The Oocytes of the Goby *Pomatoschistus minutus*

IV. Electron microscopic autoradiography of [³H]-proline incorporation

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In three previous papers not only egg-membrane and follicle of the marine goby *Pomatoschistus minutus* were examined by light and electron microscopy (Riehl 1978a), but also the morphology of the micropyle by light microscopy (Riehl 1978b) and the amino acid components of the eggs by amino acid analysis (Riehl 1980). As the most remarkable feature, oocytes and eggs of *Pomatoschistus minutus* show up to 220 attaching-filaments, which are connected with the egg-membrane only at the animal pole. After spawning, the eggs are fastened to the substratum by these attaching-filaments.

During the genesis of the attaching-filaments rough endoplasmic reticulum (RER) strongly increases in all cells of the follicle epithelium. This fact allows the opinion that the follicle epithelium decisively participates in the formation of the attaching-filaments. This hypothesis is supported by demonstrating that the material of the attaching-filaments chiefly consists of proteins and glycoproteins (Riehl, unpublished data).

Autoradiography with L-[³H]-proline should show whether or not this presumption is justified. Furthermore autoradiography allows the identification of the follicular and oocytous compartments which are involved in the synthesis, transport, and secretion of the egg-membrane.

Materials and methods

The animals used in this study were adult females of the marine goby, *Pomatoschistus minutus*, which were captured in List on the island Sylt in northern Germany.

Electron microscopy

The fishes were anaesthetized with 0.5-1% Anestasin (=4-aminobenzoid-acid-ethylester; Serva, Heidelberg, F.R.G.). The ovaries were placed in 3% glutaraldehyde buffered with phosphate buffer or sea water (Riehl 1978b) (pH 7.4; 4°C) for 2 to 4 h. After rinsing with adequate buffering solution, pieces of ovary were postfixed in 2% osmium tetroxide. Each piece was embedded via propylene oxide in Araldite and Vestopal W. A Reichert ultramicrotome OM U 3 was used to cut ultrathin sections, which were double stained in uranylacetate and phosphotungstic acid en bloc together (Wohlfarth-Bottermann 1957) or in uranylacetate and lead citrate (Reynolds 1963). Ultrathin sections were viewed with a Zeiss EM 9S.

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Electron microscopic autoradiography

After narcosis with Anestasin each fish received a 5 μ l intraperitoneal injection of L-5-[3 H]-proline (Amersham-Buchler, Braunschweig, F.R.G.), specific activity 10–20 Ci/mmol, reconstituted to an aqueous solution of 5 μ Ci/5 μ l. At all times three fishes were sampled after 15 and 30 min, and 1, 3, 6, 12, 24 and 48 h. At each interval the ovaries were removed and fixed in 2% osmium tetroxide in phosphate and veronal acetate buffer at pH 7.4, at room temperature for 30–60 min. The tissues were dehydrated in ethanol and embedded in Araldite, Vestopal W and Epon 812.

Ultrathin sections of gold interference were obtained from a Reichert ultramicrotome OM U 3 with glass and diamond knives. The sections were picked up on Formvar-covered, carbon coated 75-mesh copper grids. The grids were then attached to the surface of chromic acid-cleaned glass slides according to methods described by Caro (1962, 1969). The following steps were performed mostly according to the method of Tesoriero (1978). In brief: an Ilford-L-4 nuclear research emulsion was used, which was melted at 45°C and diluted 1:1 with 45°C twice-distilled water. Thin films of the emulsion were applied to the slides by the loop method (Caro 1962). After drying overnight the slides were stored in lightimpervious boxes containing CaCl₂ as a desiccant. The slides were exposed to 4°C for 50–90 days. They were developed in Kodak Microdol-X solution and fixed in Kodak F-24 buffered thiosulfate formula. After air-drying of the slides the grids were stained with aqueous uranylacetate and lead citrate (Reynolds 1963). Control slides for background and chemographic effects were prepared in the same manner as the above samples using thin sections of *Pomatoschistus minutus* ovaries without [3 H]-proline incubation that were fixed in osmium tetroxide.

Statistical analysis

Statistical analysis of the electron microscopic autoradiographs was carried out by grain counts from randomly selected samples. The number of grains over the various compartments for each experimental group was determined. Silver grains appearing upon the micrographs were reduced in complexity by taking the midpoint of the long axis of the grain and placing a 300 nm circle around the point. This circle has a 50% probability of enclosing the site of disintegration (Blackett and Parry 1977). Structures were scored according to the frequency with which they appeared within the 300 nm circle.

For purposes of analysis several categories of organelles were pooled so that areas of cytoplasmic matrix, mitochondria, ribosomes and endoplasmic reticulum were combined as cytoplasm. The miscellaneous compartment represents pooled data from yolk, cortical alveoli and nuclei.

The percentage distribution of grains in each compartment was analysed by the chi-square method to determine the probability of the frequency being randomly distributed. The analysis is included in the table.

Results

Electron microscopy

Oocytes of stages I–III were examined by electron microscopy (stage-classifi-

Figs. 1–5. Formation of the egg-membrane. 1, peripheral portion of a stage I oocyte. Microvilli at the oocyte surface and cell protrusions from the follicle epithelium are formed. Thus, an intercellular space develops. $\times 18,000$. 2, first material of the egg-membrane appears in the intercellular space and is deposited at the oocyte surface between the bases of the microvilli (late stage I). $\times 19,500$. 3, the material of the egg-membrane becomes electron-denser and grows in thickness (early stage II). $\times 21,000$. 4, in stage II interwoven fibrils appear at the base of the egg-membrane forming the “zona radiata interna” or “zone 3” described by other authors. $\times 19,500$. 5, radial section through the periphery of a late stage III oocyte showing the egg-membrane with the radial canals, the attaching-filaments and the quadrilateral follicle cells. $\times 5,500$. AF=attaching-filaments, BM=basement membrane, EM=egg-membrane, FE=follicle epithelium, I=internum of an attaching-filament, IS=intercellular space, M=matrix of an attaching-filament, MV=microvillus, N=nucleus of a follicle cell, O=oocytoplasm, OL=oolemma.

cation by Arndt 1956). Stage IV is not very important, because the formation of egg-membrane and attaching-filaments is completed in late stage III. In stage IV the egg-membrane increases only in thickness whereas the attaching-filaments grow in length.

Oocytes of stage I are surrounded by the oolemma, which peripherally faces an

Figs. 6-9. Formation of the attaching-filaments. 6, an electron-dense material forming the attaching-filaments is deposited in the intercellular fissure found between the follicle cells (stage II). $\times 18,500$. 7, increase of the attaching-filament (stage II). $\times 18,500$. 8, developing attaching-filament in early stage III. At present the first deposited osmiophilic homogeneous material is surrounded by a granular matrix. $\times 15,600$. 9, cross-section of a mature attaching-filament showing both the homogeneous internum and the surrounding granular matrix (late stage III). $\times 11,500$. AF=attaching-filament, B=border of follicle cells, BM=basement membrane, EM=egg-membrane, FE=follicle epithelium, I=internum of an attaching-filament, M=matrix of an attaching-filament.

intercellular space of variable width. Microvilli of the oolemma and microvillous-like processes project into this space. At the bases of the microvilli, pinocytotic vesicles are found. The cells of the follicular epithelium are clearly flattened and are sometimes interconnected by desmosomes. A basal membrane separates the follicular epithelium from the surrounding theca folliculi. The cytoplasm of the follicular cells contains free ribosomes, mitochondria but only a few endoplasmic reticulum. (Fig. 1).

The formation of the egg-membrane (=vitelline envelope of Dumont and Brummett 1980) begins late in stage I. During this time a dense and rather homogeneous material, closely associated with the oolemma, appears in the intercellular (=perivitelline) space and surrounds the microvilli (Fig. 2). This substance is almost invisible if the sections are stained with lead citrate only. Contrast is due to binding with uranyl acetate. This extracellular deposit is the earliest morphological manifestation of the developing egg-membrane.

During stage II the egg-membrane increases in size rapidly (Fig. 3) and after reaching 0.5 μm in thickness a zone becomes visible at its base, which is formed from bundles of interwoven filaments. This is the inner part of the egg-membrane (=“zona radiata interna”). The follicle cells increase in number and undergo a change in shape, becoming elongated and spindle-shaped. At the same time their cytoplasmic organization changes insofar that a massive increase of the endoplasmic reticulum appears.

During this time the synthesis of the attaching-filaments initiates. The narrow intercellular fissures found between the follicle cells widen and form tubular spaces. An electron-dense, granular material is deposited in these spaces, which is the initial deposit that forms the attaching-filaments characteristic of *Pomatoschistus minutus* (Figs. 6+7).

At the end of stage III the egg-membrane is fully developed and differentiated, now measuring 6 μm in width. The outer part of the egg-membrane (=“zona radiata externa”) is very thin only reaching a thickness of 0.5 μm . Microvilli of the oocyte and follicle cells traverse radial canals through the egg-membrane (Fig. 5).

The transition from stage II to III is accompanied by several major changes in follicle cell organisation. Follicle cells remain spindle-shaped, but their profile becomes trapezoid. They are completely filled with rough endoplasmic reticulum (Fig. 5). Numerous mitochondria are found between RER.

Attaching-filaments grow in length and width. They generally lie parallel to the egg-membrane following the contortion of oocytes and are attached to the egg-membrane only at the animal pole. Aside from this they have no contact with the egg-membrane. In stage III they consist of an osmiophilic homogeneous internum, which is surrounded by a granular matrix (Figs. 8+9). Preliminary histochemical experiments have shown that the attaching-filaments contain a high degree of proteins and carbohydrates.

In stages II and III a striking feature is the occurrence and rapid increase of numerous pinocytotic vesicles and dictyosomes. They are mostly found in the peripheral cytoplasm and pinocytotic vesicles in the immediate vicinity of oolemma.

Figs. 10-16. Electron microscopic autoradiography of $[^3\text{H}]$ -proline. 10, electron microscope autoradiograph of an oocyte, 15 min post-injection. Grains are scattered throughout the oocyttoplasm. The follicle epithelium is labeled sporadically. $\times 20,000$. 11, EM autoradiograph, 30 min post-injection. Autoradiographic grains are associated with cisternae of endoplasmic reticulum. The egg-membrane is unlabeled. $\times 20,000$. BM=basement membrane, DCV=dense-core vesicle, EM=egg-membrane, FE=follicle epithelium, N=nucleus of a follicle cell, O=oocyttoplasm, TF=theca folliculi.

Dense-cored vesicles are found as well. Fusion of dense-cored vesicles with the oolemma was observed frequently.

Electron microscopic autoradiography

Qualitative analysis of autoradiographs reveals that at 15 min after injection autoradiographic grains are scattered throughout the cytoplasm of stage I, II, and III oocytes, whereas the cytoplasmic label is random. Egg-membrane, pinocytotic vesicles, dense-cored vesicles and dictyosomes are unlabeled. The follicle epithelium is labeled sporadically (Fig. 10).

At 30 min post-injection a high number of autoradiographic grains are associated with cisternae of the peripheral oocytous endoplasmic reticulum. A smaller number of grains has been seen in the dictyosomes. In stage II and III oocytes, the follicular endoplasmic reticulum is weakly labeled. Egg-membrane, pinocytotic and dense-cored vesicles remain unlabeled (Fig. 11).

By 1 h after injection all membranous components of the oocytoplasm contain labeled material. A concentration of grains is found in the Golgi bodies (Fig. 12). At this period most of the dictyosomes are labeled, while egg-membrane and the vast majority of pinocytotic vesicles and dense-cored vesicles remain unlabeled. The more mature follicle cells of stage II and III oocytes have a somewhat higher amount of label than after the 30 min injection. In comparison the oocytous microvilli and the follicular microvilli-like projections show no autoradiographic grains.

At 3 h post-injection grains appear within many of the pinocytotic vesicles and most of the dense-cored vesicles (Fig. 13). Most of the Golgi bodies are still labeled. Some label is seen adjacent to oolemma and egg-membrane of stage II and III oocytes. The material of the egg-membrane reveals only a very small number of grains (Fig. 13). Oolemma of stage I oocytes is unlabeled. Some label remains within the oocytoplasm and appears to be associated with the endoplasmic reticulum. The follicle cells show a considerable amount of autoradiographic grains. At this point some of the attaching-filaments are also weakly labeled.

By 6 h and 12 h after injection the number of grains in egg-membrane and attaching-filaments increases. During this time nearly 50% of the Golgi bodies are still labeled. The non-Golgi oocytoplasmic label is still more reduced and remains without an apparent organization. Endoplasmic reticulum of oocytes appears to contain significant amounts of this labels. The endoplasmic reticulum of follicle cells is strongly labeled (Fig. 14).

Sections of oocytes at 24 h post-injection have a significant amount of label in egg-membrane and attaching-filaments (Fig. 15).

In attaching-filaments both the homogeneous internum and the surrounding granular matrix show autoradiographic grains, but their number is significantly higher for the granular matrix. Not only the oocytous microvilli reveal labeled parts, but also the microvilli-like projections of follicle epithelium. Most of the Golgi bodies and dense-cored vesicles are devoid of any labeling, while the distribution of grains of the random oocytoplasmic label resembles that seen after 6 h and 12 h incubation. The follicular endoplasmic reticulum shows a high amount of autoradiographic grains.

Figs. 12-13. 12, EM autoradiograph of an oocyte, 1 h post-injection. Note that the Golgi body is labeled, whereas the egg-membrane remains unlabeled. $\times 20,000$. 13, EM autoradiography of an oocyte, 3 h post-injection. Note that the label appears in the egg-membrane and associated with dense-cored-vesicles (arrow). $\times 22,000$. EM=egg-membrane, FE=follicle epithelium, G=Golgi body, N=nucleus of a follicle cell, O=oocyttoplasm.

Figs. 14–15. 14, EM autoradiograph of an oocyte, 6 h post-injection. Note the label associated with dense-cored vesicles and the egg-membrane. Some Golgi bodies are still labeled. $\times 20,000$. 15, EM autoradiography of an oocyte, 24 h post-injection. Egg-membrane, attaching-filaments (AF) and follicle cells are heavily labeled. The homogeneous internum of AF is mostly unlabeled. $\times 6,000$. BM=basement membrane, DCV=dense-cored vesicle, EM=egg-membrane, FE=follicle epithelium, G=Golgi body, I=internum of an attaching-filament, M=matrix of an attaching-filament, N=nucleus of a follicle cell, O=oocyttoplasm.

Fig. 16. EM autoradiograph of an oocyte, 48 h post-injection. The egg-membrane is heavily labeled. $\times 5,500$. BM=basement membrane, FE=follicle epithelium, EM=egg-membrane, M=matrix of an attaching-filament, N=nucleus of a follicle cell, O=oocyttoplasm, V=vessel, Y=yolk.

Chart: Distribution of $[^3\text{H}]$ -proline in oocytes. Histogram depicting the distribution of grain count data from the table.

Table 1. Distribution of [³H]-proline in *P. minutus* oocytes¹⁾

Time (h)	Percentage distribution							Grain total
	Follicle cells	Attaching- filaments	Egg- membrane	Pinocytotic vesicles	Dense-cored vesicles	Golgi	Cytoplasm	Miscellaneous
0.25	1±0.2	0	0	0	0	0	91±1.8	8±1.1
0.5	3±0.4	0	0	0	0	13±1.2	77±1.5	7±0.9
1	5±0.6	0	0	2±0.4	4±0.5	25±1.3	59±1.3	5±0.8
3	17±0.9	1±0.2	2±0.3	14±1.1	19±1.2	20±1.5	24±1.6	3±0.4
6	23±1.4	5±0.6	6±0.8	13±0.8	15±1.1	16±1.3	18±0.8	4±0.3
12	25±1.8	8±0.7	9±0.6	9±0.7	11±1.2	13±1.2	19±1.0	6±0.5
24	30±1.7	20±1.6	18±1.5	2±0.3	3±0.5	5±0.5	17±1.3	5±0.6
48	16±1.1	13±0.9	49±1.8	<1	<1	3±0.4	13±1.1	6±0.9

$\chi^2 = 1736$, $N = 16$, $P = < 0.001$.

¹⁾ From grain count data from oocytes from each of three female fishes for each time period. Six oocytes per fish were recorded. The data are presented as the percentage of cumulative grains for all oocytes within each compartment of each time interval. Standard errors of the proportion for a multinomial distribution are included for each compartment. The miscellaneous compartment represents pooled data from yolk granules, cortical alveoli and nuclei.

By 48 h post-injection egg-membrane is heavily labeled, while the number of grains in the attaching-filaments has decreased. Only a few of the Golgi bodies still show some label, whereas in dense-cored vesicles labeling is no longer observed. The cytoplasmic label is scattered and reduced further but mostly it remains associated to the oocytous endoplasmic reticulum. At this point the label of the follicular endoplasmic reticulum has decreased significantly (Fig. 16; compare table and chart).

Discussion

In recent years isotopically labeled amino acids have been used to study the sequence of synthesis in many materials in the oocytes of some teleostean fish (Tsusaka and Nakano 1968, Nakano and Ishida-Yamamoto 1965, Nakano and Yamamoto 1972, Siebers and Rosenthal 1977, Riehl 1978d, Shackley and King 1979, Tesoriero 1978, 1980). This technique allows the identification of these cellular components which participate in the processes of synthesis, transport and secretion of cellular products at the subcellular level (Tesoriero 1978).

On account of the three following reasons, L-[^3H]-proline was chosen as a tracer to research the synthesis of the egg-membrane and the attaching-filaments in the marine fish *Pomatoschistus*: 1) It has been shown by previous investigators that this amino acid is very effective in demonstrating dynamics of cellular synthesis (Petri and Wyman 1974, Weinstock and Leblond 1974, Tesoriero 1978). 2) Tesoriero (1978) employed [^3H]-proline to label the oocytes of the limnic fish *Oryzias latipes*. Thus, greater accuracy is attained when using the same amino acid for comparing the results obtained from a limnic species with those of a marine one. 3) According to Riehl (1980) proline is one of the most common amino acids found in *Pomatoschistus* oocytes.

Nakano and Yamamoto (1972) found that in *Oryzias latipes* proline exists predominantly as a protein-bound fraction. This result is in good agreement with that of Riehl (1980) found in *Pomatoschistus minutus*. The amino acid analysis of *Pomatoschistus* oocytes has shown that more than 60% of proline exists as protein, whereas only 0.21% of proline is free and 0.9% is peptide-bound.

The results of the electron microscope autoradiography of *Pomatoschistus minutus* oocytes confirm earlier findings of Tesoriero (1978) obtained from *Oryzias latipes*. The egg-membrane of *P. minutus* is formed in the following way: first label appears at 15 min post-injection in the cytoplasmic compartment pointing to a synthesis of egg-membrane precursor material in the oocytoplasm. By 30 min post-injection the cytoplasmic label begins to decrease. This decline is followed by a rising, cellular activity in the Golgi bodies, which suggest a movement of material from the cytoplasm to the dictyosomes by pinocytotic vesicles. This material then appears to be concentrated in the Golgi bodies. The Golgi bodies lose their label within 48 h. The Golgi compartment releases the egg-membrane precursor material mostly into dense-cored vesicles, which migrate from the Golgi bodies to the oolemma. There the dense-cored vesicles discharge their contents—consisting of proteins and polysaccharides—into the intercellular space, thus forming the egg-

membrane.

These findings correspond in detail with those of Tesoriero (1978). Formation of egg-membranes is nearly identical in the limnic *Oryzias latipes* and the marine *Pomatoschistus minutus*. Therefore the present results support the hypothesis that the egg-membrane in fish is secreted by the oocyte. Tesoriero (1978) believes that the attaching-filaments of *Oryzias latipes* are formed from the same source (=cytoplasm) as the egg-membrane. This finding is in contrast to that found in *Pomatoschistus minutus*.

The follicle cells of developing *Pomatoschistus minutus* oocytes demonstrate an incorporation of [³H]-proline. This incorporation is correlated with the developmental stages of oocytes and the formation of the attaching-filaments. Labeling of follicle cells begins at 3 h post-injection in early stage II, reaches its maximum at 24 h post-injection in late stage II/stage III and decreases 48 h after injection in late stage III. It is exactly at this point that formation of attaching-filaments takes place. This suggests that the attaching-filaments of *Pomatoschistus minutus* are formed by the endoplasmic reticulum of follicle cells. This results, made on the basis of morphological evidence, confirm to the assumption of Wourms and Sheldon (1976), that the Golgi compartment plays no role in the secretion process of the secondary envelope in *Cynolebias melanotaenia* and *Cynolebias ladiges*.

Although up to this time data of autoradiographic labeling are lacking, there is general agreement by means of morphological examinations that the attaching-filaments (=chorionic fibrils, after Dumont and Brummett 1980) of teleost fish are produced by the surrounding follicle cells, as in *Fundulus heteroclitus* (Anderson 1967, 1974, Flügel 1967, Dumont and Brummett 1980), *Oryzias latipes* (Tsukahara 1971), *Cichlasoma nigrofasciatum* (Busson-Mabillot 1973, 1977), *Cynolebias melanotaenia* and *C. ladiges* (Wourms and Sheldon 1976) and *Chaenogobius isaza* (Takahashi 1978). The present study represents the first attempt to check morphological observations by means of autoradiography. Results indicate agreement.

In fish, two processes are discussed, how egg-membrane and attaching-filaments are formed: 1) they are synthesized by the oocyte itself; 2) they are formed by the follicle epithelium. The present results indicate that for *Pomatoschistus minutus* both possibilities are verified. The egg-membrane is chiefly secreted by the oocyte, whereas the attaching-filaments are synthesized by follicle cells.

In 1906 Waldeyer had already made the classical definition of primary, secondary and tertiary egg-membranes. This definition was renewed by Ankel (1933) and employed by Anderson (1967, 1974), Götting (1967) and Riehl (1978c). The latter made the attempt to bring order into the confusion of egg-membrane terminology for fish. According to Waldeyer (1906) and Ankel (1933) primary egg-membranes are synthesized by the oocyte itself, secondary egg-membranes from the follicle epithelium, whereas tertiary egg-membranes are formed by the oviduct. A chorion is, in conformity with this definition, always a secondary egg-membrane. Therefore the terminology used by some authors (Flegler 1977, Shackley and King 1977, Tesoriero 1977, 1978, Laale 1980) is not exact. In *Pomatoschistus minutus* the egg-membrane is a primary envelope, while the attaching-filaments represent a secondary "envelope" or chorion.

Dumont and Brummett (1980) have shown in their excellent paper that the application of these terms presents difficulties when the origin of the envelope in question is not firmly established and when there is the added problem of what to call an envelope that appears to derive structural components from both the oocyte and the follicle cells. Therefore in a forthcoming paper the ambiguity concerning the terminology of egg-membranes in fish will be discussed again in detail.

Summary

Formation of the egg-membrane and the attaching-filaments of the marine goby *Pomatoschistus minutus* was studied with electron microscopic autoradiography using the tracer L-[³H]-proline. The egg-membrane is formed in the chronological sequence as follows: first labeled material is found in the oocytoplasm by 15 min post-injection, in Golgi bodies by 30–60 min, in dense-cored vesicles and pinocytotic vesicles by 3 h and the substance of the egg-membrane by 12–24 h post-injection. This suggests that the material forming the egg-membrane is synthesized in the oocytoplasm, concentrated in the Golgi bodies and then transferred via dense-cored and pinocytotic vesicles to the developing egg-membrane. In the follicle epithelium first label appears in stage II and III oocytes at 30 min post-injection and reaches the maximum concentration in the endoplasmic reticulum by 24 h. These findings suggest that the egg-membrane of *Pomatoschistus minutus* is produced by the oocyte itself, whereas the attaching-filaments are synthesized by the endoplasmic reticulum of the follicle cells. Therefore according to the classical definition of Waldeyer (1906) the egg-membrane of this species is a primary envelope, while the attaching-filaments represent a secondary envelope or chorion.

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