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Egg Membrane Lytic Activity of Sperm Extract and its Significance in Relation to Sperm Entry in *Hydroides hexagonus* (Annelida)*.†

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**PLATES 158 TO 162**

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**ABSTRACT**

Previous electron microscope studies indicated that the individual spermatozoön of *Hydroides hexagonus* forms a hole in the vitelline membrane by means of lysis. Other observations established that the hole is real, being visible in living material during sperm entry.

During the present investigation sea water extracts from frozen-thawed sperm were tested for lytic effect on the membrane. In normal living eggs the membrane appears as a single thick envelope, but in electron micrographs of sections it is seen to consist of a narrow outer border layer, a wide principal or middle layer, and a narrow inner border layer. After immersion in sperm extract the outer border layer elevates but does not dissolve, the middle layer liquefies and disappears, and the inner border layer seems not to change. This is interpreted as lysis of the middle layer. The extract exerted the same effect on fertilized and unfertilized eggs.

In electron micrographs the sections treated with extract greatly resemble that part of the membrane which has been penetrated by the individual spermatozoön. It is concluded that the individual spermatozoön, too, exerts a lytic effect. Together, the present and two earlier studies are considered clearly to demonstrate that in *Hydroides* the individual spermatozoön does indeed make an entry hole in the egg membrane by applying lytic material to that part of the membrane in its own vicinity.

**INTRODUCTION**

The existence of egg membrane lysins in sperm extracts has been established in a number of species and some authors have postulated that such lysins would enable the spermatozoa to cross the egg membrane (reviewed by Tyler (23) and Mann (18)). In the annelid *Hydroides hexagonus* the spermatozoön forms a hole in the vitelline membrane as can be seen in living material (15) and from electron micrographs it has been deduced that the individual spermatozoön makes this hole by applying lysin locally to the egg membrane (13 to 15). The finding of egg membrane lytic material in the sperm of *Hydroides* would, of course, lend strong support to this deduction and studies were undertaken in this direction. A chemical characterization of the sperm extract (12) will be presented later. The present paper deals with the effect of sperm extract on the egg membrane as observed by light and electron microscopy. These findings have been reported briefly in abstracts (10, 11).

**Materials and Methods**

The sperm and eggs used for this study were of the annelid *Hydroides hexagonus*. The animals were obtained in the vicinity of Woods Hole, Massachusetts. Sperm extracts were prepared by collecting “dry” sperm from many animals over a period of several weeks. As collected, the sperm was frozen rapidly by a mixture of acetone and dry ice and stored in the freezer compartment of a refrigerator. When preparations amounting to about 5 ml. had been accumulated, they were

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thawed and ground with sand in a minimum amount of filtered sea water in a chilled mortar, centrifuged either at room temperature or in a refrigerated Spinco, and the resultant supernatant used. When samples of the supernatant were dialyzed against filtered sea water, it was found that the lytic material remained within the dialyzing membrane. The extract was frozen and stored in the refrigerator. For use, the extract was thawed. The desired amount was withdrawn and added to the desired amount of eggs in sea water and allowed to act for various lengths of time. The concentrations of sperm were not identical in the initial preparations and, as might be expected, the different supernatants differed somewhat in their lytic strength. An indication of the relative strengths of some supernatants can be had by comparing the exposure times required to effect the changes seen in various specimens shown in Figs. 26 to 40.

Living material was studied on slides with supported coverslips and in depression slides. Photomicrographs were made with low, high, and chiefly oil immersion lenses of a conventional (non-phase) light microscope through a Leitz Micro-Ilso apparatus and enlarged photographically to the desired magnification.

Material for thin sectioning was fixed in 2 per cent osmium tetroxide in sea water for approximately ½ hour and then passed successively through sea water, diluted sea water, distilled water, and increasing concentrations of ethyl alcohol. The material was then infiltrated in three changes of a mixture of 85 per cent n-butyl and 15 per cent methyl methacrylate monomer containing 2 per cent luproco as catalyst. Polymerization was carried out in an oven at 63°C. Thin sections were cut with a Porter-Blum microtome and examined with an RCA model EMU-3C electron microscope. The electron micrographs were made at original magnifications of approximately \( \times \) 5,600 to \( \times \) 11,000 and enlarged photographically to the desired final magnification.

OBSERVATIONS

The vitelline membrane or envelope of the egg has the same general appearance in fertilized as in unfertilized eggs. This was found to be true in sections as well as in living material. Since, in addition, fertilized and unfertilized eggs gave the same reactions to the treatments used in this study, the ensuing account will not generally distinguish between the two conditions of the egg. The appropriate information, however, will be given in the explanations of figures.

A. Egg Membrane before Treatment:

In living eggs the vitelline membrane or envelope appears as a single layer of translucent homogeneous material approximately 3 to 4 \( \mu \) in thickness (Figs. 1 to 16). Its outer boundary is well defined. The inner boundary, too, is well defined if seen in areas in which it fails to lie directly against the cell. Thus before fertilization it may sometimes be seen in specimens in which the germinal vesicle has broken down (Fig. 2); soon after fertilization it is readily visible in the vicinity of the polar body (Fig. 3), and later it shows well in the region above furrows between blasomeres (Fig. 4). Both boundaries are well defined in plasmolyzed eggs (Fig. 7).

The consistency of the envelope is firm. This is demonstrated in several ways: (a) When routinely handled eggs are jostled against each other on the slide, the envelopes of adjacent eggs are not greatly reduced in thickness in the areas in which they are pressed together (Figs. 5, 6). (b) The thickness of the envelope is reduced very little when an egg is compressed gently beneath the coverslip (Fig. 17). (c) When the sperm entry hole in the envelope (15) is examined the envelope substance which adjoins the completed hole is never seen to move into the hole like a liquid or to escape through the hole. Instead, the envelope retains the hole in approximately its original shape and size. (d) If the egg is compressed strongly beneath the coverslip, hyaline material exuded from the egg passes through the envelope and forms globules outside it (Figs. 13, 18, 19). Still the envelope appears as a single thick layer of firm translucent material.

Electron micrographs of sections show that this envelope consists of at least three elements (Fig. 20). A wide principal or middle layer is closely bounded between a much narrower outer border layer and inner border layer. As seen in radial sections of the egg the salient structural feature of the outer border layer is a group of small round bodies which closely adjoin each other and form a single regular row attached to a thin basal sheet. The middle layer is somewhat uniformly felt-like in appearance. The inner border layer, too, is felt-like in appearance and would seem to be continuous with the middle layer, but it is more compact and shows a greater electron density than the middle layer. Although the inner border layer shows well in areas in which it is somewhat removed from the cell (Fig. 31), it can also be seen in areas closely adjoining the cell (Figs. 31, 34, 26). This layer is clearly visible in tangential sections, in which the plane of the section enhances the apparent width of all the layers (Fig. 32). When, as happens occasionally, a
region is found in which the inner border layer is not apparent (Fig. 30) other regions in the same section usually show this layer as well defined.

Although the three layers were not distinguished in untreated living eggs under the light microscope it is probable that the material of the middle layer was the chief substance seen as the vitelline membrane and that the border layers merely contributed to the sharp delineation of this envelope. Hereafter, the terms outer border layer, middle layer, and inner border layer will be applied, when it seems appropriate, to living eggs as well as to fixed and sectioned ones.

Thread-like microvilli which are extensions of the egg proper project far into the middle layer and some, but perhaps not all, reach to the outer edge of this layer (Figs. 26, 30). They appear to be more or less evenly spaced when seen in tangential sections of the envelope (Figs. 32, 34). There is some evidence that there are fewer microvilli in fertilized eggs than in unfertilized ones. Although microvilli were not observed in living eggs it is possible that they were responsible, in part, for the results observed when untreated living eggs were strongly compressed. In these eggs, as described above, material exuded by the eggs forms globules outside the envelope. The microvilli may have provided channels through which the exuded material could traverse the firm substance of the middle layer. Since, as seen in electron micrographs, the distal ends of the microvilli have the least amounts of middle layer substance surrounding them, it might be expected that the exudate would be released at these rather weakly supported points.

B. Egg Membrane after Treatment in Sperm Extract:

Living Eggs.—A number of preparations of sperm extract were tested with many different samples of eggs in sea water. Usually one or two drops of the extract were mixed with a drop or less of sea water containing the eggs. Often the full effect was obtained within a few minutes. Briefly, the response was as follows (Figs. 8 to 11; 20 to 22): the outer border layer elevated, the inner border layer remained as close to the egg as before treatment, and the firm middle layer became invisible.

In some eggs the outer border layer expanded like a balloon and broke and was shed (Fig. 9). Within a given culture (Fig. 8) it might expand greatly in some cases but in others expand less and remain loosely about the egg. The persisting inner border layer showed especially well in cleaving eggs (Fig. 8) and in plasmolyzed eggs (Figs. 10, 21). The extract caused neither the outer nor the inner border layer to disappear.

Elevation of the outer border layer is so easily seen that it was used as the first criterion for judging whether or not response to the extract had occurred. Most eggs responded. Thus, for example, when a group of 129 unfertilized eggs with unelevated outer border layers was treated with extract, 128 outer border layers became elevated within 2 minutes. Again, when a group of 116 plasmolyzed fertilized eggs was treated with extract, this layer was elevated in all within 2½ minutes; 100 similar eggs treated with sea water as controls, showed no elevation whatever.

The change undergone by the middle layer was one of liquefaction as the following observations show. (a) Part of a loosened outer border layer would sometimes touch or could be pushed against the inner border layer (Figs. 20 and 21). Indeed, in a given specimen the two border layers could be brought together and separated repeatedly. Thus it was evident that a liquid and not a substance of firm consistency now intervened between the two layers. (b) Eggs which had shed the outer border layer frequently stuck together. In many cases the inner border layer of one egg touched the inner border layer of another, thus demonstrating that little or no firm material of the middle layer was present between them (Fig. 11). (c) When eggs were compressed strongly the hyaline material which they exuded formed globules which remained in the site formerly occupied by the middle layer (Figs. 14, 15, 23, 24); hence the material of the middle layer must have lost its firm consistency and perhaps even have become dispersed. Presumably the microvilli, if not firmly supported, fail to conduct the exuded material beyond the area of the middle layer. Upon being jarred the exuded globules moved about in the middle layer area as though in a liquid. Sometimes they pushed the outer border layer outward and often it broke (Figs. 23, 24). (d) Ciliated blastulae were treated with sperm extract. Before treatment the cilia had projected far beyond the envelope and their protruding portions, only, were beating. Soon after immersion in sperm extract the outer border layer moved outward. The cilia, which remained behind, continued to beat and even those parts which had formerly been immobile in the firm middle layer...
began to move as though in a liquid medium. Clearly, the middle layer material had become liquid (Fig. 22).

Partial response to the sperm extract was observed when the extract was too weak or the exposure time too brief to elicit the full response. The outer border layer moved outward but at least some of the material of the middle layer remained visible (Figs. 12, 25). However, with subsequent adequate exposure the middle layer gradually disappeared.

Liquefaction of the middle layer was used as a criterion for judging extent of change. When it was not possible to make this determination by inspection alone it was necessary to resort to measures like pushing or compressing the eggs as mentioned above, and if such measures were not feasible the condition of liquefaction was listed as “unknown.” Two examples of observations using liquefaction of the middle layer as the criterion are given below. Though in each case every egg had elevated the outer border layer, examination under high power gave the following results:

A. (unfertilized eggs) Liquefied—62, Partly liquefied—13, Unknown—16.
B. (fertilized eggs) Liquefied—32, Partly liquefied—0, Unknown—5.

In both cases control eggs showed no liquefaction and no elevation of the outer border layer.

In electron micrographs of sections the characteristic structural pattern of the outer border layer (Figs. 27, 28, 35, 36, 38, 39) has the same appearance as before treatment. There is no evidence that it dissolves or is removed from the egg by means other than by being shed.

In contrast the middle layer gradually undergoes a complete loss of density (Figs. 27 to 29, 35 to 37, 38 to 40). This is considered to mean that the material of the middle layer as such gradually disappears. Beginning near the outer edge and progressing inward, masses of felt-like material typical of this layer become interspersed with small areas of no density (Fig. 27). The latter areas increase in size (Figs. 35, 36) and the amount of felt-like material decreases until finally none of the material of the middle layer can be seen (Fig. 37). Sometimes a fold in the outer border layer emphasizes the condition of the middle layer. Thus in Fig. 38, which shows an early stage of treatment with sperm extract, the fold still embraces isolated felt-like masses but in Fig. 39, which shows a later stage, the fold contains none of these masses. Microvilli gradually become divested of the middle layer material surrounding them and come to project freely into the area formerly occupied by that material (Figs. 28, 29). Tangential sections which include such microvilli strikingly demonstrate the demarcation between affected and as yet unaffected regions of the middle layer (Fig. 33). The extent to which the middle layer had disappeared from an egg can be gauged in radial sections of eggs which had stuck together (cf. Fig. 11). In areas of contact between two such eggs, the maximum outer limit of either specimen clearly cannot extend beyond the recognizable outer limit of the other; the closer the two eggs lie to each other, the less there is of the material of the middle layer. Thus in the specimen shown in Fig. 40, in which two eggs lie so close together that their microvilli interdigitate and are even bent back, it is evident that virtually all of the material of the middle layer is absent.

The appearance of the inner border layer is not changed by treatment with sperm extract. Fig. 37 shows two eggs which had stuck together. Even though the surrounding middle layer has disappeared the narrow inner border layer shows the same felt-like appearance as in an untreated egg.

DISCUSSION AND CONCLUSIONS

The observations reported here indicate that the sperm extract effects a profound change in the egg envelope. As seen in living eggs, the principal material of the envelope loses its original firm consistency and becomes liquefied. From the electron micrographs of sections it is evident that the middle or principal layer of the envelope loses its initial density and progressively disappears. From both sources of information the evidence is clear that the middle layer material is not merely rendered invisible but actually removed or dispersed. These findings are interpreted to mean that lysis takes place when the eggs are treated with the sperm extract and it is concluded that the sperm extract contains egg membrane lytic material.

The above conclusion is in harmony with the findings of earlier workers, of whom a few may be cited (16, 17, 21, 22, 25, 26), that sperm extracts contain substances which will dissolve or disperse certain barriers that surround eggs. Such barriers include the several membranes, jelly coats, and masses of cells that surround the eggs of one or another of the several invertebrate
and vertebrate species in which such sperm extracts have been studied. This subject has been reviewed extensively by Tyler (23), Chang (8, 9), Mann (18), and Austin and Bishop (3). Some investigators have held that lysin from sperm would act collectively to facilitate sperm entry through the egg barrier. Others (1, 23) have held that the individual spermatozoa would use lysin to make its own way through the barrier. In species other than *Hydroides* the latter, more generally held, view has had perhaps its strongest support from the following facts and deductions:

(a) Spermatozoa come to lie completely within the egg proper even though the lysin susceptible barrier is still present and appears to be intact. The beautiful photomicrographs of living rat eggs by Austin and Smiles (5) will serve as an illustration. Obviously the cumulus oophorus, which can be dispersed by hyaluronidase, was breached by the individual spermatozoa without having been dispersed. From this material Austin (1) deduced that the local presence of the enzyme might conceivably have assisted the passage of the individual spermatozoa.

(b) The vitelline membrane in some cases appears to weaken at points at which spermatozoa are attached. In the polychaete *Pomatoceros*, for example, Monroy (20) observed an outflow of hyaline material at such points and reasoned that the attached spermatozoa had exerted lytic activity on the membrane. Then, finding that spermatozoa treated with certain—SH group inhibitors could not adhere to the vitelline membrane, he assumed provisionally that the lytic factor might be a proteolytic enzyme which, he assumed, enabled the spermatozoa to go through the vitelline membrane.

(c) An opening or hole is formed in the barrier when the spermatozoa passes through. Such an opening was demonstrated by Austin (2), with photomicrographs, in the zona pellucida of living rat eggs. Austin supposed that since the hole exists the individual spermatozoa carries a lytic agent which enables it to digest a path through the zona, but the presumed lytic agent has not yet been demonstrated (4, 7). A comparable hole in the vitelline membrane of the mollusc, *Mytilus*, was shown by Mèves (19), who made drawings from sectioned material, and confirmed by Wada, Collier, and Dan (24) who showed several sketches from living material. Berg (6) demonstrated egg membrane lysin from sperm extract in *Mytilus*, and Wada, Collier, and Dan, finding such lytic material in the supernatant of spermatozoa which had undergone the acrosome reaction, indicated support for the belief that the lytic material acts to enable the individual spermatozoa to penetrate the membrane barrier.

In *Hydroides* the individual spermatozoa forms an entry hole in the vitelline membrane. Formation of this hole has been observed repeatedly in living material (15), and the holes seen in living eggs have their counterparts in holes found in electron micrographs of sections of sperm entry sites (13, 15). As seen in sections, there is a remarkable similarity in appearance between that part of the membrane in the immediate vicinity of a penetrating spermatozoa and a portion of membrane affected by the sperm extract reported here. In both, the outer and inner border layers are present and naked microvilli project into a space from which the material of the principal or middle layer is absent (cf. Fig. 28 with Fig. 27 of the preceding paper (15)). Plainly the lytic effect of the sperm extract on the membrane of the entire egg is duplicated by the effect of the individual spermatozoa locally. From this it is concluded that the individual spermatozoa, too, exerts a lytic effect. The present findings and those of the two earlier studies (13, 15), taken together, are considered clearly to demonstrate that in *Hydroides* the individual spermatozoa does indeed make an entry hole in the egg membrane by applying lytic material to that part of the membrane in its own immediate vicinity.

REFERENCES

10. Colwin, A. L., and Colwin, L. H., Egg membrane...
Lytic activity in relation to sperm entry


EXPLANATION OF PLATES

All figures are of eggs of *Hydroides hexagonus*.

Legend

- b blastomere
- i inner border layer
- m middle layer
- pb polar body
- o outer border layer
- p perivitelline space
- v microvillus

*Exp.*: length of time egg had been in extract when fixed.

*Prep.*: sperm extract. The different preparations of extract used are indicated by different letters.
Photomicrographs of living eggs. All figures are at a magnification of X 450 except Fig. 5 which is at a magnification of approximately X 300.

Figs. 1 to 7. Eggs not treated with sperm extract.
Fig. 1. Ripe unfertilized egg with germinal vesicle. Outer boundary of vitelline membrane well defined.
Fig. 2. Ripe unfertilized egg without germinal vesicle. Inner and outer boundaries of vitelline membrane well defined.
Fig. 3. Fertilized egg. Envelope shows well where separated from egg near polar bodies.
Fig. 4. 2-cell stage. Envelope shows well above furrow between blastomeres.
Fig. 5. Envelope not reduced in thickness although lightly compressed between crowded eggs. (Unfertilized eggs.)
Fig. 6. Same as Fig. 5, but cleaving eggs.
Fig. 7. Plasmolyzed unfertilized egg. Note well defined boundaries of envelope.

Figs. 8 to 12. Eggs treated with sperm extract.
Fig. 8. Egg at left shows elevated outer border layer remaining loosely about egg. Egg at right has much more highly elevated outer border layer. Both eggs from same preparation. (Cleaving eggs.)
Fig. 9. Egg which has shed outer border layer. (Unfertilized.)
Fig. 10. Plasmolyzed cleaving egg showing intact inner border layer and highly elevated, broken, outer border layer.
Fig. 11. Eggs which had shed outer border layer and had stuck together. Inner border layers of adjacent eggs meet as though middle layers were absent.
Fig. 12. Partial reactions to sperm extract. Outer border layer partly elevated but most of middle layer still visible. (Plasmolyzed, unfertilized egg.)

Fig. 13. Strongly compressed egg not treated with extract. Globules of exuded hyaline material are outside thick, well defined apparently single envelope. (Unfertilized egg.)
Figs. 14 and 15. Eggs strongly compressed after treatment with sperm extract. Globules of exuded material are within outer border layer and occupy area formerly occupied by material of the middle layer. Middle layer not visible.
Fig. 14. Arrow: outer and inner border layers appear to meet. (Cleaving egg.)
Fig. 15. Unfertilized egg.
(Colwin and Colwin: Lytic activity in relation to sperm entry)
Photomicrographs of living eggs. Figs. 16 and 17 are at a magnification of X 1600; Figs. 18 to 25 are at a magnification of X 1100.

**Fig. 16.** Typical appearance of ripe unfertilized egg with germinal vesicle. Well defined outer boundary of vitelline membrane irregularly scalloped. Inner boundary close to egg proper.

**Fig. 17.** Egg similar to that in Fig. 16, but slightly compressed. Vitelline membrane (envelope) well defined. 

**Figs. 18 and 19.** Strongly compressed eggs not treated with extract. Globules of hyaline material are outside thick, well defined apparently single envelope.

**Fig. 18.** Cleaving egg.

**Fig. 19.** Unfertilized egg.

**Figs. 20 to 25.** Eggs after treatment with sperm extract.

**Fig. 20.** Portion of partly shed outer border layer touches inner border layer. Liquefied middle layer not seen. Unfertilized egg.

**Fig. 21.** Elevated outer border layer had been pushed against inner border layer; suggests liquefaction of middle layer. Plasmolyzed, unfertilized egg.

**Fig. 22.** Elevated outer border layer encloses cilia (between arrows) which are beating in area formerly occupied by firm material of middle layer. Blastula.

**Figs. 23 and 24.** Strongly compressed eggs. Globules of exuded material occupy area formerly occupied by material of middle layer. Elevated outer border layer, broken in some places, touches some of the globules. Unfertilized eggs.

**Fig. 24.** The arrow points to a region of the outer border layer which gives the appearance of containing granules; possibly these are the round bodies seen in electron micrographs of this layer.

**Fig. 25.** Partial response to sperm extract. Outer border layer elevated but much of middle layer material still present. The inner border layer is well shown near polar body of this cleaving egg.
(Colwin and Colwin: Lytic activity in relation to sperm entry)
Plate 160

Electron micrographs of sections of unfertilized eggs. All figures are at a magnification of about $\times 22,000$.

Fig. 26. Untreated egg showing structure of the three layers of the vitelline membrane. Portions of three microvilli protrude into middle layer.

Figs. 27 to 29. Stages of change in the vitelline membrane following treatment with sperm extract.

Fig. 27. Early stage. Outer border layer elevated (slight damage to this layer attributed to handling during preparation). In distal region, felt-like material of middle layer is interspersed with areas of no electron density. Exp. 22 seconds; Prep. A.

Fig. 28. Late stage. Microvilli project freely into area from which much of felt-like material of middle layer has disappeared. Note elevated outer border layer and unchanged inner border layer. Exp. 41/₂ minutes; Prep. A.

Fig. 29. Late stage. Outer border layer has been shed. Naked microvilli project through inner border layer. Very little of middle layer remains present. Exp. 41/₂ minutes; Prep. A.
(Colwin and Colwin: Lytic activity in relation to sperm entry)
Plate 161

Electron micrographs of sections of eggs.

Fig. 30. Untreated egg showing a microvillus that extends to outer edge of middle layer of envelope. Inner border layer not visible here. (Broken areas in outer border layer attributed to handling during preparation.) Unfertilized. About \( \times 22,000 \).

Fig. 31. Untreated cleaving egg. Inner border layer visible both in area where it adjoins the polar body and in area where it adjoins the perivitelline space between polar body and blastomere. About \( \times 22,000 \).

Fig. 32. Untreated egg. Semitangential plane of section enhances apparent width of all layers of envelope. Note fairly even spacing of microvilli. Unfertilized. About \( \times 11,000 \).

Fig. 33. Treated egg. Semitangential plane of section (cf. Fig. 32). Intermediate stage of change caused by sperm extract. Outer border layer has been shed. Microvilli are naked in region from which material of middle layer has disappeared. \( \text{Exp. } 30 \frac{1}{2} \text{ minutes; Prep. } B \). Unfertilized. About \( \times 11,000 \).

Fig. 34. Untreated egg. Very slightly tangential section. Note inner border layer; fairly regular spacing of microvilli. Unfertilized. About \( \times 22,000 \).

Fig. 35. Treated egg. Nearly in same plane of section as Fig. 34. Early stages of change caused by sperm extract. Some elevation of outer border layer. In distal region of envelope, masses of felt-like material of middle layer are interspersed with areas of no electron density. \( \text{Exp. } 22 \text{ seconds; Prep. } A \). Unfertilized. About \( \times 22,000 \).

Fig. 36. Treated egg. Later stage of change than that shown in Fig. 35. More extensive disappearance of felt-like material of middle layer. \( \text{Exp. } 4 \frac{1}{2} \text{ minutes; Prep. } C \). Early cleavage stage. About \( \times 22,000 \).

Fig. 37. Treated eggs. Full effect of sperm extract. Two eggs which had shed their outer border layers and had stuck to each other (cf. Fig. 11). At left, unchanged inner border layers of the two eggs touch each other, demonstrating absence of intervening middle layer. \( \text{Exp. } 14 \frac{1}{2} \text{ minutes; Prep. } D \). Early cleavage stage. About \( \times 22,000 \).
(Colwin and Colwin: Lytic activity in relation to sperm entry)
Electron micrographs of sections of eggs showing different stages of change in vitelline membrane following treatment with sperm extract. All figures are at a magnification of about $\times 20,000$.

Fig. 38. Early stage. Fold in elevated outer border layer contains felt-like masses of material of middle layer surrounded by areas of no electron density. Exp. 4\textsuperscript{1/2} minutes; Prep. C. Early cleavage stage. Arrow: junction of two blastomeres.

Fig. 39. Late stage. No felt-like material remains visible within fold of outer border layer. Exp. 14\textsuperscript{1/2} minutes; Prep. D. Early cleavage stage. Arrow: furrow between blastomeres.

Fig. 40. Very late stage. Eggs which had shed their outer border layers and had stuck to each other. Interdigititation and bending back of microvilli between two eggs indicate absence of material of middle layer, virtually all of which has disappeared. Exp. 4\textsuperscript{1/4} minutes; Prep. A. Unfertilized.
(Colwin and Colwin: Lytic activity in relation to sperm entry)