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The Fine Structure and Amino Acid Composition of the Envelope of the Chum Salmon Egg

By

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(With 1 Text-figure, 1 Table and 2 Plates)

Throughout early embryonic development, morphogenesis proceeds in the space enclosed by the egg envelope. In teleosts, the envelope is especially thick and tough. Besides having a protective role for the developing embryo, it inhibits the penetration of spermatozoa into the ooplasm and acts as a barrier for polyspermic fertilization (Kobayashi and Yamamoto, 1981). While the formation and fine structure of the egg envelope in growing oocytes have been repeatedly the subject of thorough investigations in various teleosts (Stahl and Leray, 1961; Anderson, 1967; Flügel, 1967; Ulrich, 1969; Busson-Mabillot, 1973; etc.), relatively little information is available on the fine structure of the envelope immediately before and after egg activation (Lönning, 1972; Hagenmaier and Wilhelm, 1972).

In contrast to the well-known case of the medaka (Yamamoto, T., 1961), the salmonid egg does not show any morphological changes in activation when inseminated in an isotonic salt solution but is parthenogenetically activated by mere contact with tap water (Kanoh, 1950; Yamamoto, K., 1951). Furthermore, the egg envelope undergoes apparent physico-chemical changes immediately after cortical vesicle exocytosis (Kusa, 1949a, b; Yamamoto, T. S., 1957a; Zotin, 1958). In order to obtain a clue to the underlying mechanism of activation peculiar to salmonids, the fine structure of the envelope in the chum salmon eggs was studied. In addition, the amino acid composition constituting the envelope was analyzed.

Materials and Methods

Females of the chum salmon, *Oncorhynchus keta*, were obtained from the Hokkaido Salmon Hatchery at Chitose, Hokkaido. Ripe eggs were immediately stripped from the females into glass vessels and stored at 1°C. After thorough washes in the Salmon-Ringer solution (Yamamoto, T. S., 1976), parts of these eggs were used for the study of unactivated eggs. The remaining eggs were immersed in tap water to induce parthenogenetic activation (Kanoh, 1950; Yamamoto, K., 1951). Those eggs which were kept in tap water for 5 and 120 minutes at 20°C

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were used for the observation of activated eggs.

The observations were carried out mainly on the envelopes dissected from living eggs but sometimes whole eggs were also used. To obtain these envelopes, eggs were cut in the Salmon-Ringer solution by means of fine scissors or a sharp razor. Small pieces of the egg envelope were vigorously washed with several changes of the solution to remove contamination from the vitellus. Observations were made principally on the envelope from the animal hemisphere of the egg.

For scanning electron microscopy (SEM), dissected envelopes or whole eggs were fixed for 16 hours in 5% glutaraldehyde in a cacodylate buffer, pH 7.4, at 4°C. In the case of the whole eggs, the envelope was separated from the vitellus after the end of the fixation. They were rinsed in the same buffer and postfixed for 1 hour in 1% OsO_4 in the same buffer at 4°C. The specimens were dehydrated in ethanol, critical point dried in CO_2 and sputter-coated with gold. They were viewed in a JEOL JSM-T20 scanning electron microscope operated at an accelerating voltage of 20 kV.

For transmission electron microscopy (TEM), dissected envelopes or whole eggs were fixed for 16 hours in cacodylate buffered 5% glutaraldehyde which contained 2.5% sucrose. In the case of the whole eggs, the envelope with the underlying thin layer of the ooplasm was separated from the remaining mass of vitellus in the fixative. They were rinsed in the same buffer and postfixed in cacodylate buffered 1% OsO_4. The specimens were dehydrated in a graded acetone series and embedded in Epon 812 (Luft, 1961). Thin sections were cut and mounted on collodion-coated copper grids. After staining in uranyl acetate and lead citrate (Reynolds, 1963), they were examined in a JEOL JEM-100S electron microscope operated at 60 kV.

The periodic acid-chromic acid-silver methenamine (PA-Cr-Ag) method was employed for the detection of polysaccharides in thin sections (Rambourg et al., 1969). The material was fixed in glutaraldehyde but no postfixation in OsO_4 was carried out. Thin sections mounted on collodion-coated stainless steel grids were passed successively through 1% periodic acid (20 min.), redistilled water (30 min.), 10% chromic acid (5 min.), 1% sodium bisulfate (1 min.) and redistilled water (30 min.). They were then treated with a silver methenamine reagent for 30 minutes in a dark at 60°C and rinsed in redistilled water. In the control preparation, sections were treated with the silver methenamine reagent without the previous immersions in periodic and chromic acids.

In the amino acid analysis, envelopes dissected from the egg were thoroughly washed in the Salmon-Ringer solution and in deionized water. Particular care was taken to avoid the contamination of the vitellus. The envelopes were then lyophilized and weighed. About 2.5 mg of the lyophilized envelope was placed in an evacuated sealed tube with 10 ml of 6 N HCl and hydrolyzed in an oven at 110°C for 24 hours. The hydrolyzate was lyophilized again and dissolved in 1 ml of 0.01 N NaOH. It was kept for 4 hours at 20°C in order to convert cysteine into cystine. After neutralization with 0.1 N HCl the solution was

diluted with redistilled water to a final volume of 10 ml. An aliquot (10 μl) of it was taken for an analysis using a Hitachi 835 high speed amino acid analyzer.

Results

In the light microscopic observation, the outer and inner surfaces of the unfixed egg envelope are smooth in appearance. After staining with 0.4% methylene blue, however, the outer surface shows a particulate structure which is more distinct when observed in tap water than in the Salmon-Ringer solution (Fig. 1). The structure is easily removed by a slight rubbing of the outer surface with the fingers. Since it is not observed on the outer surface near the micropylar apparatus, the animal pole is easily distinguished from other regions of the egg (Fig. 2).

In SEM, a fibrillar layer is found on the outer surface of the envelope (Fig. 3) and removed by a slight rubbing of the surface with the fingers. It is not detected in the vicinity of the vestibule of the micropylar apparatus (Fig. 4). These observations indicate that the fibrillar layer is no other than the particulate structure of the outer surface seen in light microscopy. In the vicinity of the micropylar apparatus, an underlying homogeneous layer of the envelope is exposed (Fig. 4). This homogeneous layer bears a number of small knobs on the surface; about 60 knobs per 100 μm^2 area are counted. In contrast to the fibrillar layer, the knobs of this region are not removed by a rubbing with the fingers.

The inner surface of the envelope is not homogeneous when viewed by SEM but shows a texture consisting of intertwisted fibers (Fig. 5). There are pores on the texture; they are distributed uniformly over the inner surface (60–65/100 μm^2), and contain minute cytoplasmic clots, particularly when the removal of the vitellus is insufficient (Fig. 5). Each fiber constituting the inner surface shows branching and is about 0.4 μm in diameter at the thicknest portion.

The unfixed envelope is semitransparent in the Salmon-Ringer solution. However, it becomes transparent after immersion in 5×10^{-3} N HCl. At the same time, the envelope increases in adhesiveness, and sticks on the bottom of the petri dish. Immediately after these changes, a considerable swelling occurs in the inner region of the envelope dissected from unactivated eggs but not from activated ones. With a further immersion, the envelope of unactivated eggs dissolves leaving only an outer transparent film. No dissolution is observed in the envelope dissected from activated eggs. These observations indicate that the solubility in HCl of the envelope is eliminated during the first 5 minutes of the eggs' immersion in tap water; at the same time, the cortical vesicle exocytosis occurs all over the ooplasmic surface of the egg. A similar solubility change is also induced by mere immersion in deionized water of the envelope dissected from unactivated eggs; in this case, however, the change is only recognized after immersion for more than 10 hours.

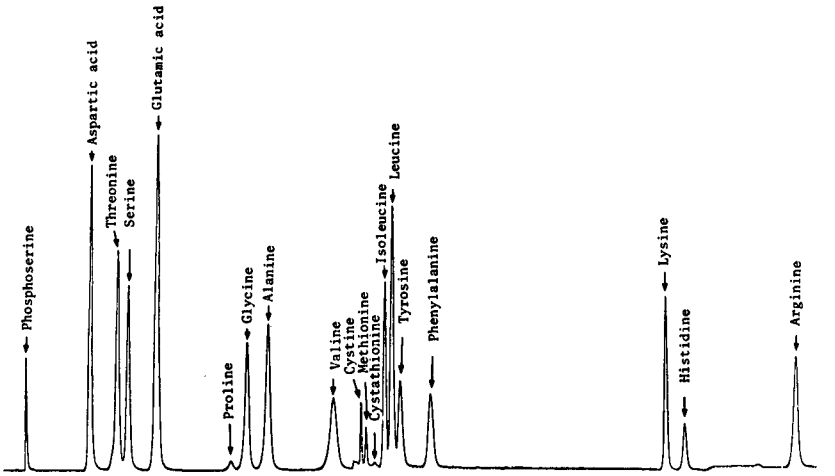
Transverse sections of the envelope reveal the presence of three concentric layers showing different fine structure; the outer fibrillar, intermediate homogeneous

and inner fibrous layers (Figs. 6 and 7). The outer fibrillar layer shows a moderate electron density and is easily removed after slight rubbing of the envelope with the fingers. It is 0.1–0.5 μm thick on the animal hemisphere of the egg but thickens gradually toward the vegetal hemisphere. The thickness of the layer attains about 2 μm at the vegetal pole. The fibrillar layer is not detected on the restricted area of the egg surface near the micropylar apparatus. With the PA-Cr-Ag technique an intensely positive reaction is obtained in this layer (Fig. 8). Therefore, the fibrillar layer is polysaccharide in nature. In 5×10^{-3} N HCl, a marked swelling of the layer is observed (Fig. 9).

The homogeneous intermediate layer shows a high electron density. It is not uniform in thickness but thickens considerably at intervals (Fig. 6); in the thinnest portion, it measures about 0.1 μm. In sections, the thickened portion of the intermediate layer appears as if the layer bears conical-shaped bodies of a high electron density on its inner surface (Fig. 7). Since the fibrillar outer layer does not exist on the region near the micropylar apparatus, the intermediate layer constitutes the outer border of the envelope in this region, where the thickened portions of the layer are visible as knobs in SEM of the outer surface. A moderately positive reaction for the polysaccharide test is observed in the layer (Fig. 8). In 5×10^{-3} N HCl, the intermediate layer swells markedly with the disappearance of conical-shaped bodies (Fig. 9).

The inner fibrous layer of the envelope is directly faced to the ooplasm and shows a moderate electron density (Fig. 6). The thickness of the layer is relatively uniform and measures about 50 μm except in the region near the micropylar apparatus where it is about 30 μm. Intertwisting fibers constituting the layer are distinct in the region near the ooplasmic surface but lose their individuality and form a more or less continuous sheet in the outer portion. The inner layer is characterized by the presence of a number of radial canals; they perforate through the entire width of the layer and contain microvilli of the ooplasmic surface in unactivated eggs (Figs. 10 and 11). These observations indicate that pores observed in SEM of the inner surface (Fig. 5) are no other than the inner openings of radial canals. At the outer opening of each canal, there is a thickening of the intermediate layer; it appears as if the opening is plugged by a conical-shaped body of electron dense, homogeneous materials (Fig. 7). A weak positive reaction for the polysaccharide test is seen in the fibrous constitutent of the inner layer (Figs. 8 and 12). In the envelope dissected from unactivated eggs, the inner one fifth or third of this layer swells markedly and dissolves in 5×10^{-3} N HCl, whereas the remaining parts show no change (Fig. 13). This fact indicates that the inner soluble region of the layer differs in chemical constitution from the outer portion. No regional difference is, however, appreciable after cytochemical detection of polysaccharides. Although the soluble property of the inner region disappears in the envelope dissected from activated eggs, any morphological change of the structure is not detected in the envelope itself following the egg activation, yet no microvilli are to be seen in the lumen of radial canals.

A lyophilized envelope measures about 3.6 mg, which constitutes about 5.0 weight % of a lyophilized whole egg. The chromatogram of the amino acid analysis of the protein forming the envelope is shown in Text-fig. 1. At least 19 kinds of amino acid were found during the analysis and account for about 90 weight % of the lyophilized envelope. The ratio of the residues and the weight of each amino acid in the envelope are shown in Table 1. No change in amino acid composition of protein forming the envelope is detected after the parthenogenetic activation of the egg. In order to obtain evidence of the regional differentiation in the envelope, the amino acid analysis was performed on the HCl-soluble fraction of the inner layer. The envelopes dissected from unactivated eggs were immersed in 5×10^{-3} N HCl for 30 minutes, and the dissolved fraction was served as the sample. Although the same kinds of amino acid are detected in this fraction, the ratio of residues slightly differs from that in the whole envelope (Table 1). It should be emphasized that the ratio of cystine/cysteine to other amino acids in the HCl-soluble fraction is apparently different from that found in the case of the whole envelope.



Text-fig. 1. Chromatogram of the amino acid analysis of protein forming the egg envelope.

Discussion

The demersal egg of teleosts is equipped with a specialized apparatus which seems to be useful for the embryonic development in natural circumstances (Stahl and Leray, 1961; Götting, 1967). An adhesive layer or "Zottenschicht" enclosing the cyprinid egg (Arndt, 1960) and filamentous threads protruding from the vegetal surface of the medaka egg (Yamamoto, T., 1961) are the examples of such apparatus. Flügel (1967) described a jelly layer on the outer surface of the salmonid egg; it seems

Table 1. Amino acid composition in the envelope of chum salmon egg

Amino acid	Whole envelope				HCl-soluble fraction[4]
	Residues[1]		Weight[2]		Residues[1]
	unactivated	activated[3]	unactivated	activated[3]	
Phosphoserine	13	14	17.15±2.91	19.90±5.70	14
Aspartic acid	73	74	71.42±5.03	66.87±3.55	80
Threonine	77	75	66.42±5.47	60.81±3.34	78
Serine	52	52	41.84±2.47	37.28±2.44	63
Glutamic acid	138	138	110.51±9.09	111.94±6.27	127
Glycine	53	54	29.06±2.08	27.56±1.35	67
Alanine	67	67	43.79±2.86	40.85±2.19	74
Valine	50	50	42.75±2.70	40.17±2.10	41
Cystine/Cysteine	9	8	15.51±0.35	12.67±3.16	5
Methionine	7	7	7.60±1.47	7.07±1.61	4
Cystathionine	1	1	0.91±0.05	0.89±0.09	1
Isoleucine	47	47	45.07±3.13	42.49±2.33	36
Leucine	75	75	72.21±5.06	67.50±3.66	66
Tyrosine	37	36	49.31±3.05	44.98±2.87	40
Phenylalanine	41	41	49.00±3.23	45.85±2.25	32
Lysine	41	41	43.60±3.43	40.78±2.20	41
Histidine	16	16	18.39±1.60	17.45±1.02	18
Arginine	48	49	61.42±5.12	59.17±4.70	58
Proline	155	155	110.51±9.09	111.94±6.27	155
Total	1000	1000	896.47±56.95	856.16±42.40	1000

1) Each value is expressed as residues per thousand total and the average of three experiments.
2) Each value is expressed as μg/mg of the lyophilized envelope and the mean±S.D. of three experiments.
3) The envelope was dissected from activated egg kept in tap water for 2 hours at 4°C.
4) The envelope was dissected from unactivated egg and immersed in 5×10^{-3} N HCl for 30 minutes. The dissolved fraction of the inner layer of envelope served as the sample.

to protect the *Salmo* embryo from bacterial infections (Hagenmaier and Wilhelm, 1972). As already observed by Aoki (1941b), the outer surface of *Oncorhynchus* eggs was covered with a particulate layer. It was transparent and stained with methylene blue. The layer is especially distinct in tap water and is removed after slight rubbing of the surface with the fingers. It is probable that the non-proteineous membrane described by Bell et al. (1969) on the outer surface of chum salmon eggs corresponds to the particulate layer observed in the present study. In sections, a layer of fibrillar material constituted the outer border of the *Oncorhynchus* eggs; it was easily removed after slight rubbing of the envelope with the fingers and increased gradually in thickness toward the vegetal pole of the egg. From the view of the site occupied by the fibrillar layer in the envelope, we suppose that

the layer is included in the transparent or hyaline layer described by Aoki (1941b). Similar to the jelly layer enclosing the *Salmo* egg (Hagenmaier, 1973), the fibrillar layer of the chum salmon egg was predominantly composed of polysaccharides. The adhesive layer of the envelope in *Clupea* and *Cichlasoma* eggs is particularly rich in polysaccharides (Yamamoto, T. S., 1957b; Busson-Mabillot, 1977). It is possible that the fibrillar layer of the envelope acts as an adhesive apparatus in the chum salmon egg, because the thickness increased as the strength of the adhesion on the substratum was enhanced in an acidic environment. In view of these facts, we prefer to use the term *adhesive coat* to designate the fibrillar layer which occupies the outermost region of the envelope in the *Oncorhynchus* egg.

In the electron microscopic observation on the formative process of the egg envelope in salmonid fishes, Flügel (1967) reported that the zona pellucida (radiata) of full grown oocyte consists of concentric two layers of different electron density, outer homogeneous externa and inner fibrous interna. The same structure of the envelope was also described in other freshwater fishes (Busson-Mabillot, 1973; Riehl, 1978; etc.). Judging from its electron density, the intermediate layer observed in the present study is no other than the zona pellucida externa. It swelled markedly in HCl but never dissolved. The layer was not uniform in thickness but formed conical-shaped bodies at intervals to plug the outer openings of the radial canals. According to Flügel (1964a, b, 1967), material of these bodies in the trout is secreted from follicle cells before ovulation. Although he assumed that these bodies acts as "reinforcements" of radial canals, their significance is obscure at present.

The inner fibrous layer of the egg envelope, zona pellucida interna, was directly faced to the ooplasm and perforated with a number of radial canals. The existence of canals in the envelope is common in the growing oocytes of various teleosts including the trout (Flügel, 1967). In many species of freshwater fish, however, they disappear from the interna as the time of ovulation draws near (Yamamoto, M., 1964; Busson-Mabillot, 1973; etc.). It is however not the case in salmonid and zebra fish eggs (Hisaoka, 1958; Hurley and Fischer, 1966; Ulrich, 1969; Hart and Yu, 1980). It is of interest that the ripe eggs with the canals are parthenogenetically activated by mere contact with tap water.

During the oogenesis, the interna of the envelope is composed of alternating strata of different electron densities (Lönning, 1972; Busson-Mabillot, 1973; Manner et al., 1977; Dumont and Brummet, 1980); such an appearance of the interna is in many teleosts maintained even in the envelope of ripe eggs. In the ovulated *Oncorhynchus* eggs, the interna did not show the concentric strata, though a regional difference suggesting the presence of strata was appreciated in the chemical composition of the regions. As reported by Aoki (1941a), the inner region of the interna swelled and dissolved in HCl ("swelling layer" by Aoki), whereas the outer portion remained unchanged; each fiber constituting the interna was distinct in the HCl-soluble region, whereas it is indistinct in the insoluble portion. Furthermore, the amino acid composition of protein forming the HCl-soluble region of the interna

apparently differs from that of the whole envelope. Recent experiments with cytochemical and autoradiographic techniques have provided evidence that the zona pellucida interna of the medaka egg is a product of secretion from the oocyte itself but not from the follicle cells (Tesoriero, 1977, 1978). It is plausible that the stabilizing process of the egg envelope is initiated in the outer region of the interna and proceeds inward. The retarded stabilization in the interna of chum salmon eggs might allow the swelling and dissolution of the inner region in HCl.

Various physico-chemical changes of the envelope have been observed following activation of fish eggs; relatively soft envelope of unactivated eggs in salmonid and medaka becomes tough after cortical vesicle exocytosis (Kusa, 1949a, b; Nakano, 1956; Zotin, 1958). The acid-soluble property of the inner region of the interna of unactivated *Oncorhynchus* eggs disappears from the envelope of eggs activated in tap water (Aoki, 1941a). According to Yamamoto, T. S. (1957a), successive treatments with acid and pancreatin digest the envelope of unactivated eggs but not that of the activated eggs of the chum salmon; the difference in susceptibility to the treatments indicates the occurrence of changes in chemical property of the envelope which are induced by substances released from the egg immediately following activation. In the present study, the acid-soluble property of the inner region of the interna disappeared when the envelope dissected from unactivated eggs was kept in deionized water for more than 10 hours. Perhaps the process of stabilization in the interna spontaneously occurs in deionized water. Ohtsuka (1960), studying the underlying mechanism of the hardening of the envelope in medaka eggs, found that various oxidizing agents can induce an increase in rigidity of the envelope. In accord with his findings, disulfide bridges built in the envelope are very easy to reduce in the unactivated eggs but are fairly stable after fertilization of rainbow trout eggs (Hagenmaier, 1973). Zotin (1958) suggested presence of "membrane-hardening enzyme" in the perivitelline fluid of salmonid eggs. It is therefore plausible that the changes in the envelope observed following the activation of fish eggs are an enzyme-mediated process of the oxidation. If this holds true, then a change in the molecular configuration of the interna leading to the stabilization of the envelope might also be induced by oxygen dissolved in deionized water without participation of the "membrane-hardening enzyme".

According to the classical findings of Young and Inman (1938) and Young and Smith (1956), protein forming the envelope of salmonid eggs is classified as pseudo-keratin. In a thin layer chromatography of the constituents of envelope from medaka eggs, Ogawa and Ohi (1968) detected 13 kinds of amino acid and galactose. The cytochemical test of the envelope revealed that a major component of the adhesive coat in the *Oncorhynchus* egg is polysaccharides. Although Hamano (1949) suggested a mucoidal nature of the interna, the carbohydrate contents of zona pellucida was rather small; it was predominantly composed of protein which had nearly the same amino acid composition as that reported for the envelope of other teleost eggs (Steudel and Osato, 1923; Young and Inman, 1938; Young and Smith, 1956).

Summary

The egg envelope of the chum salmon, *Oncorhynchus keta*, was found to consist of three concentric layers; adhesive coat and zona pellucida externa and interna. The adhesive coat constituted the outer border of the envelope and increased in thickness toward the vegetal pole of the egg. It was composed of fibrils having a moderate electron density which swelled in acidulated circumstances. An intense positive result of the polysaccharide reaction was obtained in this coat. The zona pellucida externa was the intermediate layer of the envelope and showed a high electron density. It was homogeneous in appearance and considerably thickened at the outer openings of the radial canals which traverse the entire width of the zona pellucida interna. The externa showed a moderately positive reaction for the polysaccharide test and swelled markedly in HCl but never dissolved. The zona pellucida interna was the layer directly faced to the ooplasm. It was fibrous in constitution and showed a moderate electoron density. A number of radial canals traversed the interna. The lumen of each canal was invaded with microvillus protruding from the ooplasmic surface in unactivated eggs but not in activated ones. A weak reaction for the polysaccharide test was obtained in fibers constituting the interna. In HCl, the inner region of the interna swelled and dissolved in the case of envelopes dissecting from unactivated eggs but not from activated eggs.

In the analysis of protein forming the envelope, 19 kinds of amino acid were detected. No appreciable change in amino acid composition of the envelope was recognized following the egg activation. The ratio of residues in the HCl-soluble fraction of the interna varied from that found in the whole envelope. The solubility change in the interna was discussed in relation to the stabilization of the envelope after egg activation.

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Explanation of Plates I, II

Figs. 1 and 2. The outer surface of an unfixed envelope dissected from the unactivated egg and stained with methylene blue in Salmon-Ringer solution. Fig. 1, equatorial region. (Bar=50 μm); Fig. 2, animal pole. (Bar=50 μm). MP, micropylar apparatus.

Figs. 3 and 4. SEM of the outer surface of an unactivated egg showing the fibrillar layer of the envelope. Fig. 3, equatorial region. (Bar=5 μm); Fig. 4, a region near the micropylar apparatus. (Bar=5 μm). Note the absence of the fibrillar layer in the vicinity of the micropylar apparatus (MP) where the outer surface of underlying intermediate layer with small knobs (K) is seen.

Fig. 5. SEM of the inner surface of the egg envelope showing the texture consisting of fibers. Note the inner opening of the radial canal (RC). (Bar=2 μm). C, cytoplasmic clots.

Figs. 6 and 7. Transverse sections through the dissected envelope from the unactivated egg. Fig. 6, equatorial region. (Bar=10 μm). Fig. 7, higher magnification of the outer portion of dissected envelope. Thickenings of the intermediate layer (IM) forming conical-shaped bodies (CB) are obvious. (Bar=1 μm). F, fibrillar layer; IN, inner layer.

Fig. 8. Electron micrograph of a section stained with PA-Cr-Ag method for the detection of polysaccharides in the envelope. (Bar=0.8 μm). F, fibrillar layer; CB, conical-shaped body.

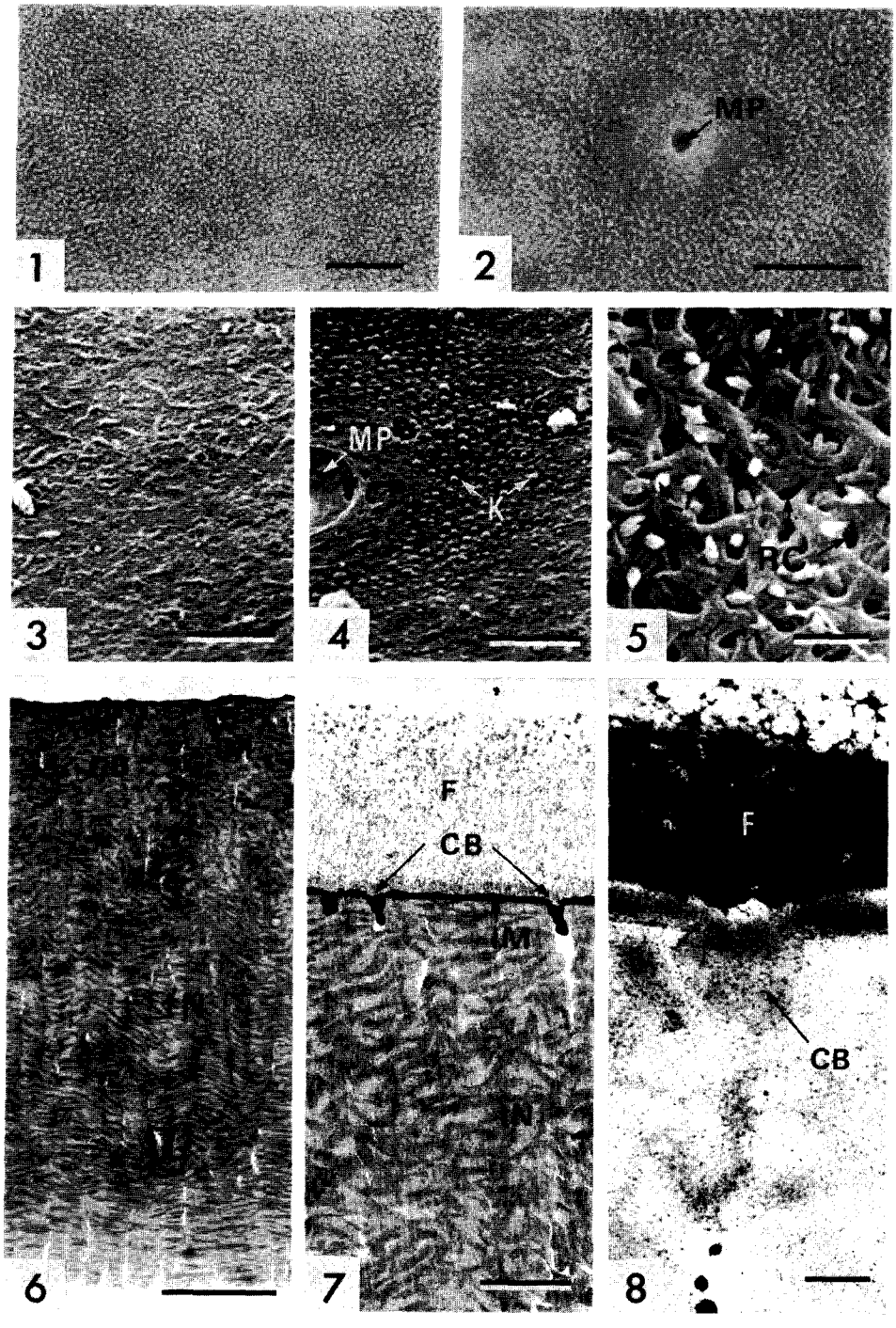
Fig. 9. Transverse section of the envelope dissected from the unactivated egg and immersed in 5×10^{-3} N HCl for 15 minutes. Note marked swellings in the fibrillar and intermediate layers. Thickenings (conical-shaped bodies) of the intermediate layer are not visible. (Bar=1 μm). IN, inner layer.

Fig. 10. A longitudinal section through the radial canal traversing the inner layer (IN) of the envelope. The canal lumen is invaded with a microvillus (MV) of the ooplasmic surface. (Bar=0.5 μm).

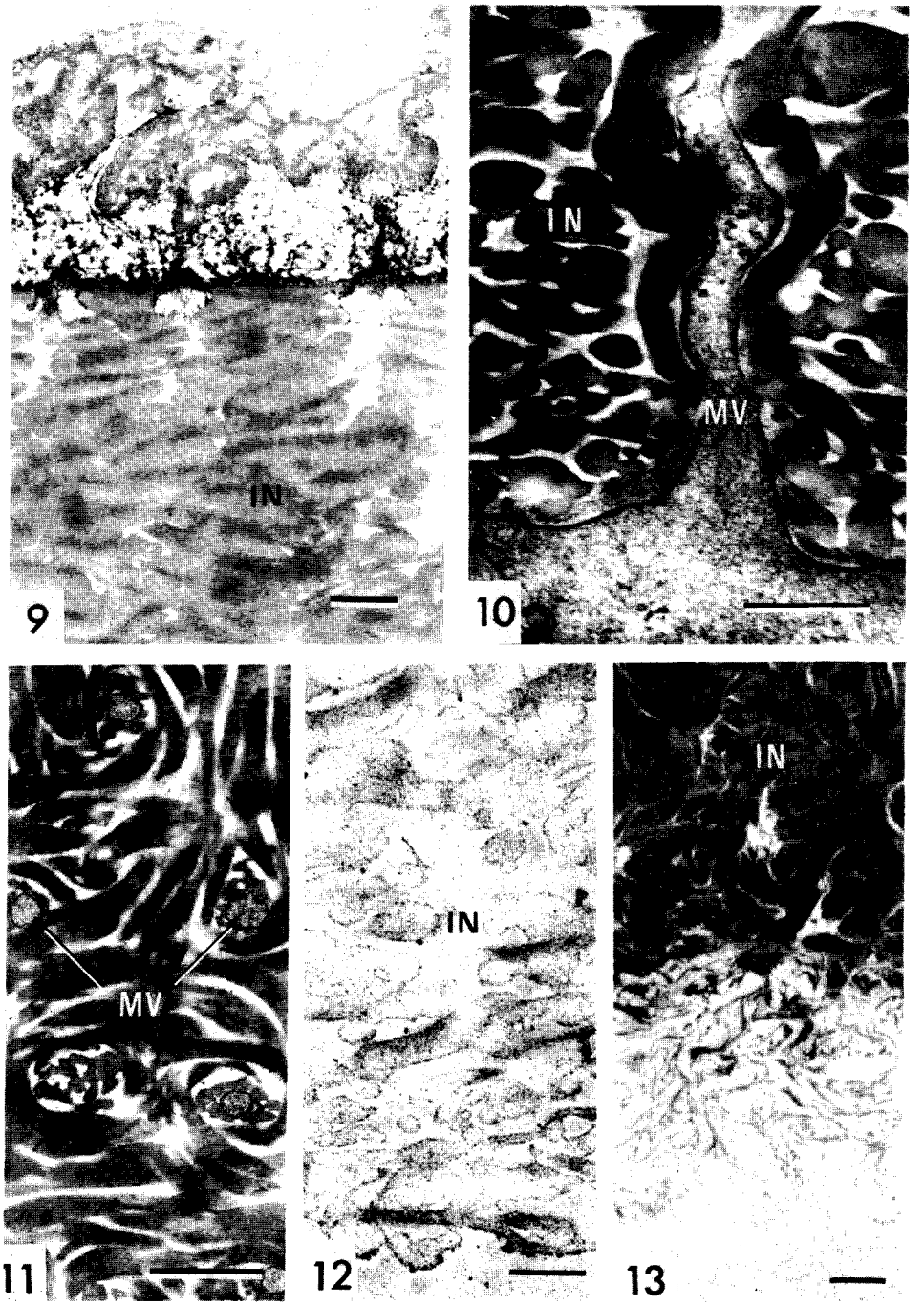
Fig. 11. A tangential section through the inner layer of the envelope showing microvillus (MV) contained in each radial canal. (Bar=1 μm).

Fig. 12. Section detected polysaccharides showing a weak positive reaction of the fibers constituting the inner layer (IN) of the envelope. (Bar=1 μm).

Fig. 13. A transverse section through the envelope dissected from the unactivated egg and immersed in 5×10^{-3} N HCl for 15 minutes showing a different susceptibility of the inner layer (IN) to acid. (Bar=1 μm).



W. Kobayashi: Fine Structure of Egg Envelope



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