Bopyridae (Stromberg, '71) seems to undergo gastrulation through polar proliferation. This phenomenon indicates both an active migration of the blastodisc blastomeres into the blastocoel, and the epibolic repulsion of these blastomeres by those near to the blastodisc. The direct result of this type of gastrulation is the absence of the blastoporic cavity in *Hemioniscus balani* found by Caullery and Mesnil ('01), and Stromberg ('71) in other Epicaridea.

The first cells migrating in the blastocoel at the beginning of gastrulation in *Hemioniscus balani* have a very specific structure. In thin sections, almost all of the cell volume is taken up by an immense vacuole of low electron opacity. Around the central vacuole there is only a thin layer of cytoplasm which contains the nucleus, some lipid droplets and a large amount of glycogen. Even though these cells are evident at a relatively young stage, they appear to correspond to the vacuolate cells pointed out by Caullery and Mesnil in older embryos of *Hemioniscus balani*.

Even without considering the ultrastructure and exact origin of these cells, they can be considered as analogous to the vitellophags which penetrate to the yolk center at gastrulation in certain Bopyridae (Stromberg, '71). One is also led to compare these peculiar cells with the huge vacuolate cells appearing at the concave surface of the blastodisc during the forma-

Fig. 1 Synoptic table of embryonic stages in *Hemioniscus balani*. Envelopes of fertilized egg and embryonic envelopes are outlined and numbered. I, first envelope of fertilized egg; II, second envelope of fertilized egg; ①, ②, ③, ④, embryonic envelopes; ⑤, fifth external coating secreted by surface embryonic cells; A–J, embryonic stages; ex. em, epithelial layer forming the extraembryonic area; microvilli, various appearances of apical plasma membrane of the extraembryonic cells (first stages) and ectodermal cells (older embryos) during embryonic envelope secretions; germ, germ-band.

tion of the blastoderm of *Peripatopsis balfouri*, a viviparus onychophoran, in which the egg is deprived of its yolk in the course of evolution (Manton, '49; Anderson, '66, '73). The same cells found as well in still another onychophoran, *Peripatopsis capensis*, make up the final endoderm in this species whereas they are merely transitory in *Peripatopsis balfouri* (Manton, '49). Cells of the same type have been observed in the blastocoel during gastrulation in *Chrysaora hysocella* (Teissier, '29).

To conclude, stage D of *Hemioniscus balani* embryology is a young gastrula measuring about 110 μ by 120 μ which has not noticeably grown since stage C and which shows cellular migration starting on the inside of the blastocoel. During stage D, at the pole opposite the blastoporal area, that is, at the level of the future dorsal side of the embryo, a zone of intense cellular proliferation appears. Finally, stage D is only covered by one embryonic envelope①, since lysis (fig. 7) of extrinsic envelopes I and II seen in thin sections brings about their disappearance. This loss of egg envelopes I and II allows precise definition of stages C and D.

(5) *Definition of stage E* (fig. 1)

The continuation of gastrulation is marked by intense cell proliferation and migration under the superficial layer of the blastoporal area. This phenomenon winds up in the formation of the classic "mesendodermal cell plug" described for all isopods and especially in certain Bopyridae (Stromberg, '71), and containing the mesodermal and endodermal cell material of the future larva. Stage E is also characterized by the large vacuolate cells extending inside te blastocoel, and by a very slight invagination of dorsal cleaving blastomeres. During morphogenesis, stage E undergoes enormous size increase, to 200 μ by 190 μ. There is also a new relatively thick protective lining located between the embryonic envelope① and the gastrula itself. In order to simplify the data from here on, the term embryonic envelope② has been given to this new thick layer.

(6) *Definition of stage F* (fig.1)

Even though embryonic stage F, still globular, has not increased in size greatly from stage E, it possesses a much better developed germ band. Apart from the germ band, the remaining superficial blastomeres that are arranged in a single cell epithelial layer forming the "extraembryonic area" apparently similar to that seen in Bopyridae (Stromberg, '71) as well as other isopods (Stromberg, '65, '67).

Invagination in the dorsal zone of the extraembryonic area seen in the preceding stage, is at this time much more noticeable and corresponds to the beginning of dorsal furrow formation, a characteristic structure of certain stages of embryonic development in Isopoda. At this point, a number of vacuolate cells concentrate at the base of the extraembryonic blastomeres at the forming dorsal furrow.

Ultrastructural examination of the apical zone of the extraembryonic cells and of the superficial germ band cells, shows embryonic envelopes① and② on stage F as well as initiation of secretion of the third coating. The new embryonic envelope③ is directly attached to the embryo, following the dorsal furrow invagination.

(7) *Definition of stage G* (fig. 1)

Stage G has increased slightly in size compared to the former stage and shows the beginning elongation corresponding to the future antero-posterior axis of the embryo. In addition, there is more clearly accentuated dorsal furrow in the extraembryonic area than in the preceding stage. The thin sections show both that embryonic envelope① still exists in stage G and also the almost complete disappearance of embryonic envelope②. Also at this stage, embryonic envelope③ seems entirely secreted and appears slightly unstuck from the embryo surface.

(8) *Definition of stage H*

Having undergone considerable growth of the germ band, the embryo at stage H has quite an elongated form. The very irregularly formed and remarkably big vacuolate cells occupy practically all of the embryonic cavity, forming a weakly-bonded network.

The roughly outlined pair of liver lobes of the mid-intestine often seen in *Hemioniscus balani* (Caullery and Mesnil, '01) as well as in some Bopyridae (Stromberg,

'71) is at this stage clearly differentiated. With this stage of growth, embryonic envelope① is lost. The shedding of envelope① is the first ecdysis that the embryo has undergone and delimits stages G and H in an obvious manner. From thin sections, stage H thus appears to be protected only by embryonic envelope③. The dorsal furrow now further widened takes the characteristic form of a saddle. This final phase of dorsal furrow evolution has been observed by Caullery and Mesnil ('01) in *Hemioniscus balani.*

(9) *Definition of stage I* (fig. 1)

Even though stage I resembles stage H both in size and general form, it is distinctive due to a gradual development of the germ band and to the presence of two envelopes around the embryo. Embryonic envelope③ becomes lined internally by a new protective layer called embryonic envelope④, secreted by the apical zone of the superficial embryonic cells.

(10) *Definition of stage J* (fig. 1)

Stage J includes the later embryonic stages, and ends with the formation of Epicaridium larva. The constantly changing embryo grows slightly; the dorsal furrow seems less pronounced and tends to disappear steadily. Thin sections expose two embryonic envelopes③ and ④ during stage J, with fifth lining starting to be secreted by the superficial embryonic cells. This fifth layer very likely corresponds to the cuticle of the future Epicaridium larva.

At hatching, the simultaneous shedding of embryonic envelopes③ and ④ permitting the Epicaridium larva to go free, represents a second ecdysis which clearly separates embryonic development from forthcoming larval life.

B. *Envelope structure of the fertilized egg*

(1) *Structure of envelope I*

Envelope I is of relatively low electron opacity and directly enrobes the egg during the first segmentation and stages A and B of embryonic development (figs. 1, 4, 5). Exceedingly wrinkled, it is generally separated from the apical surface of the blastomeres (fig. 4).

At stage C envelope I is superimposed on embryonic envelope① and has a smooth appearance. Although difficult to estimate because of its thinness, the envelope must measure about 50–100 Å thick. Some sections were treated according to Thiéry's method ('67). Envelope I does not increase its contrast through interaction with silver proteinate (fig. 5). On the same sections, in contrast, the test is in fact positive for the glycogen particles, compared to control sections not having undergone previous oxydation by periodic acid.

(2) *Structure of envelope II*

Envelope II encloses envelope I and surrounds the egg from the beginning to segmentation as well as during embryonic development stages A, B, C (figs. 1, 4, 5, 6).

It has low electron opacity (fig. 6) and has a thickness of about 500 Å. Like envelope I, envelope II is somewhat wrinkled while enclosing A and B, but smoother when around C. The external surface still seems granular (figs. 4, 6). Envelope II does not react to Thiéry's test ('67) (fig. 5). What is more, it seems affected by pronase for the 24 hour duration of the experiment. These last results were compared to control sections having remained on 0.1 M Tris buffer. It is also attacked by 10% KOH solution at 70°C.

C. *Investigation of the embryonic envelopes*

(1) *Embryonic envelope①*

a. Formation. Near the end of stage B, very short microvilli appear fairly regularly spread out over the surface of the blastomeres. In places the microvilli are slightly larger and grouped together (fig. 6). Next, a small-grained dense material manifests itself both in the apical cytoplasm of the microvilli and on the outside of the plasma membrane, always accumulating at the tips of microvilli (fig. 6). Attached by the apex of the microvilli, envelope① is definitely made through the granular material densifying.

In stage C, the microvilli elongate in small groups while envelope①, attached to their tips, gradually detaches from the actual apical surface of the blastomeres.

b. Structure. Envelope① has a granular structure and low electron opacity. It is about 100 Å thick at its formation, but when fully formed at stage C it is about 300 Å thick. It is surrounded by egg envelopes I and II and folds somewhat (figs. 1, 7). After lysis envelopes I and II, at stage D, envelope① remains relatively wrinkled. Only at the time of enormous embryonic growth witnessed between stages D and E does envelope① unwrinkle. In stage F it forms a perfectly smooth enclosure, stretched about the underlying envelopes and embryo (figs. 1, 8). It is resistant to 25% KOH solution at 70°C.

c. Cytology of the apical region of stage D blastomeres. At the end of stage C, apical Golgi complexes are made up of isolated dictyosomes consisting of some densely-filled, flat saccules. Small coated vesicles are budded off the latter. In this apical area are multivesicular bodies with a loose matrix. Some sections show the fusion between the Golgi-type small coated vesicles and the multivesicular bodies (fig. 7). The apical area is also rich in large coated vesicles seen near the plasma membrane, and in large semi-transparent vesicles situated close to the multivesicular bodies (fig. 7).

(2) *Embryonic envelope*② (the thick enveloping layer)

From the end of stage D and during all of stage E, all of the space between envelope① and the surface of the blastomeres of the young gastrula is filled with granular material (figs. 1, 8). This matrix, called embryonic envelope②, might be compared to the albumin capsule which encircles the embryo of *Niphargus* as soon as the pleon is formed (Ginet, '60). During stage E, this envelope thickens and pushes embryonic envelope① slightly away from the gastrula surface.

(3) *Embryonic envelope*③

a. Formation. When stage F commences, both the apex of the germ band's superficial blastomeres and that of the extraembryonic blastomeres manifest fairly short and regularly distributed microvilli on the cell surface. The microvilli then increase in their dimension, becoming 0.3–0.4 μ long and 0.09–0.1 μ wide (figs. 8, 9). Now they conceal very slender and longitudinal filaments, observed from transversal sections (fig. 10).

The apical cytoplasm of microvilli is composed of dense and granular material which can also be seen on the outside of the cell, located at the apex of each microvilli. The formation of embryonic envelope③ starts in the form of tiny convex patches of matter with dense opacity found above the microvilli (figs. 8–10). These patches are most likely made through granular material (considered as the forerunner of envelope③) densifying. The microvilli lengthen while envelope③ thickens. Both give the impression of slender stems, measuring 0.7–1 μ long, at the conclusion of the envelope③ secretion cycle (fig. 15).

b. Structure. Envelope③ when entirely constructed is 0.4–0.5 μ thick. Its make-up is relatively complex compared to the embryonic envelopes already described. In fact, this envelope appeared very folded in the figures obtained. It does not react positively to Thiéry's test (fig. 21). Pronase partially attacks envelope③, of which only a quite indistinct web remains after enzymatic digestion (Monneron and Bernhard, '66) (fig. 22). The control sections were treated with 0.01 M Tris buffer. Envelope③ is resistant to 25% KOH solution at 70°C, and like envelope① may be considered as an embryonic cuticle.

c. Envelope form. Envelope③ has protuberances already observed by Caullery and Mesnil ('01). These measure about 10 μ wide and are mainly located around the dorsal furrow (figs. 2, 3, 8). When the envelope is first being formed, each protuberance covers an extraembryonic cell (fig. 8). It seems plausible that the initial form of the cell surface determines the form of envelope③, as found for secretion of the epicuticle of the adult *Tenebrio molitor* (Delachambre, '70). The folds persist in stages H and I and then their form like that of the dorsal furrow begins to fade as stage J proceeds. In fact, envelope③ unfolds to its maximum by the end of embryonic development to adapt to the extension of the embryo.

d. Cytology of the epithelial layer of the extraembryonic area during envelope secretion. Even though all stage F superficial blastomeres participate in the enve-

lope's elaboration, the figures illustrating this study were selected from the most convenient extraembryonic area. In the cell of the extraembryonic layer, the median position of the nucleus distinguishes the infranuclear area from the apical supranuclear area.

Golgi apparatus. From the start of envelope③ formation, this is composed of apical Golgi complexes and basal Golgi complexes. On the sections, each complex contains one or two dictyosomes made up of a few flat and fenestrated saccules (figs. 12–14), which in turn bud off both small coated vesicles and a few large vesicles with smooth membrane and dense contents (figs. 13, 14). By the end of envelope③ secretion, large vesicles alone are budded off in a great number with smooth membrane and dense contents from the flat saccules of dictyosomes (fig. 18).

Rough endoplasmic reticulum. This is formed of long flat saccules frequently juxtaposed, which take up the infranuclear area of the cell from when envelope secretion begins. Their content is cloudy and seems closely related to the basal Golgi complexes (fig. 14). Near termination of the envelope, the rough endoplasmic reticulum enters the apical zone and then seems to be related to apical Golgi complex dictyosomes (fig. 18).

Mitochondria. These occupy the apical supranuclear area, especially close to the end of envelope secretion (fig. 15).

Microtubules. Spread throughout the volume of the cell, these remain parallel to the cell median, except in the apical zone where their arrangement is less orderly (fig. 13). In this last zone some figures show clearly that the microtubules are attached to the apical plasma membrane (fig. 32). This cytoskeleton first acts as a framework providing mechanical stability to the cell (Dustin, '72) and secondly, traces specific pathways, similar to those described for the epidermal cells in insects (Locke, '66, '69; Zacharuk, '72), necessary for discharging the small coated vesicles secreted by the Golgi complex (fig. 11).

Apical vesicles. At the beginning of envelope③ formation, the apical region is occupied by large coated vesicles with dense content. Afterwards, when the envelope is all formed, the apical zone is filled with large coated vesicles and dense vesicles with smooth membrane. A few figures show these two types of vesicle attached directly underneath the plasma membrane (fig. 16).

Multivesicular bodies. These are few and not typical, and seem to remain in the area at the upper side of the nucleus (fig. 17).

Plasma membrane. As soon as envelope③ starts to form, the plasma membrane produces invaginations at the cell apex (figs. 8, 15) between the microvilli, comparable, for example, to those designed by the oöcyte oölemma of *Xenopus laevis* (Dumont, '72) and of both *Hyla cinerea* and *Hyla arenicolor* (Massover, '73).

On the apical side, the cells are unified by a zonula adherens type of junction (Farquhar and Palade, '63) which in this case is split into two parts (fig. 23): the most apical part is quite reduced but is associated with another much more developed part. The zonula adherens exhibits in its principal part an accumulation of dense matter which traces a broken outline differing from the faint median line generally described (Farquhar and Palade, '63). Furthermore, the zonula adherens is always preceded by a profound invagination zone formed by the apical plasma membranes of adjoining cells.

(4) *Embryonic envelope④*

In stage H, the apical plasma membrane microvilli are still elongated and are approximately 3 μ in length (fig. 18). Their apex is filled with dense matter, and on the outside of the cell forms envelope④, attached to the tips of the microvilli. Envelope④ is made of a somewhat irregular layer of particularly dense matter adhering to the inner side of the envelope③ (figs. 19, 20). On thin sections, envelope④ responds negatively to Thiéry's test ('67) (fig. 21) and is not attacked by pronase (Monneron and Bernhard, '66) (fig. 22).

(5) *Fifth external coating secreted by surface embryonic cells*

a. Formation and structure. At stage J, the apical surface of ectodermal cells in developing Epicaridium larva is characterized by very small microvilli, spread out regularly and at their apex filled with

dense granular matter (figs. 24, 25, 28). The dense matter is also noticed outside the cell, on top of each microvilli (figs. 25, 28). The first layer of coating⑤ is secreted uniformly above the microvilli (figs. 24, 25, 28). Closely attached to the microvilli tip, it has a rough structure and seems dense. It is apparently produced by a densifying of the diffused matter just over the tip of the microvilli. Coating⑤ adopts the shape of the underlying cell surface, and notably in the dorsal region of the future larva where the lobes designed by already distinct somites are traced (fig. 31). In fact, the coating⑤ has little crests (figs. 25, 31) which during its secretion correspond to the large microvilli zones of the apical plasma membrane. These large microvilli are generally located near to apical cell junctions (figs. 25, 31). At this same level, the early coating⑤ secretion has gaps (fig. 25), sometimes nearby the apical cell junctions which are like growing points of the envelope similar to those noted at the formation of "cuticulin" in *Calpodes ethlius* (Locke, '66).

b. Cytology of the ectodermal cell of stage J dorsal zone. The cell is filled by one or several lipid matrix droplets of great size that are not outlined by a real membrane (figs. 24, 31). The nucleus is often deformed by the lipid droplets in the midst of the cytoplasm and is frequently pushed to the cell periphery (fig. 31).

Golgi apparatus. This is made of isolated dictyosomes located either at the apex or in the basal region of the cell, yet apparently always in close relationship to the lipid droplets (figs. 24, 29). Each dictyosome is made of flat saccules containing dense matter which bud off small coated vesicles at the same time as large smooth-membraned vesicles densely filled (figs. 24, 29).

Rough endoplasmic reticulum. Although undeveloped at this stage, it includes a few flat saccules encircling lipid droplets. (fig. 24).

Smooth endoplasmic reticulum. Also poorly developed, it is distinguished only by small clusters of smooth-membraned vesicles containing matter of low electron opacity (fig. 25).

Mitochondria. These are situated very regularly under the apical surface of the ectodermal cells (figs. 26, 31).

Apical cell junctions. The epidermal cells of future Epicaridium larva are united at their apical pole (figs. 25, 26, 27) by a zonula adherens-type junction exactly identical to that uniting secretive cells of the preceding embryonic envelope④. Following the Zonula adherens, septate and gap junctions unite the epidermal cells of the young larva. The coexistence of these two junction varieties has once before been observed in an epithelium of Crustacea (Hudspeth and Revel, '71).

Apical vesicles. Apical cytoplasm of cells encloses two distinct types of vesicles. First, those with a smooth membrane and dense contents, and second, those with a coated surface membrane containing cloudy matter, called large coated vesicles (figs. 26, 27, 30).

Cytolysomes. At times cytolysomes can be seen which contain fragments of still recognizable cytoplasmic organelles.

Multivesicular bodies. Only multivesicular bodies are observed in the apical cytoplasm, which are composed of an exceedingly dense, granular matrix and which are thought to be dense multivesicular bodies (figs. 25, 30).

Microtubules. Scattered throughout the cell, these are most often situated parallel or slightly obliquely to the major cell axis (fig. 25). The microtubules often seem attached to the principal part of the zonula adherens (fig. 25); yet, in agreement with Ashhurst's theory ('70), they would not be attached to the zonula adherens, but would lie parallel to it in the adjoining cytoplasm.

DISCUSSION

A. *Envelopes of the fertilized egg*

Envelope I may correspond to the primary egg membrane (membrane of fertilization) and thus is analogous to the "vitelline membrane" observed about the fertilized egg in Bopyridae (Stromberg, '71), other isopods (Van Beneden, 1869; McMurrich, 1895; Nair, '56; Stromberg, '65, '67, '72) and decapods (Cheung, '66). However, for all of these as well as for the egg of *Hemioniscus balani*, it is not easy to identify this envelope as primary mem-

brane without precise proof regarding the manner in which it is formed.

Similarly, even though there is a tendency to consider envelope II as a secondary membrane of the egg (or chorion), like that cited in some Bopyridae (Stromberg, '71), this is actually only a presumption requiring study on the secretion of this envelope.

B. *Embryonic envelopes: the concept of an embryonic molting cycle*

These envelopes most likely serve a protective role and should theoretically succeed one another around the embryo in order to be able to handle the embryo's increases in volume. At least four exuviations should therefore be seen during development, parallel to succeeding cycles of embryonic envelope secretion.

In reality, only two successive exuviations were observed. These allow the embryo to shed embryonic envelope① initially and at the end of embryonic development, envelopes③ + ④ simultaneously. In relation to this phenomenon, some sections with at times three superimposed envelopes enclosing the embryo, confirm the existence of successive embryonic molting cycles in *Hemioniscus balani*, certain of which manifest ecdysis at the end of the following molting cycle. The capacity for fertilized egg envelopes I and II and embryonic envelopes① and③ to unfold permits them to accommodate these changes and the large size increase of the embryo.

In Crustacea, the phenomenon of embryonic molting is already known, namely in Leptostraca (Manton, '34), in Mysidacea (Manton, '28; Davis, '66), in Amphipoda (Le Roux, '33; Ginet, '60; Turquin, '67; Graf, '72), and in Isopoda (Van Beneden, 1869; Forsman, '44; Kjennerud, '50; Naylor, '55; Jensen, '55; Ellis, '61; Holdich, '68). In these studies, the ultrastructure and means of elaboration of the envelopes which intervene during embryonic molting were not examined, rendering it impossible to draw comparisons between the various cases. An attempt to relate the fact that the *Hemioniscus balani* embryo undergoes exuviations to other Crustacea can nevertheless be made.

Exuviations experienced by *Hemioniscus balani* embryo do indeed seem different from those seen in the embryos first, of Leptostraca, Mysidacea, Isopoda, in each of which exuviations are regularly spaced during the course of embryogenesis and end at the conclusion of each molting cycle; and secondly, different from those in the Ostracoda embryo (Tétart, '70) where protective envelopes are shed almost simultaneously with hatching. In fact, the peculiar rhythm of exuviations noted during *Hemioniscus balani* embryonic development can be compared to that running through *Orchestia cavimana* development (Graf, '72). In this Amphipoda, the loss of the naupliar cuticle seems retarded and happens at the same time as shedding of the next envelope or intermediate cuticle.

C. *Embryonic envelope secretion*

(1) *Relationship between coated vesicles and multivesicular bodies during secretion of embryonic envelopes③, ⑤*

The cells responsible for secretion of envelopes③, ⑤ have little Golgi-associated coated vesicles in their cytoplasm migrating toward the apical area of the cell, large coated vesicles often attached to the plasma membrane, and multivesicular bodies with varying traits. These last sometimes fuse with small coated vesicles. There is at present no experimental proof confirming the roles of the various vesicles and multivesicular bodies. However, the figures here suggest that there may be invaginations of the absorptive plasma membrane (fig. 27), uptake of exogenous material, and formation of large coated vesicles (Roth and Porter, '64; Anderson, '69) which detach themselves from the plasma membrane (fig. 27). In addition, either type of coated vesicle as well as the multivesicular bodies can participate in the type of lytic cycle described on one hand for rat vas deferens cells (Friend and Farquhar, '67), and on the other, for epidermal cells secreting the protein epicuticle in *Calpodes ethlius* and the cuticle in *Tenebrio molitor* (Locke, '69; Delachambre, '71). The precise origin of uptake in the apical plasma membrane has

not been determined. It is probable, however, that the cells involved in embryonic envelope formation could reabsorb part of the matter they had just secreted. Therefore, these cells regulate their own secretion and act like epidermal cells in insects, where they reabsorb a part of the epicuticle precursor (Locke, '69; Delachambre, '71).

(2) *Role of the Golgi apparatus in the secretion of embryonic envelopes*③ and ⑤

Dense, smooth-membraned vesicles come from Golgi saccules and can be seen in the apical cytoplasm at the base of the microvilli, often touching plasma membrane. They contain the same matter that apparently acts in forming embryonic envelopes③ and ⑤.

The Golgi secretion is much more abundant when envelope③ is constructed. Rather than a basic constituent, it might represent a substance which permits the envelope to adopt its final structure. A similar hypothesis was considered for the same type of vesicles at the time of internal epicuticle formation in *Tenebrio molitor* (Delachambre, '71).

(3) *Changes in plasma membrane during embryonic envelope formation*

One of the most interesting changes observed in the superficial embryonic cells and extraembryonic cells during envelope secretion involves the apical plasma membrane (fig. 1). Very short microvilli appear at the cell surface during envelope① secretion, and then seem bigger and irregularly arranged during stage C and envelope② formation. Next, they probably disappear. The apical plasma membrane become microvillate once again when the first patches of secretion③ appear at the beginning of stage F. Later on, during stage G, they become longer and reach their maximum size as envelope④ starts to be secreted. Although these large microvilli may be related to envelope④ elaboration, they probably participate in another phenomenon in nutritive substance absorption (inducing a great increase of apical cell surface retained during the next stage I). Only at the end of this later stage, the apical plasma membrane becomes smooth. Last, when embryogenesis is nearly terminated, the apical plasma membrane of ectodermal cells becomes microvillate once again with the fifth external coating secretion.

(4) *Role of apical plasma membrane microvilli in the secretion of embryonic envelopes*①, ③, ④, ⑤

Microvilli are described as being found at the apex of the cells responsible for secreting envelopes. It is without any doubt the dense and granular substance always noticed at the tips of these microvilli in the cytoplasm as well as outside the cell which is precursor of the envelopes.

The first elements of the embryonic envelope are created from the densification of the granular material, recalling the elaboration of the epicuticle in *Calpodes ethlius* (Locke, '66, '69) and in *Tenebrio molitor* (Delachambre, '70, '71), of the proctodeal epi- and endocuticle in *Kalotermes flavicollis* (Noirot and Noirot-Thimothée, '71), of the first larval cuticle in *Drosophila melanogaster* (Hilman and Lesnik, '70) and of the secretion of the larval cutile in some Elateridae (Coleoptera) (Zacharuk, '72).

These first elements elaborated at the extremity of the microvilli may go on to molecular rearrangement and thereby influence the embryonic envelope in attaining its final structure.

To date, only the present study, done with the electron microscope, shows the construction of protective envelopes by the embryo of *Hemioniscus balani*, and proves the existence of several successive embryonic molting cycles in this Epicaridea.

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PLATE 1

EXPLANATION OF FIGURES

2 External side view of stage C, beginning of stage E, stage F and stage H of embryonic development in *Hemioniscus balani* with identical magnification; shape of the fertilized egg indicated by dotted line; df, dorsal furrow. × 250.

3 External side view of stage H dorsal furrow. See details of protuberances outlined by embryonic envelope③. df, dorsal furrow; verr, protuberance. × 735.

4 Apical region of a coeloblastula = Stage B of *Hemioniscus balani* embryonic development. I, first envelope of fertilized egg; II, second envelope of fertilized egg. × 14,600.

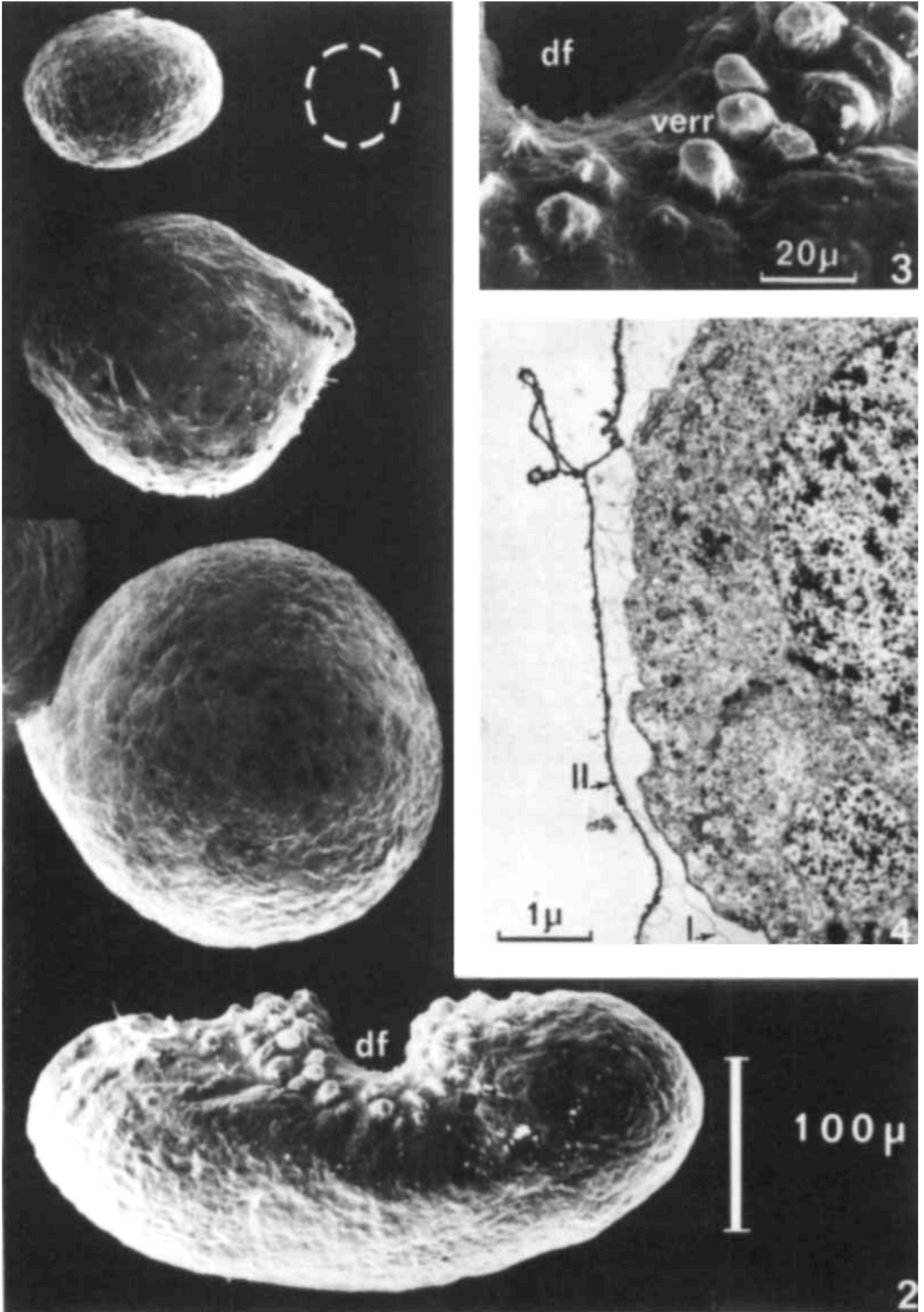
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PLATE 2

EXPLANATION OF FIGURES

5 Stage A embryo. Apical region of blastomere. I, first envelope of fertilized egg; II, second envelope of fertilized egg; gy, glycogen; ML, matrix lipid droplet. × 50,200.

6 End of stage B embryo. Apex of blastomere during embryonic envelope① secretion. I, first envelope of fertilized egg; II, second envelope of fertilized egg. × 32,000.

7 Stage D embryo. Apical region of an epithelial cell of the extraembryonic area during second embryonic envelope formation. I, first envelope partly lysed of fertilized egg; II, second envelope partly lysed of fertilized egg; ①, embryonic envelope①; Gcv, large coated vesicle; Gv, large semi-transparent vesicle with smooth membrane; mv, irregularly arranged microvilli; Mvb, multivesicular body; scv, small coated vesicle. × 25,000.

8 Stage F embryo. Epithelial layer of extraembryonic area near dorsal furrow, during embryonic envelope③ secretion. ①, embryonic envelope①; ②, embryonic envelope②; ③, embryonic envelope③; verr, protuberance outlined by envelope③; inv, invaginations produced by apical plasma membrane. × 9,200.

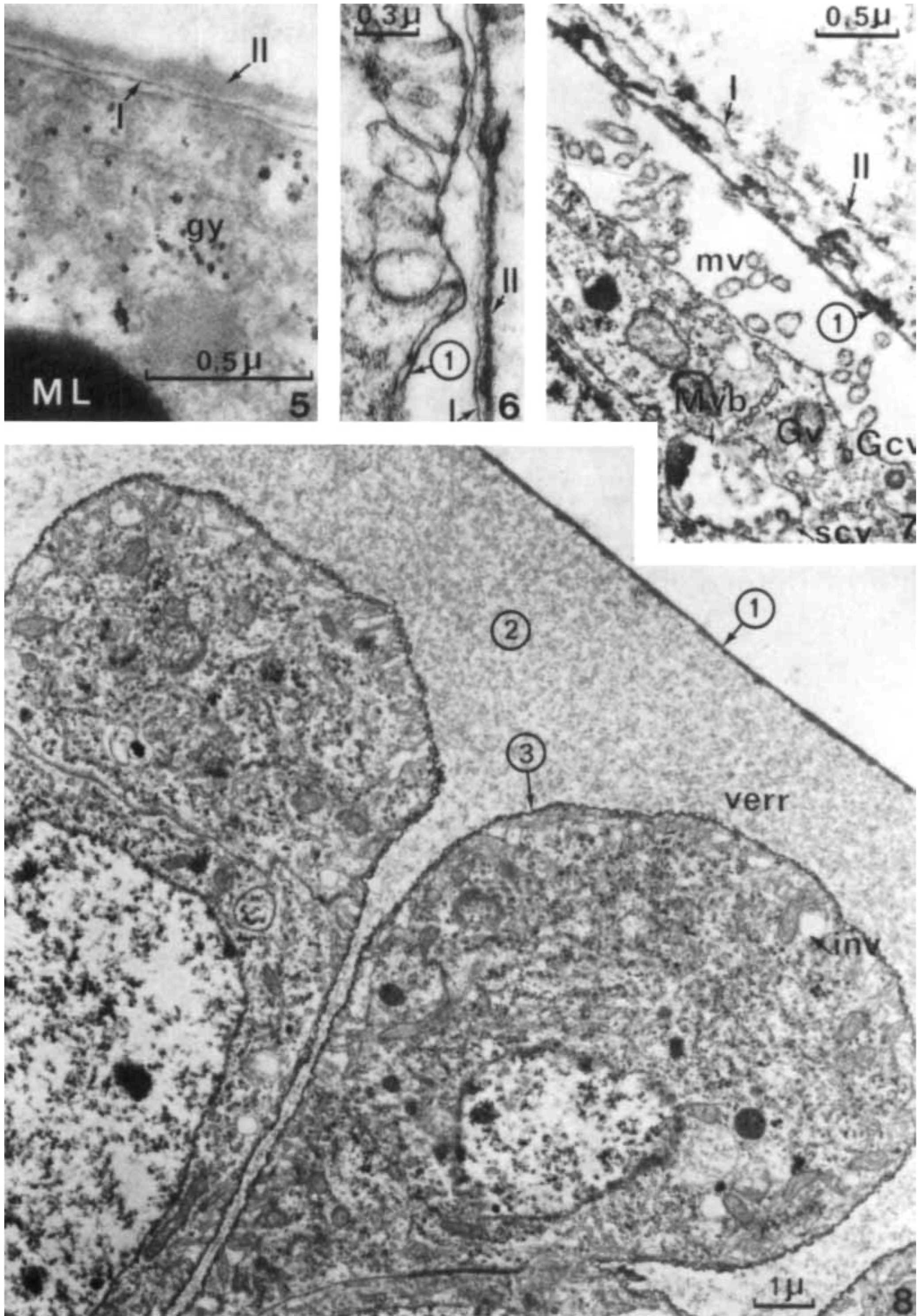
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PLATE 3

EXPLANATION OF FIGURES

Cells of the epithelial layer forming the extraembryonic area.

9 Stage F embryo. Apical region of a cell; parasagittal-section of microvilli during secretion of embryonic envelope③. × 41,000.

10 Stage F embryo. Apical region of a cell; cross-section of microvilli during secretion of embryonic envelope③. × 41,000.

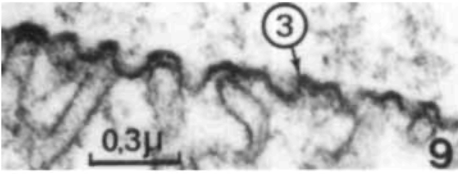
11 Stage F embryo. Part of apical cytoplasm. mt, microtubules; scv, small coated vesicles. × 30,000.

12 Stage F embryo. Apical Golgi complexe of a cell; thin section, tangential to fenestrated saccule. × 32,000.

13 Stage F embryo. Apex of a cell.③, embryonic envelope③; Gap, apical Golgi complex; mt, microtubule; scv, small coated vesicle forming. × 32,000.

14 Stage F embryo. Basal Golgi complex of a cell. dv, dense vesicle with smooth membrane; ER, saccules of rough endoplasmic reticulum; Gb, basal Golgi complex. × 32,000.

15 Stage G embryo. Apex of a cell.③, embryonic envelope③; ER, saccules of rough endoplasmic reticulum; inv, invaginations produced by apical plasma membrane; M, mitochondria; mv, microvilli of apical plasma membrane (more elongated than those observed in stage F. × 20,600.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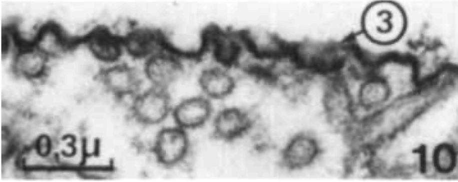
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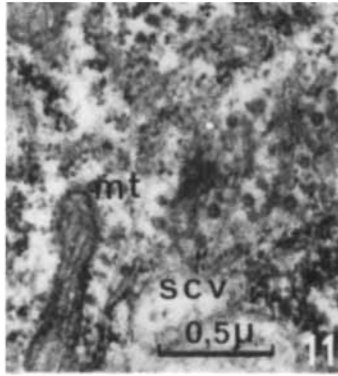
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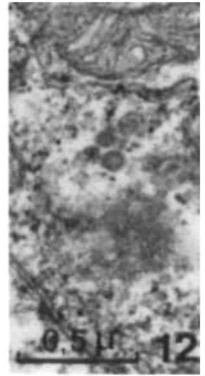
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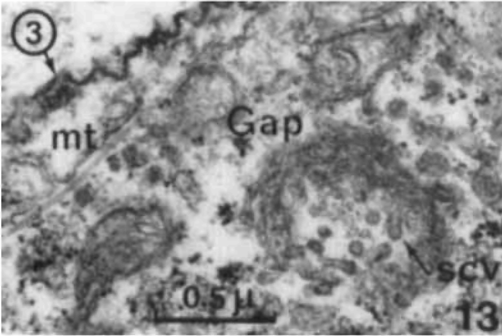
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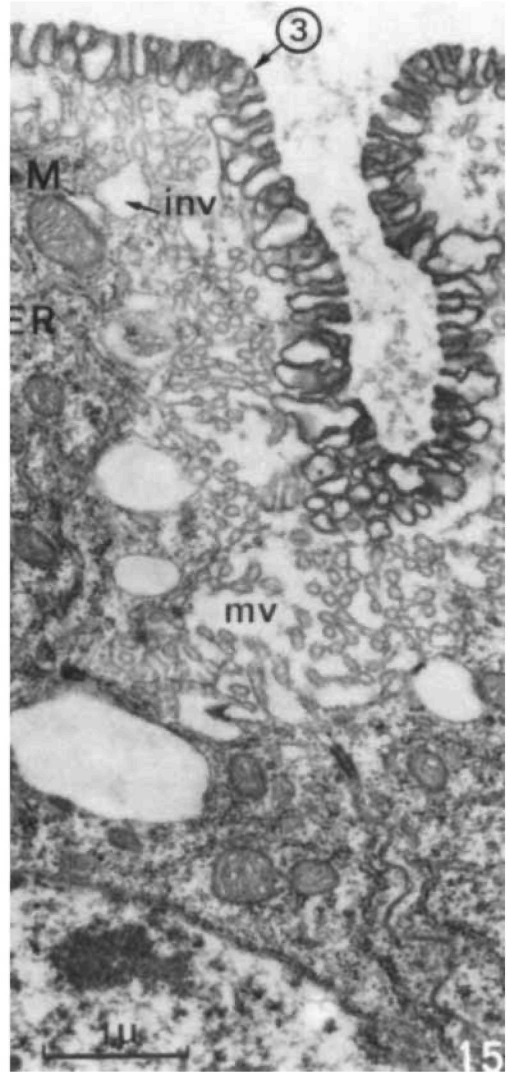
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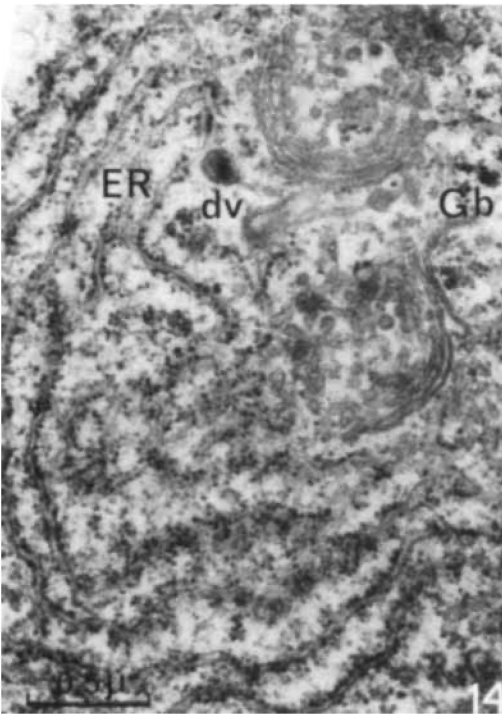
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PLATE 4

EXPLANATION OF FIGURES

Details of the extraembryonic area.

16 Stage G embryo. Apex of a cell. dv, dense vesicle with smooth membrane; Gcv, large coated vesicle; mv, microvilli of apical plasma membrane. × 40,600.

17 Stage G embryo. Apex of a cell. Part of cytoplasm showing multivesicular-body. × 25,000.

18 Stage G embryo. Apical Golgi complex of a cell. dv, dense vesicle with smooth membrane. × 32,000.

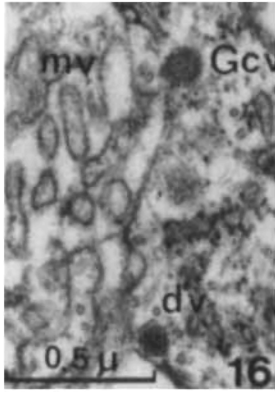
19 Stage I embryo. General view of epithelial layer of the extraembryonic area. ③, embryonic envelope③; ④, embryonic envelope④; gy, glycogen; inv, invaginations produced by apical plasma membrane between clustered microvilli; ML, lipid droplet; mv, microvilli of apical plasma membrane. × 9,200.

20 Stage I embryo. Apex of a cell.③, embryonic envelope③; ④, embryonic envelope④. × 20,000.

21 Stage I embryo. Detail of embryonic envelopes③ and ④, tested by Thiéry's method. × 50,200.

22 Stage I embryo. Detail of embryonic envelopes③ and ④, treated with pronase. × 16,200.

23 Stage I embryo. Apical region of a cell. Detail of zonula adherens split into two parts. Arrows, apical part of zonula adherens. × 75,000.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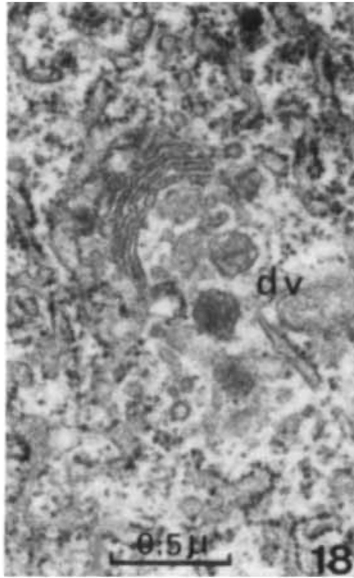
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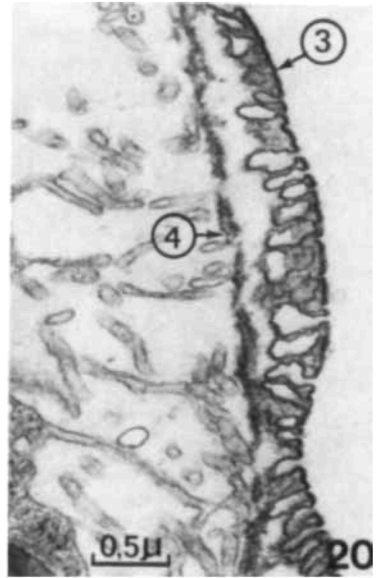
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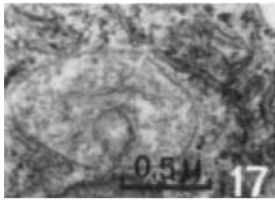
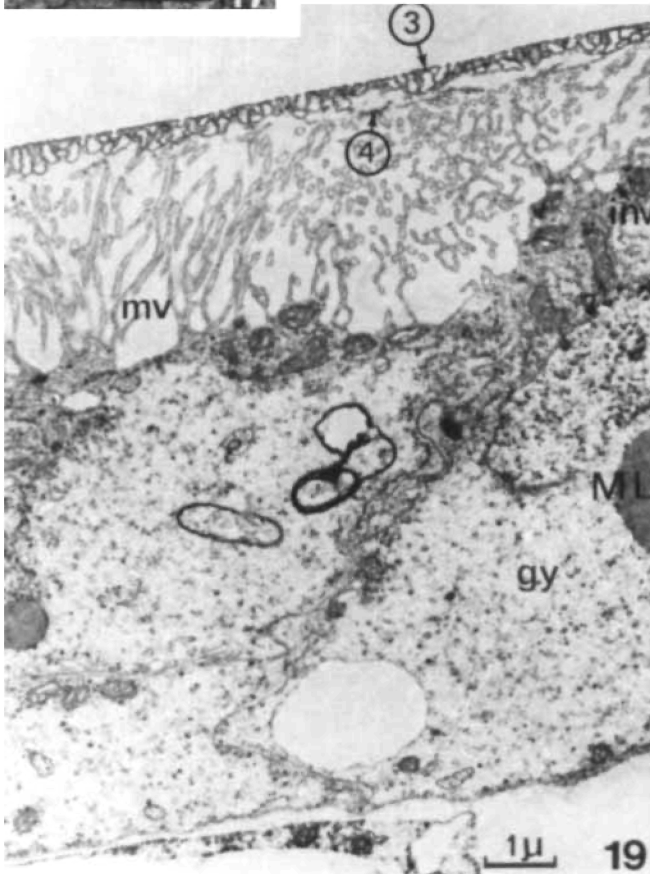
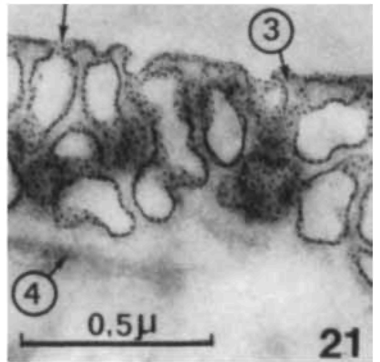
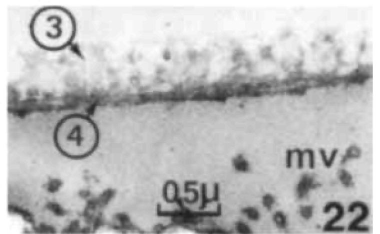
20


0.5μ
17


3
4
mv
inv
ML
gy
1μ
19


3
4
0.5μ
21


3
4
mv
0.5μ
22

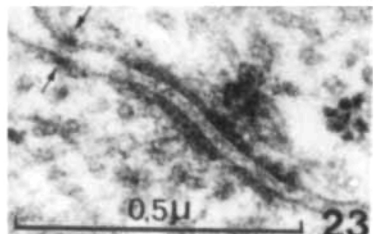

0.5μ
23

PLATE 5

EXPLANATION OF FIGURES

Stage J embryo. Apical region of a dorsal epidermal cell.

24 View of three superimposed coatings secreted by embryonic cells. ③, embryonic envelope③; ④, embryonic envelope④; ⑤, fifth external coating secreted by surface embryonic cells; dv, dense vesicle with smooth membrane; ER, saccules of rough endoplasmic reticulum; G ap, apical Golgi complex; ML, lipid droplet; mv, microvilli. × 25,000.

25 See the growing points (arrows) of⑤. cr, little crests outlined by⑤; db, dense residual body; mt, microtubule; SER, smooth endoplasmic reticulum. × 25,000.

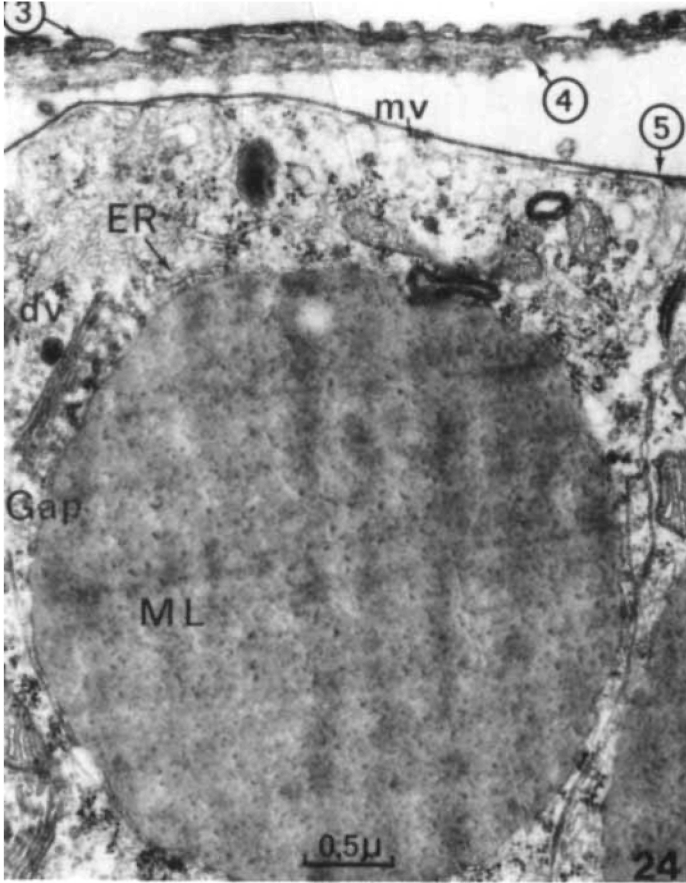
26 Detail of apical region. dv, dense vesicle with smooth membrane; Gcv, large coated vesicle; M, mitochondria; za, principal part of zonula adherens; arrows, apical part quite reduced of zonula adherens. × 37,500.

27 Detail of⑤. Gcv, large coated vesicle; za, principal part of zonula adherens; arrows, large coated vesicle forming. × 50,000.

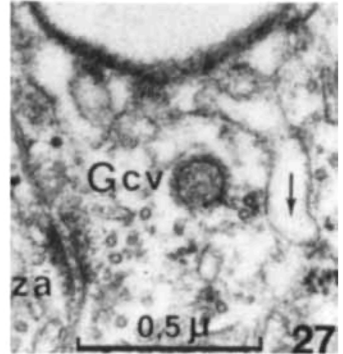
28 Detail of⑤ secreted by surface embryonic cells. dm, dense material at tips of microvilli. × 50,000.

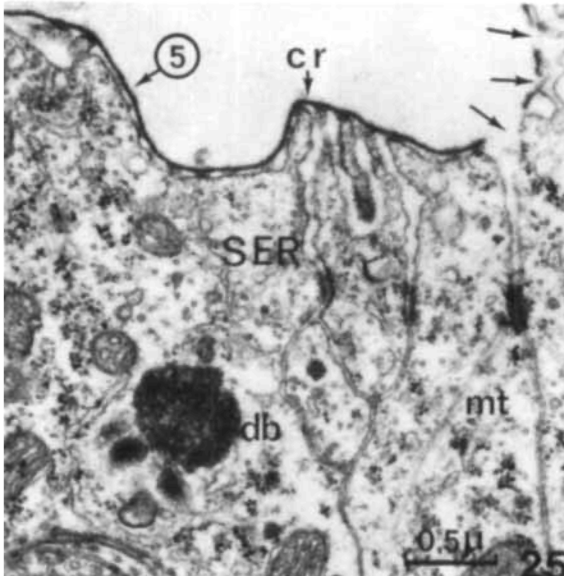
29 See Golgi complex next to lipid droplet. dv, dense vesicle with smooth membrane; scv, small coated vesicle. × 25,000.

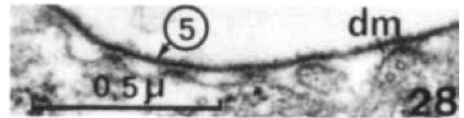
30 Sample of apical cytoplasm. db, dense residual body; G ap, apical Golgi complex; Gcv, large coated vesicle. × 25,000.

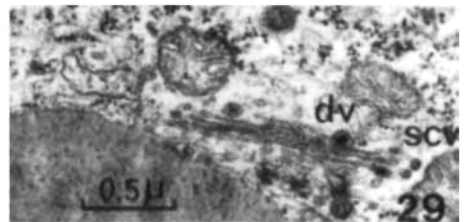

3
4
mv
5
ER
dv
Gap
ML
0,5µ
24

dv
Gcv
M
0,5 µ
za
26


Gcv
za
0,5 µ
27


5
cr
SER
db
mt
0,5µ
25


5
dm
0,5µ
28


dv
0,5µ
29

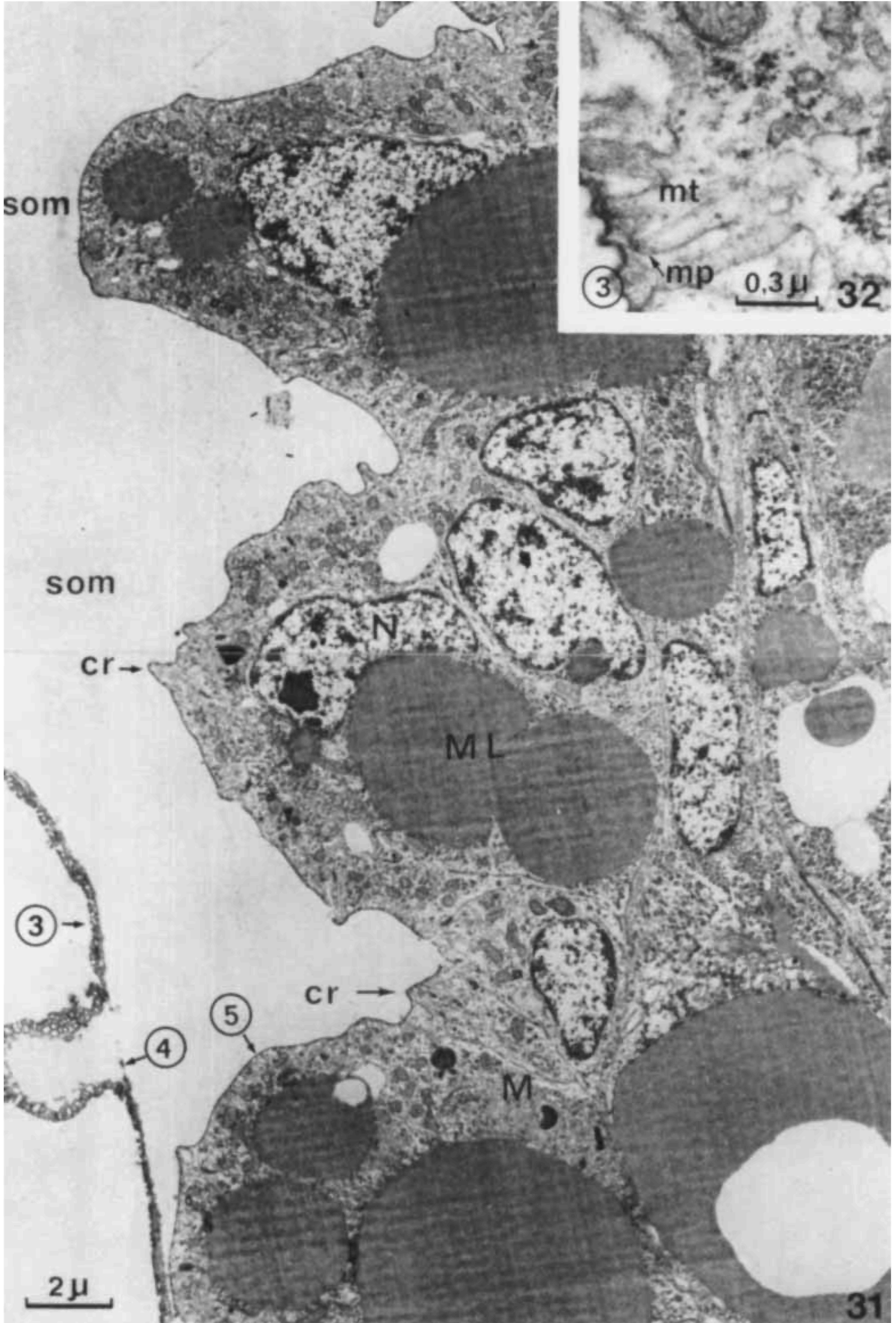
Gap
Gcv
db
0,5µ
30

PLATE 6

EXPLANATION OF FIGURES

31 Parasagittal-section of Stage J. Epidermal cells of dorsal zone. ③, embryonic envelope③; ④, embryonic envelope④; ⑤, fifth external coating; cr, crest outlined by⑤ ; M, mitochondria; ML, lipid droplet; som, somite. × 6,200.

32 Stage F embryo. Apex of a cell in epithelial layer of extraembryonic area.③ embryonic envelope③ forming; mp, apical plasma membrane; mt, microtubule. × 41,000.


som
som
cr
N
ML
cr
M
3
4
5
2µ
31
mt
mp
3
0,3µ
32