FERTILIZATION IN BROWN ALGAE. I. SEM AND OTHER OBSERVATIONS ON *FUCUS SERRATUS*

MAUREEN E. CALLOW, L. V. EVANS, G. P. BOLWELL AND J. A. CALLOW

Department of Plant Sciences, The University, Leeds LS2 9JT, England

SUMMARY

The cell wall secreted immediately following sperm entry into an egg can be visualized by the fluorescent dye Calcofluor white. Cell wall secretion precedes nuclear fusion by 10–20 min. SEM observations of the surface of unfertilized and fertilized eggs and sperm attachment to eggs are described. These results are discussed in relation to fertilization in sea urchins and the biochemical phenomena associated with egg-sperm recognition in *Fucus*.

INTRODUCTION

Eggs of *Fucus* spp. are bounded only by a plasma membrane (Pollock, 1970; Matthews, Evans & Callow, 1976; Brawley, Wetherbee & Quatrano, 1976), but following fertilization there is rapid secretion of a cell wall. This has been detected by various methods such as treatment with zinc chloride (Pollock, 1970), plasmolysis and birefringence (Quatrano & Stevens, 1976). However, these methods are relatively insensitive since they depend on a substantial degree of structural and mechanical integrity having developed in the new cell wall. The first formed wall component is alginic acid which can be detected chemically within minutes of fertilization (Quatrano, personal communication). Cellulose becomes detectable after 1 h and from 4–24 h the composition of the wall is stable, consisting of alginic acid (60%), cellulose (20%) and sulphated fucan (20%) (Quatrano & Stevens, 1976).

In *Fucus*, sperm are attracted to eggs by chemotaxis (Cook, Elvidge & Heilbron, 1947/48). The conjugated octatriene fucoserraten has been isolated from *F. serratus* eggs (Müller & Jaenicke, 1973; Jaenicke & Seferiadis, 1975), but the attraction is not species specific (Müller & Seferiadis, 1977). Although, there do appear to be biochemical barriers to cross fertilization amongst *Fucus* spp. (Bolwell, Callow, Callow & Evans, 1977, 1978).

The structure of *F. serratus* spermatozoids has been described in detail (Manton & Clarke, 1950, 1951, 1955). The posterior flagellum is very elongated, greatly exceeding the Flimmer-bearing anterior one in length. The anterior end of the cell is prolonged into a flattened structure, the proboscis which possesses an interior framework of microtubules derived from a modified flagellar root. Spermatozoids attach to the egg surface by the anterior flagellum, leaving the posterior one free thus causing the egg to rotate (Thuret, 1854; Thuret & Bornet, 1878; Strasburger, 1897; Levring, 1947).

The present study is concerned with sperm attachment and the morphological changes in the egg surface which occur following fertilization in *F. serratus*. The timing of such changes is correlated with the timing of nuclear fusion. Since the term fertilization in the literature is widely used to refer to sperm entry rather than nuclear fusion *per se*, for uniformity fertilization will be used synonymously with sperm entry in this paper.

MATERIALS AND METHODS

Mature plants of *F. serratus* were collected from the East Coast of Yorkshire, England. Gametes were released as described by Callow, Coughlan & Evans (1978).

Calcofluor staining

To 5000 eggs, 2 cm^3 sperm suspension were added at a concentration of 2000 sperm per egg. At various times after gamete mixing, sperm were inactivated by addition of 20 μl of 0·2 % I_2 in 2 % KI. Seawater was pipetted off the eggs and a saturated solution of Calcofluor White ST in seawater added (American Cyanamid Co.). After 10 min the eggs were washed with 2 changes of seawater. Staining and washing procedures were performed at 0 °C. Cells were examined with a Reichert Diapan fluorescence microscope using exciter filters BG38 and UGI and barrier filter GG13 + W2B. Photographs were taken using the Reichert photo-automatic system on Kodak 35-mm Tri-X.

Scanning electron microscopy

Cells were fixed in 5 % glutaraldehyde in 0·1 M cacodylate buffer containing 0·25 M sucrose at pH 7·0. Dehydration was carried out using an ethanol series with 30-min changes (30, 50, 70, 85, .95, 100, and 100 %). Cells were pipetted into perforated Beem capsules lined with 10-μm nylon mesh and critically point dried from liquid CO_2 in a Polaron E3000 critical point dryer. The contents of the nylon mesh containers were shaken on to stubs covered with glue, coated with gold and examined in a Cambridge Stereoscan 600 electron microscope.

Assessment of nuclear fusion

Cells were fixed in freshly mixed glacial acetic acid/absolute ethanol (1:3, v/v) overnight and then transferred to 70 % ethanol for storage. Prior to staining the cells were washed in distilled water then in a dilute solution of ferric chloride. The cells were squashed in aceto-carmine and treated as described by Evans (1966) until the nuclei were stained dark red. The number of nuclei per cell was recorded, a minimum of 100 cells per sample being counted.

RESULTS

The difference in appearance between fertilized and unfertilized eggs after staining with Calcofluor can be seen in Fig. 3 A, B. Fertilization was detected within minutes of adding sperm to eggs and was essentially complete after 10 min (Fig. 2).

Repeated attempts to prepare stained nuclei using Feulgen reagent (Evans, 1966) for subsequent quantitation by microspectrophotometry all failed, presumably due to the presence of interfering substances (Jensen, 1962) or the frequent dispersion of DNA in egg nuclei (Ruthman, 1970). Thus, the method used to determine the timing of nuclear fusion was indirect. Fig. 1 shows the distribution of cells with different numbers of nuclei over a 60-min period after mixing eggs and sperm. After 10 min
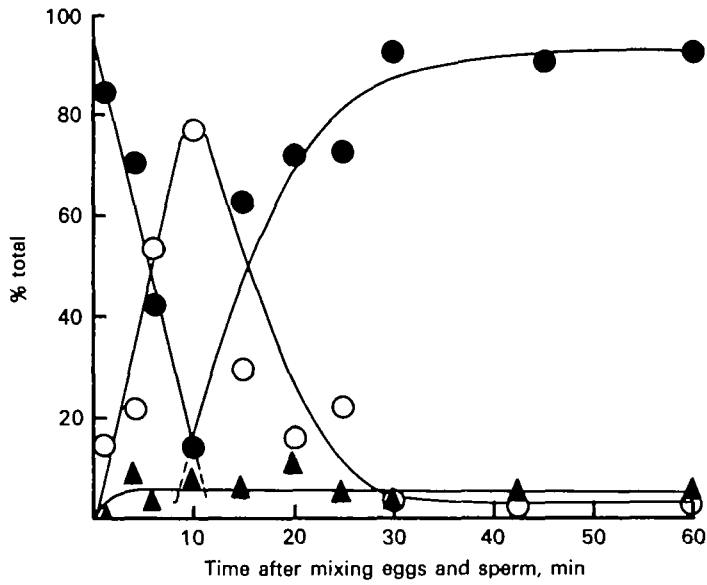
Fig. 1 Distribution of cells with 1 nucleus (●), 2 nuclei (○), and more than 2 nuclei (▲) per cell, at various times after mixing egg and sperm.
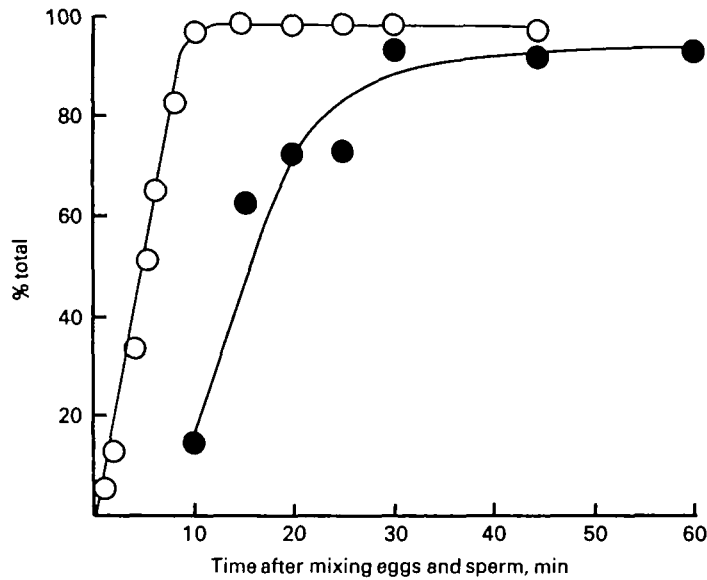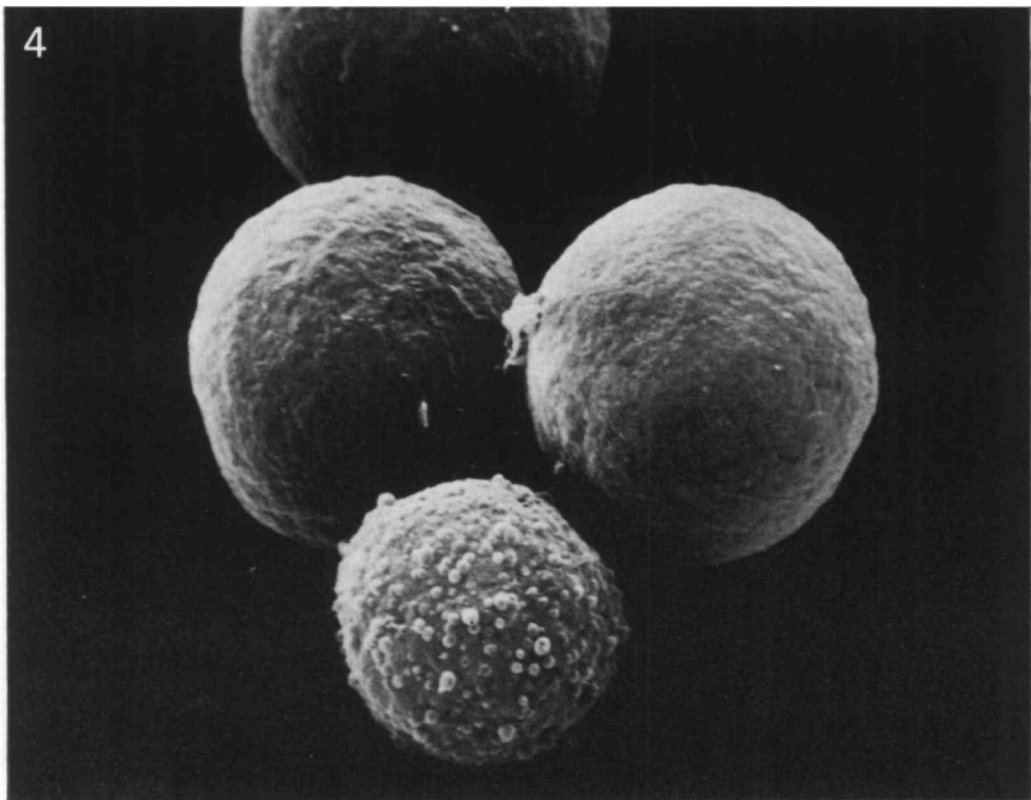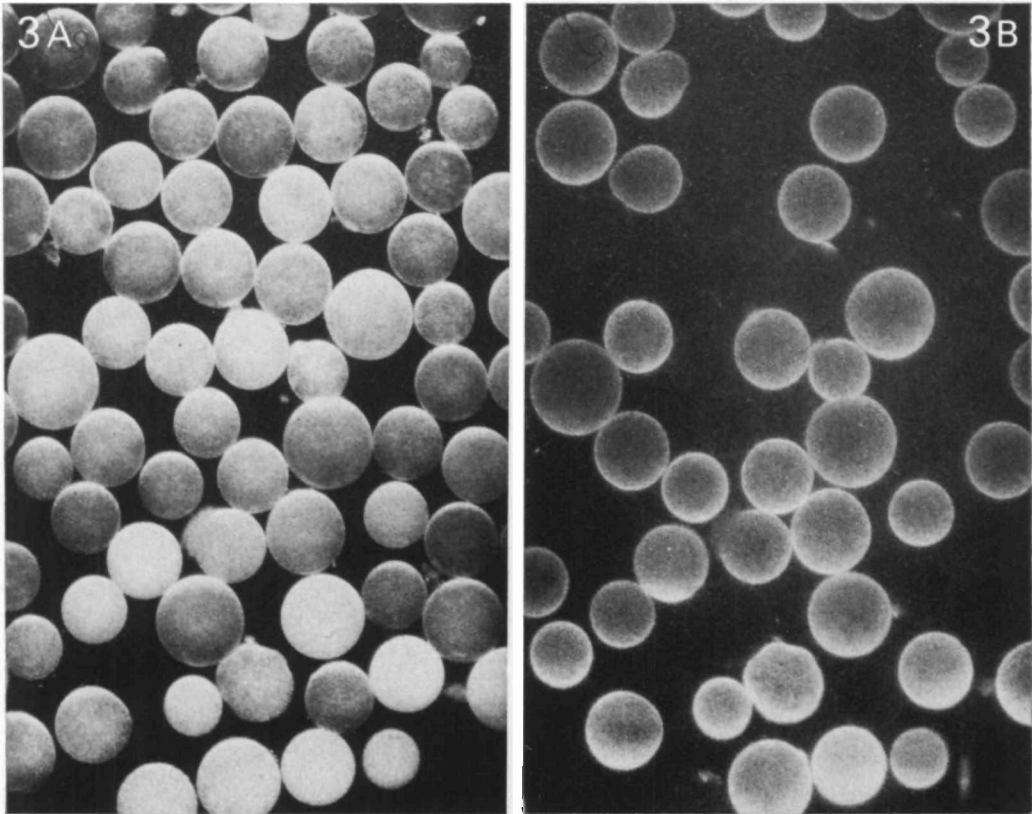

Fig. 2. Distribution of cells stained with Calcofluor (○) and with fused egg and sperm nuclei (●) at various times after mixing. Both sets of data were obtained from the same batches of egg and sperm. Each point is based on a minimum count of 100 cells.

the increase in the percentage of cells with 2 nuclei (1 egg + 1 sperm) corresponds well with the percentage of cells stained by Calcofluor. After 10 min the percentage of cells with 2 nuclei declines, with a concomitant increase in the percentage of cells with one nucleus. This increase in the percentage of cells with a single nucleus has been taken to represent those cells in which nuclear fusion has occurred. Fig. 1 also shows that in approximately 5–10% of cells more than one sperm can enter an egg, i.e. polyspermy may occur. The number of sperm nuclei in an egg varied from 2 to 8. Unfertilized eggs were occasionally (1 in 400) found to contain 2 or 3 nuclei.

Fig. 2 shows that sperm entry triggered the secretion of a cell wall as evidenced by Calcofluor staining, and this was essentially complete before nuclear fusion began. Calcofluor-stained cells invariably gave rise to segmented zygotes if cultured.

Scanning electron microscopy

The difference in the surface topography of unfertilized and fertilized eggs is shown in Fig. 4. The surface of the unfertilized egg is characteristically rough, due to protrusion of the cytoplasmic vesicles lying beneath the plasma membrane (Fig. 5). On sperm entry those vesicles which contain alginic acid presumably fuse with the plasma membrane thus discharging their contents by exocytosis (Fig. 6). The secreted cell wall is relatively smooth (Figs. 7–9) and its appearance does not change from 10 min to 16 h.

Variable numbers of sperm attach sufficiently firmly to the egg surface to be retained in intact form throughout specimen preparation. Entry of a sperm into the egg appears not to affect others which may be bound to the egg. The anterior flagellum of each sperm attaches to the plasma membrane and following fertilization the anterior flagellum of any attached sperm becomes encased in secreted wall material. The unattached sperm body and posterior flagellum remain on the surface of the secreted wall material (Figs. 8, 9). Agglutinated clumps of sperm are frequently found attached to the surface of unfertilized and fertilized eggs (Fig. 9).

DISCUSSION

Egg-sperm recognition in *Fucus* as in mammalian (Nicolson, 1974) and sea-urchin fertilization (Aketa, 1973) appears to be based on the association of surface-localized complementary macromolecules (Bolwell *et al.* 1977, 1978). However, there are many differences between the fertilization process in *Fucus* and, for example, sea urchins. *Fucus* eggs are naked cells, without vitelline membranes or jelly coats (Lillie, 1914) outside the plasma membrane. Although the presence of 2 layers external to the

Fig. 3. Cells of *Fucus serratus* stained with Caleofluor white ST (A) with barrier filters GG13 + W2B and (B) with barrier filters GG13 + W2B + exciter filters BG38 + UG1. In B only fertilized cells which are stained by Calcofluor fluoresce. ×95.

Fig. 4. Group of cells taken 10 min after mixing eggs and sperm seen in the SEM. Note the smooth surfaces of the fertilized cells and the rough surface of the unfertilized egg (lower). ×700.
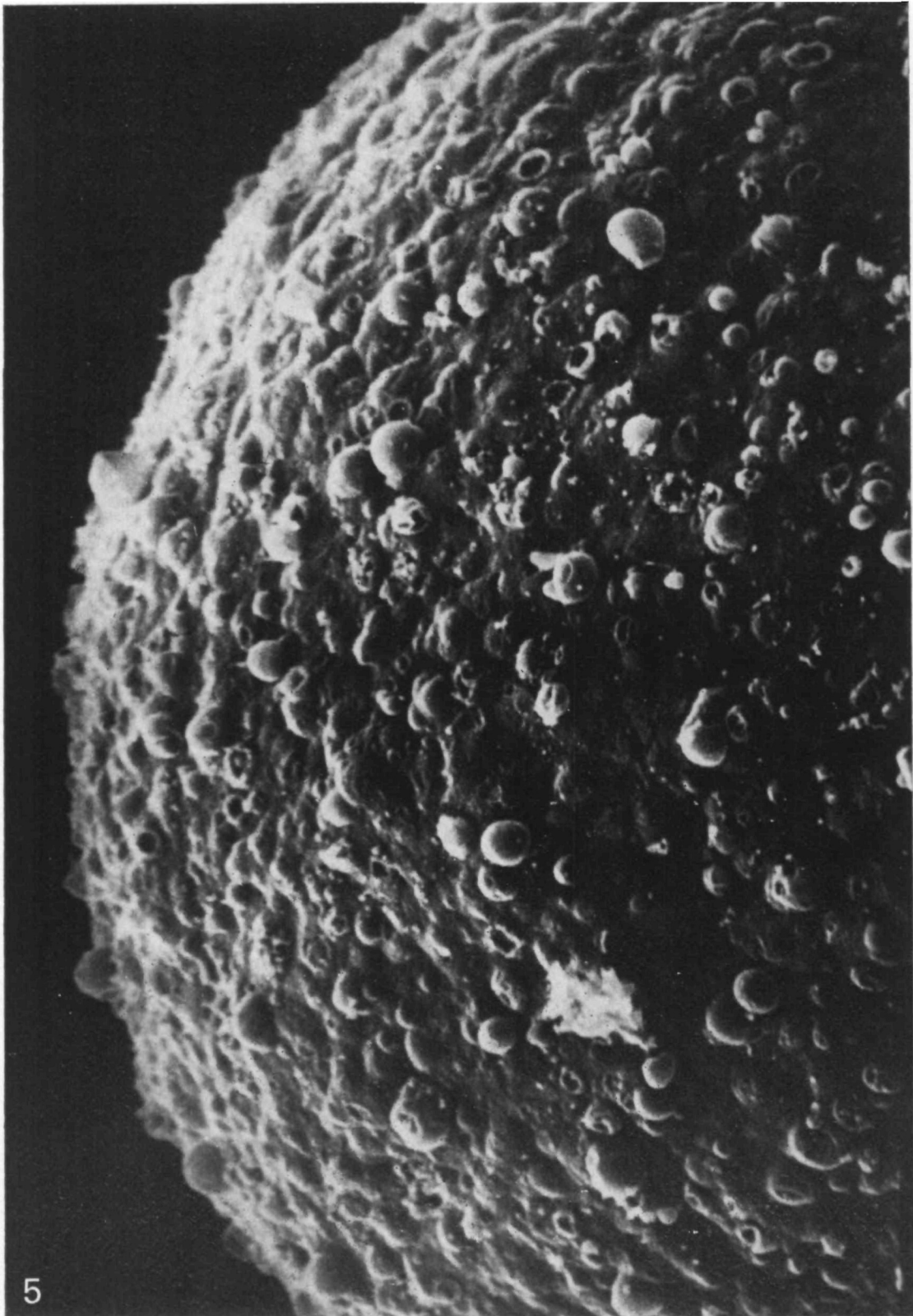
Fig. 5. SEM photograph of the surface of an unfertilized egg showing the irregularity due to the protrusion of cytoplasmic vesicles. × 3500.

plasma membrane in *Fucus* eggs, an 'egg membrane' and an outer gelatinous coat, have been described (Levring, 1952; Takamura, 1976) the present authors consider these to represent remnants of oogonial mucilages (see McCully, 1968) since repeated washing of eggs removes all traces of adhering mucilage.

In animals, sperm attachment and penetration is mediated through an acrosome reaction (see Monroy, 1965). Although the proboscis of the *Fucus* sperm could have some acrosomal function (see Manton, 1969) the initial attachment to the egg surface is through the anterior flagellum. In a detailed cinemicrographic study of *Ascophyllum*, Friedmann (1961) observed initial attachment to and subsequent penetration of the egg membrane by the tip of the anterior flagellum. Since sperm also attached to glass slides he postulated that a non-specific stickiness of the flagellar tip may establish the first contact between sperm and egg surface. Pollock (1970) noted a swelling at the tip of the anterior flagellum of *Fucus* and speculated on a possible acrosomal function. Although no particular role could be ascribed to the proboscis, Friedmann noted it was always pointed towards the egg surface. The observations reported here also show that sperm attachment is mediated through the tip of the anterior flagellum. Since current biochemical evidence is consistent with the presence of mannosyl and fucosyl residues on the egg surface being recognized by appropriate carbohydrate binding proteins on the sperm (Bolwell *et al.* 1978), and since attachment takes place by the flagellar tip, it is possible that the carbohydrate-binding proteins are located in this region of the *Fucus* sperm. The carbohydrate-containing residues on the egg may also be localized in discrete regions since there are a finite number of sperm-binding sites (Bolwell *et al.* 1978) and the sperm probes the egg surface with its anterior flagellum prior to attachment (Friedmann, 1961). Localization of the recognition sites on the egg surface is currently being investigated using labelled lectins.

Following fusion of the egg and anterior flagellar membranes it has been proposed that the body and then the posterior flagellum are drawn into the egg (Friedmann, 1962). At this stage wall material is rapidly secreted. Following sperm entry in sea urchins, cortical granules release material which is incorporated into the vitelline membrane to form the fertilization membrane (Endo, 1952, 1961 *a, b*). The fertilization membrane is rigid and prevents further entry of sperm into eggs (Eddy & Shapiro, 1976). The secreted wall in *Fucus* also performs the same function. The ultrastructure of nuclear fusion in *Fucus* has been described by Brawley *et al.* (1976) and the results presented here clearly show that this takes place after cell wall formation. Calcofluor white stains β-linked polysaccharides (Maeda & Ishida, 1967; Hughes & McCully, 1975) hence its affinity for the wall components rapidly secreted after fertilization, namely alginic acid (β1–3-linked) and cellulose (β1–4-linked).

The incidence of polyspermy recorded here appears high (5–10 %). This may be the result of a high sperm/egg ratio (2000 : 1), although polyspermy in *Fucus* has been observed previously (Yamanouchi, 1909). Whether the additional male nuclei degenerate or take part in multipolar spindle formation (Yamanouchi, 1909) is not known. Likewise the fate of nuclei in multinucleate eggs, also observed by Farmer & Williams (1896) is unknown.
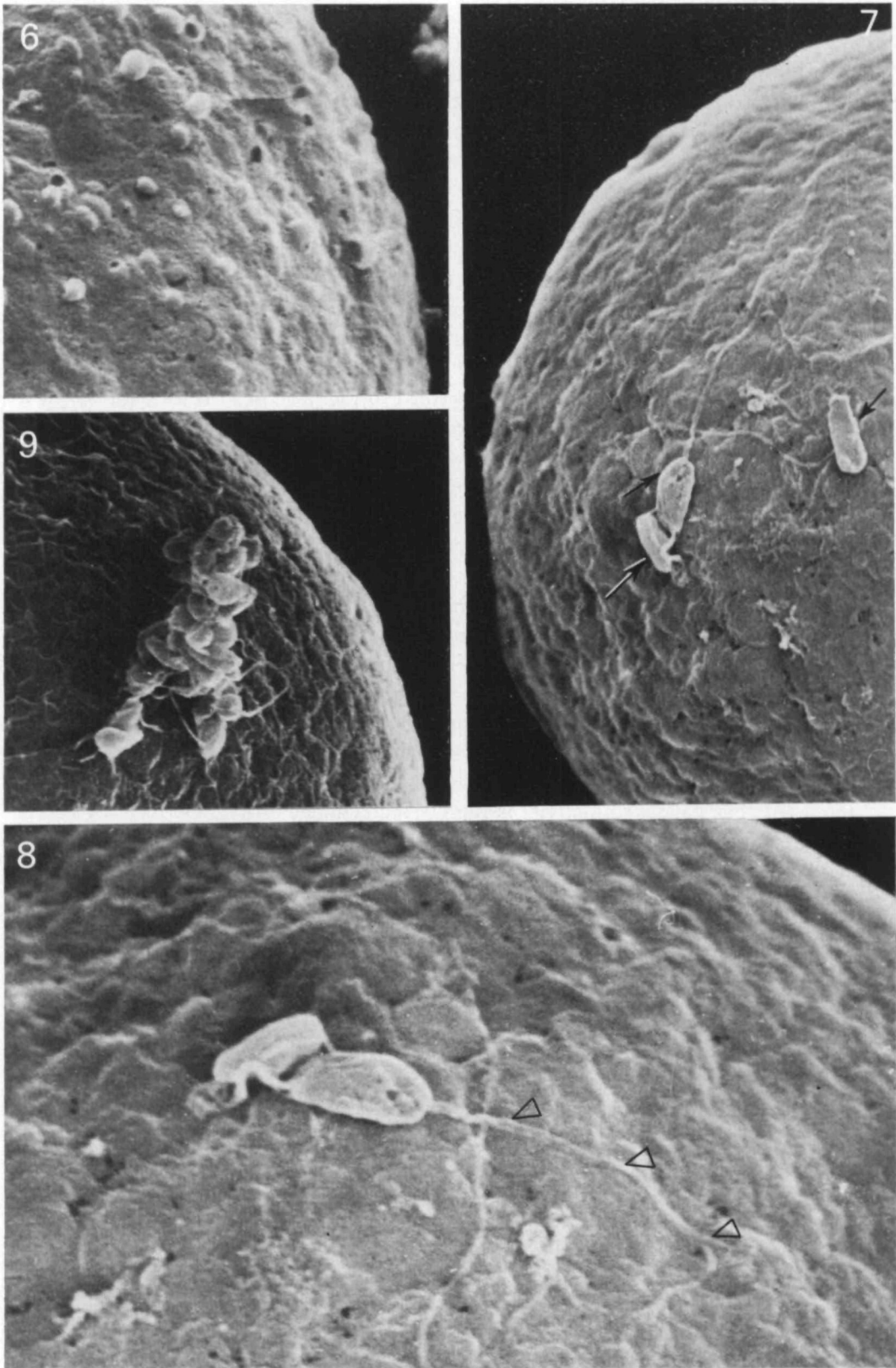
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Fig. 6. SEM photograph showing the surface of an egg in the process of secreting cell wall material, following sperm penetration. The protruding surface vesicles have largely disappeared. Cell fixed 4 min after mixing eggs and sperm. × 1400.

Fig. 7. SEM photograph of a fertilized egg, fixed 10 min after mixing egg and sperm. Note the uniformity of the cell wall and 3 attached sperm (arrows). × 2500.

Fig. 8. Enlarged view of Fig. 7 to show the curved anterior flagellum, its tip embedded in secreted cell wall material and the long posterior flagellum (arrowheads). × 5000.

Fig. 9. SEM photograph of group of agglutinated sperm attached to surface of fertilized egg. × 2500.

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