ULTRASTRUCTURAL AND IMMUNOCYTOCHEMICAL DEMONSTRATION OF GAMETOPHYTIC PROTEINS IN THE POLLEN TUBE WALL OF THE PRIMITIVE GYMNOSPERM CYCAS

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SUMMARY
Ripe pollen of the primitive gymnosperm Cycas armstrongii germinates 8–15 days after sowing on a suitable artificial medium. During the pre-germination interval, the tube cell of the tricellular male gametophyte is actively engaged in the synthesis and export of polysaccharide material. This polysaccharide is laid down as a continuous microfibrillar stratum between the tube cell plasmalemma and the pollen wall intine. While the polysaccharide layer is forming, numerous evaginations arise as cytoplasmic extensions from the tube cell and penetrate the component. The structural link between the cell and the evaginations is eventually lost and the isolated fragments of cytoplasm immediately break up to form discrete particles. These particles remain within the channels previously occupied by the evaginations.

At germination, the exine and intine layers of the pollen wall are disrupted and the tube cell emerges through the germinal aperture to establish the pollen tube. Polysaccharide deposition is resumed at about this stage, and consequently, a second microfibrillar layer begins to form beneath the first. The wall of the early pollen tube, therefore, has two distinct zones: the outer stratum is a loose superficial reticulum of fibrils and contains cytoplasmic inclusions; the inner stratum is structurally compact and is without inclusions.

Throughout the period of tube development, the presence of protein, including the enzyme acid phosphatase, can be demonstrated in the outer wall zone with cytochemical methods. Immunofluorescent staining using pollen antiserum immunoglobulin G in an indirect technique with fluorescein isothiocyanate-labelled secondary antibody shows that one component in the pollen tube wall is immunologically related to the male gametophyte, and results from immunoelectron microscopy support the conclusion that this consists of the inclusions rather than the polysaccharide fabric of the tube wall itself.

There are remarkable similarities between the cytoplasmic inclusions in the tube wall in Cycas and those found in the intine layer of flowering plant pollen. Both are of haplophase derivation, although the inclusions in the flowering plants are introduced via evaginations into the intine, the incipient pollen tube wall, at a much earlier stage in pollen development than they are in Cycas. The difference in the time of incorporation represents, perhaps, one more episode in the progressive evolutionary trend towards rapid pollen tube initiation and growth, and hence rapid fertilization, in spermatophyte pollen. This possibility is discussed, taking into account the view that the cycads and flowering plants are groups with a common ancestor.
INTRODUCTION

During the deposition of the polysaccharide intine layer of the pollen wall in flowering plants, proteinaceous inclusions are inserted into the layer as tangentially arranged lamellae or, more commonly, as radially disposed microvillus-like extensions of the gametophyte plasmalemma and peripheral cytoplasm. Where the process of protein incorporation has been followed, three separate phases can usually be distinguished: (1) deposition of the early intine without inclusions; (2) deposition of intine with included proteins; (3) termination of intine growth when the inclusions are sealed off by the formation of a continuous polysaccharide substratum. Significantly, in aperturate pollen (pollen that possess one or more specialized areas for germination) the principal concentration is in the region of these structures, while in inaperturate grains (grains without structurally circumscribed germinal sites) the protein is incorporated into the intine over the entire circumference of the grain (J. Heslop-Harrison, 1975; Y. Heslop-Harrison, 1977; Pacini & Juniper, 1979). The investigations of Knox & Heslop-Harrison (1970, 1971a), Knox (1971), Heslop-Harrison (1975, 1978 a) and Ashford & Knox (1980) have revealed some properties of the intine proteins. A number of hydrolases can be identified cytochemically, and high-resolution methods have clearly shown the activity of one of these, acid phosphatase, to be associated with the cytoplasmic inclusions. It was found, in addition, that while the hydrolases occupy the same general sites in the intine as mobile antigens, the enzymic activity in the extracted antigenic fraction is relatively low for the total amount of protein present. At the qualitative level, although there is remarkable uniformity among flowering plant species in respect of cytochemically detectable intine enzymes, immunological and electrophoretic techniques reveal considerable complexity and heterogeneity in the total proteins. These findings are taken to suggest that the catalytic moiety comprises only part of the protein located in the pollen intine and, given that there is no immunological difference between proteins having the same enzymic properties, that it does not contribute significantly to the total antigenic activity of the leachate.

Several experiments have shown that following pollination and after attachment of the pollen to the stigma the intine proteins are released from the wall as the grain hydrates. They flow out through the apertures of aperturate pollen or through the exine layers of inaperturate grains and reach the receptive stigma surface. Germination and penetration of the stigma by the pollen tube follow immediately in a compatible combination (Knox & Heslop-Harrison, 1971b; Heslop-Harrison, 1975). From a detailed study of this interval in the grasses it appears that, in these plants at least, protein is secreted at the tip of the pollen tube itself (Knox & Heslop-Harrison, 1970; Heslop-Harrison, 1979a; Heslop-Harrison & Heslop-Harrison, 1981).

A number of generalized functions has been proposed for the intine-borne proteins in the biology of the flowering plant pollen grain and it is believed that they have a part to play in, or are a special provision for, germination, early tube nutrition or stigma penetration (Heslop-Harrison, 1975, 1976, 1979b; Heslop-Harrison & Heslop-Harrison, 1981). However, a different, but not contrary, role has been con-
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sidered for the rather special situation found in the grasses. There are indications, consistent with what is known of the general working of compatibility reactions regulated by the sporophyte in flowering plants, that in this group the protein fraction localized at the pollen tube tip, which encounters the female receptive surface directly, could be implicated in pollen-stigma recognition (Heslop-Harrison, 1976, 1978a; Shivanna, 1979; Sastri & Shivanna, 1979).

The evidence summarized here has prompted an intriguing conjecture concerning the utility of the gametophytic proteins in the biology of flowering plant pollination: that their presence in the pollen wall was a necessary concomitant to the evolution of siphonogamy in the group (Heslop-Harrison, 1975, 1979b). This paper gives the results of a study of pollen germination in a primitive gymnosperm, which revealed the presence of proteins of haplophase derivation in the pollen tube wall.

MATERIALS AND METHODS

Pollen source

Ripe pollen of *C. armstrongii* Miq. was collected from the strobili of plants growing in the vicinity of Darwin, Northern Territory, Australia. The pollen was shaken from the cones, sifted to clean it of plant debris and insects, sealed in glass vials over silica gel and stored at -20 °C.

Pollen germination

*Cycas* pollen was germinated at room temperature on Tulecke's (1957) sterile yeast extract medium solidified with agar but with the following supplements: 500 mg penicillin G; 500 mg streptomycin sulphate; 48 × 10⁶ units nystatin (mycostatin) per 1 liquid medium. The grains were briefly rinsed in ethanol, surface-sterilized in 0.01% mercuric chloride and washed five times with sterile distilled water before sowing.

Preparation of pollen extract

Ungerminated *Cycas* pollen was homogenized in chilled 150 mM-sodium chloride containing 50 mM-Tris and 1 mM-calcium chloride (pH 8.4). The supernatant obtained from centrifuging the homogenate at 4 °C was dialysed at 4 °C against distilled water until free from sodium chloride, and freeze-dried. Analysis of the extract by sodium dodecyl sulphate (SDS)/polyacrylamide electrophoresis (Laemmli & Favre, 1973) after dissociation in 2-mercaptoethanol and SDS at 100 °C revealed a minimum of 13 bands staining with Coomassie Blue, suggesting that they mark the position of proteins. The spectrum is dominated by protein subunits with molecular weights between 14,000 and 45,000 (Fig. 1). Three of the components with molecular weights between 24,000 and 40,000 showed a faint reaction following periodic acid–Schiff staining for carbohydrates. This extract was used as the antigen without purification.

Antiserum production

Antiserum against the *Cycas* pollen extract, reconstituted to 2 mg/ml in 0.15 M-saline, was raised in rabbits by subcutaneous injection in the neck of 1 ml antigen with 1 ml Freund's complete adjuvant (first challenge) or incomplete adjuvant (subsequent challenges), following the schedule given by Knox & Clarke (1978). Antiserum obtained from the bleeds was distributed between several vials and either stored as solution at -20 °C or freeze-dried and stored at -20 °C until required.

The immunoglobulin G (IgG) fraction was prepared from the antiserum by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The packed column was stabilized with 0.1 M-phosphate buffer (pH 7.0) at 4 °C and the rabbit antiserum was applied, diluted 1:2 with buffer. Loading was controlled so that
the binding capacity for IgG (20 mg/ml gel) was not exceeded. Unbound material was washed through the column with 10 bed vol. of buffer and discarded. The bound material was eluted with 1 M-acetic acid and immediately dialysed against repeated changes of distilled water at 4 °C and freeze-dried. The IgG population of normal (pre-immune) rabbit serum was isolated by the same method.

**Immunodiffusion and immunoelectrophoresis**

The pollen extract was examined for antigenic components by immunodiffusion and immunoelectrophoresis using the corresponding antiserum. For immunodiffusion a medium of 1% agarose (Bio-Rad Laboratories, Richmond, California, U.S.A.) in 0.15 M-saline containing 0.02% (w/v) NaN₃ was plated onto microscope slides and the concentration of the antigen was 2 mg/ml (freeze-dried weight) in 0.15 M-saline. The immunoelectrophoresis medium was 1% agarose in Gelman high-resolution Tris/barbital/sodium barbital buffer (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.), I = 0.05, pH 8.5, on microscope slides, and the antigen was reconstituted to 2 mg/ml and immunoelectrophoresis run in the same buffer. A constant current of 2 mA per gel slide was applied for 3 h. Antiserum for both immunodiffusion and immunoelectrophoresis was reconstituted from freeze-dried material to 100 mg/ml in 0.15 M-saline. Four major antigens, three anodic and one cathodic, were consistently revealed by immunoelectrophoresis of the pollen extract using serum raised against the extract (Fig. 3). Four major bands were also obtained by immunodiffusion (Fig. 2). Control experiments in which pre-immune rabbit serum was used to replace the antiserum showed no precipitin bands.

**Immunofluorescent staining**

Pollen was removed from the growth medium and incubated for 30 min at room temperature in either the IgG preparation of the antiserum (test) or the IgG preparation of the pre-immune serum (control) reconstituted to 2 mg/ml IgG in 0.15 M-sodium chloride to 0.01 M-phosphate buffer, pH 7.4 (PBS). After incubation the pollen was washed four times with PBS and then incubated for 30 min at room temperature in fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG prepared in goat (FITC-goat anti-rabbit IgG; Miles-Yeda Ltd, Rehovot, Israel, lot no. S751), working titre, adjusted with PBS, 1:25 the concentration supplied. The pollen was washed four times with PBS and mounted on microscope slides in 1% glycerol/PBS. The preparations were examined by incident-light fluorescence microscopy using a Leitz Ploem illuminator equipped with FITC filter system 12 (exciter KP500 + 1 nm GG455; dichroic mirror TK510; suppression filter K515). Photomicrographic records were taken on Ilford HP5 film to a standard exposure so that the intensity of the fluorescence emission in the test and control preparations could be directly compared.

**Electron microscopy**

Germinating pollen intended for examination by transmission electron microscopy was fixed for 12–15 h at 4 °C in 2.5% glutaraldehyde in 0.1 M-sodium cacodylate-HCl buffer (pH 7.2), 450 mosm. The material was rinsed in three changes of the cacodylate vehicle, the osmolarity of which had been adjusted with sucrose to correspond to that of the fixative, and then post-fixed in 1% aqueous osmium tetroxide for 1.5 h at room temperature. Post-fixation was followed by dehydration through an acetone series and embedding in Spurr's (1969) resin mixture. Thin sections were cut on a diamond knife and stained with uranyl acetate and lead citrate for general enhancement of contrast.

For study by scanning electron microscopy, specimens were removed from the glutaraldehyde/osmium-treated material at the final stage of the dehydration sequence and dried by the critical-point method using carbon dioxide as the transitional fluid. The dried pollen was dusted onto small coverglasses, the surfaces of which had been covered with a very thin smear of Araldite, the coverglasses were attached to stubs and these were placed in an incubator at 60 °C until the resin had set. A coating of conducting metal was then applied.
Immunoelectron microscopy

The first incubation for both test and control was the same as for immunofluorescent staining and this was followed by washing the pollen six times with PBS over a period of 45 min with occasional vortexing. The grains were then incubated for 30 min at room temperature in ferritin-conjugated anti-rabbit IgG prepared in goat (ferritin-goat anti-rabbit IgG; Miles-Yeda Ltd, lot no. S630), working titre, adjusted with PBS, 1:50 the concentration supplied. After this incubation the pollen was again washed, with vortexing, six times with PBS over a period of 30 min and then processed for transmission electron microscopy according to the procedure given above. Thin sections were mounted on uncoated copper grids and either stained with methanolic uranyl acetate and lead citrate or examined without recourse to section staining. However, to increase the electron opacity of the ferritin detected at intermediate and high electron-optical magnifications, the unstained sections of test and control material were subsequently treated with bismuth subnitrate (Ainsworth & Karnovsky, 1972).

Cytochemical methods

Cytochemical methods were used to establish some characteristics of the Cycas pollen wall and pollen tube. Polysaccharides containing vicinal 
\( \alpha / \beta \)-glycol groups were localized with the standard periodic acid-Schiff (PAS) procedure (Pearse, 1968). Controls were not subjected to periodate oxidation. Protein was detected in pollen germinated in vitro by mounting the grains directly in the Coomassie Blue stain-fixing medium of Heslop-Harrison, Heslop-Harrison, Knox & Howlett (1973). The Coomassie Blue stain employed by Cawood, Potter & Dickinson (1978) was used to detect protein in pollen tubes penetrating the nucellus tissue of pollinated C. armstrongii ovules. Pollinated ovules were sliced and fixed in buffered glutaraldehyde. Slices having an undamaged micropyle and nucellus apex were selected for embedding in purified hydroxyethyl methacrylate (Bennett, Wyrick, Lee & McNeil, 1976), following the procedure of Feder & O'Brien (1968). Sections were cut at 2 \( \mu \)m on glass knives, mounted on coverglasses and examined by phase-contrast optics. Those in which germinated pollen could be seen were reserved for staining. Callose (=\( \alpha \) \( \beta \)-glucan) was detected in unfixed grains germinated in vitro by its characteristic fluorescence in 0.005% aniline blue in 0.15 M-potassium phosphate (decolourized aniline blue), and mixed \( \alpha \beta \)- and \( \beta \beta \)-linked glucans were revealed with the fluorescent whitener Calcofluor White MR2 New (Takeuchi & Komamine, 1978) in 0.001% aqueous solution.

Acid phosphatase activity was localized in unfixed grains germinated in vitro using naphthol AS-BI phosphoric acid dissolved in dimethylformamide (10 mg/ml) in a reaction mixture at pH 5.0 with hexazoitated paraarosanilin (Barka & Anderson, 1962). The reaction was stopped after 30 min by rinsing in water and fixation in buffered glutaraldehyde. Controls were: (a) the reaction mixture with the substrate omitted; (b) pollen pre-incubated in 0.01 M-NaF for 5 min before incubation in the complete reaction mixture containing 0.01 M-NaF. The pollen was rapidly dehydrated in ethanol, cleared in xylene and mounted in a permanent synthetic medium for examination by light microscopy.

For acid phosphatase localization by transmission electron microscopy, a modification of the Gomori lead-capture reaction was used (Bitensky & Cheyen, 1977). Unfixed, germinating pollen was incubated in the reaction medium for 1 h at room temperature, the medium was withdrawn, the pollen rinsed in several volumes of distilled water and then fixed in chilled 2.5% glutaraldehyde buffered in 0.1 M-sodium cacodylate-HCl (pH 7.2), 450 mosM. The conversion treatment with H\(_2\)S-saturated water, necessary when the procedure is for light microscopy (Bitensky & Cheyen, 1977) was omitted. Following the dialchyd fixation the grains were processed as described above. The thin sections were examined before and after staining in methanolic uranyl acetate and lead citrate. Methods for controls were the same as for the paraarosanilin coupling procedure.

Non-specific esterase activity was detected in unfixed germinating grains using \( \alpha \)-naphthyl acetate in a coupling reaction with Fast Blue B Salt (tetrazoitated \( o \)-dianisidine) at pH 6.5 (Pearse, 1972). The reaction was terminated by glutaraldehyde fixation after rinsing in water. Fixed grains were rinsed and mounted in polyvinylpyrrolidone medium (Burstone, 1977) for examination by light microscopy.
**Periodate oxidation of pollen tubes**

*Cycas* pollen germinated *in vitro* was suspended in 0.05 M aqueous sodium metaperiodate and shaken intermittently. Samples were withdrawn at 15, 30 and 60 min intervals, concentrated by centrifugation, washed six times with 0.1 M-phosphate buffer (pH 7.0) and incubated for 30 min at room temperature in the IgG preparation of the pollen antiserum containing 2 mg/ml IgG in PBS. The pollen was then washed four times with PBS, incubated in FITC-goat anti-rabbit IgG for 30 min at room temperature, washed four times with PBS and mounted in 1% glycerol/PBS for examination by incident-light fluorescence microscopy using the filter system specified above.

**Method with Lilium pollen tubes**

Ripe pollen of *Lilium regale* was successfully germinated on a freshly-prepared, semi-solid agar medium containing sucrose, calcium and boron (Medium A of Heslop-Harrison, 1979a) cast onto microscope slides. When the cultures showed a high percentage of grains with well-developed pollen tubes, the pollen was carefully harvested and incubated for 30 min at room temperature in either the IgG preparation of *Cycas* pollen antiserum or the IgG preparation of pre-immune rabbit serum reconstituted to 2 mg/ml IgG in PBS. The pollen was gently centrifuged to a pellet, the supernatant was removed and the pellet washed four times over 30 min with PBS and then incubated for 30 min at room temperature in FITC-goat anti-rabbit IgG. After four post-incubation washes with PBS the pollen was mounted in 1% glycerol/PBS and examined by incident-light fluorescence microscopy using Leitz filter system 12.

**Observations**

**Pre-germination stage**

The organization of the male gametophyte contained in the pollen grain of *Cycas*, ready for release from the sporangium but before pollination and germination, is presented diagrammatically in Fig. 4B and is the same in all species. The gametophyte is comprised of an axial row of three cells: (1) a prothallial cell, basally situated (or proximally with reference to the orientation in the original tetrad configuration), which is the cell farthest removed from the germinal site or sulcus; (2) a centrally placed generative (or antheridial) cell; (3) a large tube cell positioned distally, above the sulcus, and extending laterally past the equator of the grain. The three gametophyte cells are entirely surrounded by the pollen wall, the primary subdivision of which is into an inner polysaccharide layer, the intine, and an outer sporopollenin layer, the exine. The exine, in turn, is structurally differentiated and the thickness of the layer is reduced at the distal sulcus (Audran & Masure, 1976, 1977). In the normal course of events, after pollination, simultaneously with or following shortly upon emergence of the pollen tube, the generative cell divides, giving rise to a stalk (or sterile) cell and a body (or spermatogenous) cell. Two ciliated male gametes are ultimately produced by the body cell (Sterling, 1963).

Exposure of viable *C. armstrongii* pollen to suitable nutrients in an artificial medium results in germination of the grains 8–15 days after sowing. This behaviour is associated with a transition from hydration and simple expansion of the grains after 2–3 h as a consequence of imbibition, to emergence of the pollen tube through the pollen wall at the sulcus. Tube growth proper can be followed very soon after emergence
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Fig. 1. Protein pattern in SDS/polyacrylamide gel of C. armstrongii pollen extract revealed by Coomassie Blue staining. The spectrum is dominated by protein subunits with molecular weights between 14,000 and 45,000.

Fig. 2. Immunodiffusion gel of antigens from C. armstrongii pollen (top centre well) tested against anti-pollen serum (lower wells). The precipitin bands have been stained with Coomassie Blue.

Fig. 3. Immunoelectrophoresis of C. armstrongii antigen preparation (well) using antiserum raised against the preparation (trough). The anodic direction is to the right and the bands have been stained with Coomassie Blue.

and in vitro the process is rather short-lived. In the conditions of the experiment measurable activity ceased after a period of about 30 h; by this time the tubes were 100-150 μm in length, but the generative cell had not divided.

Electron-microscopic observations show that localized changes affecting the tube cell occur during the interval preceding germination. A stratum of loosely aggregated fibrils begins to form beneath the pollen wall intine. The microfibrillar content is not completely uniform in character and includes small compact masses, generally circular in profile, which exhibit greater electron contrast than the main component (Fig. 6). The microfibrils of the main component show no particular orientation and
Fig. 4. Diagrams of pollen germination in Cycas. A shows pollen in the pollen chamber of an ovule. The pollen tubes have penetrated the nucellus tissue thus securing the grains above the position of the future archegonia (represented by the flask-shaped unshaded area). B is the pollen grain at the time of release, before germination. The male gametophyte comprises three cells: a prothallial cell (pc), a generative cell (gc) and a tube cell (tc). The pollen wall has two principal strata, the exine (e) and the intine (i). C shows evaginations arising from the surface of the tube cell and penetrating the polysaccharide layer deposited beneath the intine. D is the aspect immediately before germination. The evaginations are now isolated inclusions in the polysaccharide layer. E represents the early germination stage. The exine and intine layers of the pollen wall have been ruptured and the tube cell has emerged to form the pollen tube. The outer zone of the tube wall contains the cytoplasmic inclusions. The various structures are not drawn to scale.

are not stratified. Polysaccharides are detectable cytochemically in the stratum. The polysaccharide material is deposited at the perimeter of the tube cell and soon forms an even and substantial covering over the entire surface. The distribution of the layer beneath the intine leaves little doubt that the tube cell alone is involved in production and export of the constituents. Sections show that the cytoplasm of this cell contains a category of membrane-limited vesicles of moderate size and with fibrillar, grey-staining contents (Figs. 5, 6). These are occasionally seen in fusion with the tube cell plasmalemma, an association indicative of discharge.

While the polysaccharide material is being laid down, evaginations, constituted from more or less perpendicular extensions of the plasmalemma and cortical cytoplasm, arise from the surface of the tube cell and penetrate the layer to the base of
Fig. 5. Pollen germination. The exine at the sulcus (outer, arcaded layer) is ruptured and the intine (compact fibrillar layer beneath exine) shows signs of structural disorganization. The thick fibrillar layer between the intine and the tube cell (left) contains numerous proteinaceous cytoplasmic inclusions and beneath this a second stratum is beginning to form. Membrane-bound vesicles are being expelled from the tube cell. × 22,000.
the intine (Figs. 4 c, 7–9). Conventional methods of preparation show a clear halo or, usually, a fenestrated zone in the fibrillar texture around each evagination (Fig. 9). Structural association between the cell and the evaginations is eventually severed, presumably by constriction and fusion of the plasmalemma across the base. However, in the period before this is effected a limiting membrane cannot always be resolved (Fig. 9), and after severance the evaginations do not remain as intact units, but immediately break up to form discrete electron-dense particles (Fig. 7). These particles take the form of spheres, cylinders or sheets, doubtfully membrane-enclosed, and they occur in the polysaccharide layer within the electron-lucent channels previously occupied by the evaginations (Figs. 5, 8, 35). The channels are sealed off below by an unpenetrated layer of the fibrillar component, clearly indicating that deposition of this continues for a time after dissociation of the evaginations (Fig. 35).

At the conclusion of the pre-germination stage, therefore, fragments of the tube cell cytoplasm are contained as inclusions in the matrix of a moderately thick polysaccharide layer that has developed between the tube cell plasmalemma and the pollen wall intine (Fig. 4 D).

Germination and characteristics of the pollen tube wall

The start of germination in Cycas pollen in culture is marked by rupture of the exine layer at the sulcus (Fig. 16) and first disorganization and then complete separation of the intine fabric beneath as the tube cell begins to emerge (Figs. 5, 35). Only viable grains proceed to this stage and in the conditions of the experiment the opening of the germinal aperture is caused by growth of the tube cell. The circumstances suggest that it is purely a physical accomplishment, attributable to simple mechanical stress in consequence of tube cell growth, rather than a process requiring enzymic degradation of the pollen wall layers. Once the determinative stage is reached, there is no extended interphase between pre-germination and germination, and as the tube cell enters the aperture, deposition of polysaccharides is resumed. The relative timing of the various events in the developmental sequence at this stage is difficult to establish and is clearly dependent on adequate sampling. There may not, in fact, be any interval separating pre- and post-germination polysaccharide deposition, which may be a continuous process. The new structure is microfibrillar in aspect and is characterized by having parallel microfibrils arranged more or less tangentially in relation to the

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Fig. 6. Pre-germination. Microfibrillar polysaccharide material is being laid down beneath the intine layer of the pollen wall. Notice that the material is substructurally heterogeneous. × 16 000.

Figs. 7–9. Pre-germination. Evaginations arise from the surface of the tube cell and penetrate the new fibrillar material to the base of the intine. Fig. 9 shows an evagination still connected to the tube cell and Figs. 7, 8 illustrate how the evaginations break up once the connection is severed. There is a lucent halo or fenestrated zone in the fibrillar matrix around each evagination and the particles formed from the evaginations occur in these channels. Figs. 7, 8, × 40 000; Fig. 9, × 60 000.

Fig. 10. Early germination. The tube cell has emerged through the germinal aperture to form the pollen tube initial. × 3000.
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plasmalemma. However, it grades in to the previously formed stratum above and material is incorporated at a constant rate as the tube cell proceeds through the aperture (Figs. 5, 10). During the earliest stage of tube emergence, membrane-bound vesicles enclosing fibrillar masses are expelled from the tube cell and evidently contribute to the layer (Fig. 5). At later stages in development direct associations between vesicles having a microfibrillar content and the plasmalemma of the cell are frequently seen in the tip region of the tube (Fig. 18).

Throughout the active period of tube growth in vitro the first-formed polysaccharide stratum and its associated inclusions constitute the outer zone of the pollen tube wall (Fig. 4B). It first forms a conspicuous cap at the tube tip (Figs. 10, 35), but as the tube extends the stratum becomes a loose superficial reticulum of fibrils most prominent below the tip and thinning towards the germinal aperture.

At all stages in pollen germination, from early emergence of the tube to maximum extension in vitro, the presence of protein in the outer zone of the tube wall is readily demonstrated by simple staining procedures (Figs. 11-13) and treatment with the appropriate fluorochromes reveals the presence of some callose - the resolution of the method is insufficient to differentiate the two zones (Figs. 27-29) - as well as mixed 1,3-β- and 1,4-β-linked glucans. When germinated pollen is reacted with the IgG preparation of the pollen antiserum, followed by FITC-goat anti-rabbit IgG, the fluorescence is localized on the pollen tube wall, the localization at all periods corresponding exactly to the distribution of the cytoplasmic inclusions in the outer zone revealed by electron microscopy (Figs. 20-22). Accordingly, in older tubes the intensity of the fluorescence emission is considerably more in the apical and sub-apical region than elsewhere (Fig. 22). The earlier cap stage, although very responsive to the treatment, produces a surprisingly low level of emission in comparison (Fig. 20). Since the ultrastructural observations and Coomassie staining show that the outer

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Figs. 11-13. Germinating grains mounted directly in Coomassie Blue stain-fixing medium. The method is detecting protein in the tube cell wall as the tube emerges and grows. Fig. 13 suggests that there may be some protein diffusion from the tube tip region. x 520.

Fig. 14. Non-specific esterase localization. The enzyme occurs in the exine and intine of the pollen wall; there is no activity in the tube. x 740.

Fig. 15. Control for esterase test. x 700.

Fig. 16. Germination. The exine and intine at the sulcus of the grain are ruptured and the tube cell is about to emerge. x 1300.

Fig. 17. Pollen tubes penetrating the nucellus tissue of the ovule. Coomassie staining reveals the presence of protein in the tube wall. x 125.

Fig. 18. Acid phosphatase localization in the pollen tube wall using a modified Gomori method. Most of the lead phosphate end-product occurs in the outer wall zone. There are vesicles with a microfibrillar content fusing with the plasmalemma in this region. x 15000.

Fig. 19. Acid phosphatase control. The stratification of the tube wall is clearly revealed by the additional magnification, although in the area photographed the outer zone does not contain any large inclusions. x 39000.
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zone of the tube wall at this period in germination contains a concentrated load of proteinaceous inclusions, and that no loss is involved (Figs. 12, 35), such a result is explained if the more compact state of the microfibrillar fabric of the cap is impeding antiserum penetration. No tube wall fluorescence is observed in control preparations consisting of germinating grains incubated in the IgG preparation of pre-immune rabbit serum followed by the FITC-conjugated antibody (Figs. 23–26).

Pollen antiserum binding to **Cycas** pollen tubes, estimated by the characteristic FITC-fluorescence emission of the secondary antibody in an indirect labelling procedure, is not inhibited by oxidation of the tubes with 0.05 M-sodium metaperiodate for periods up to 1 h. Metaperiodate treatment would be expected to affect the pollen tube wall carbohydrates and result in general modification by oxidation of both terminal and internal monosaccharide residues, except those in non-terminal positions joined in 1,3-glycosidic linkage (Hinch & Clarke, 1980). It would appear, therefore, that these molecules are not of significance as principal antigens in **Cycas**.

Germinated pollen of **L. regale** reacted with the IgG fraction of **Cycas** pollen antiserum or with the IgG population of pre-immune rabbit serum and then with FITC-goat anti-rabbit IgG exhibits weak fluorescence of the pollen tube wall after both treatments. Intense FITC emission is produced in the two preparations from a small aggregation of coarsely granular material located on the surface of the tube at the extreme tip. While binding immunoglobulin non-specifically, the material is insensitive to Coomassie Blue staining and appears to be a non-proteinaceous contaminant collected from the aperture at germination. The results from this experiment with **Lilium** help establish the specificity of the antibody binding in **Cycas**. They suggest that the antigens detected by immunofluorescence in the pollen tube wall of the gymnosperm are not present on the pollen tube of the flowering plant and, therefore, that the molecules concerned are not common to all spermatophyte pollen tubes.

When germinating **Cycas** grains are treated with ferritin-labelled secondary antibody after incubation in pollen antiserum IgG, numerous ferritin particles are found attached to the cytoplasmic inclusions derived from the tube cell. Although there is evidence that in older tubes the ferritin-conjugated antibody has penetrated for some distance into the loose fibrillar substructure of the outer wall zone, the heaviest

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**Figs. 20–22.** Germinating pollen has been incubated in pollen antiserum IgG and then in FITC-labelled secondary antibody and photographed by incident-light fluorescence microscopy. The FITC fluorescence emission is localized on the pollen tube wall; the exine layer of the pollen wall shows faint autofluorescence. × 700.

**Figs. 23–26.** Control preparations for Figs. 20–22. The germinating pollen was incubated in pre-immune rabbit IgG and then in FITC-labelled secondary antibody. There is no fluorescence emission associated with the tube wall. Figs. 24, 26 are, respectively, phase-contrast images of Figs. 23, 25. × 700.

**Figs. 27–29.** Callose localization by aniline blue fluorescence. Callose is detected in the wall of the tube cell as the cell begins to emerge through the aperture (Fig. 27) and then as a weakly fluorescing stratum of the tube wall during tube growth (Figs. 28, 29). The intine layer of the pollen wall also shows a faint reaction. × 700.
and most consistent labelling usually occurs on those inclusions located at or near the surface of the tube (Figs. 30, 31). Only rarely are ferritin particles seen attached to the tube wall microfibrils themselves. Control preparations incubated in the IgG population of pre-immune rabbit serum and then in ferritin-labelled secondary antibody show comparatively few ferritin particles associated with a majority of the inclusions and some are completely clean (Figs. 32–34).

Standard localization methods for light and electron microscopy reveal the presence of acid phosphatase in the pollen tube wall, the highest concentration being at all times in the outer zone (Figs. 18, 19). Cytochemical staining shows that throughout the entire germination period non-specific esterase activity occurs only in the pollen grain wall, in the inner stratum of the exine and in the intine (Figs. 14, 15). With regard to enzymes and related molecules, there appears to be no cytochemical difference between a pollen tube obtained in culture and that which develops in the ovule in consequence of normal pollination. Thus, Coomassie staining is able to reveal the presence of protein in the wall of pollen tubes penetrating the nucellus tissue in *C. armstrongii* (Fig. 17).

**DISCUSSION**

**Pollen germination in vivo**

Under natural conditions, the pollen in the cycads germinates on the nucellus tissue of the ovule after the grains have entered the pollen chamber, a receptacle formed before pollination by degeneration of the nucellus cells in the micropylar region, or the intermediary chamber, a cavity developed as a post-pollination chalazal extension of the pollen chamber. A pollen tube emerges from the sulcus of the grain, penetrates and grows deeply in to the nucellus tissue, thereby anchoring the gametophyte to the ovule above the position of the future archegonia. Male gametogenesis then proceeds (Fig. 4A). There is a long-established, though never demonstrated, belief that the pollen tube in the cycads has a nutritive role and is capable of absorbing metabolites from the nucellus during the long interval required to complete gametogenesis – several months in some genera (Pant & Mehra, 1962; Singh, 1978). It is generally agreed, however, that the structure has no direct commitment to fertilization, it does not convey the sperm to the egg, and in this respect differs fundamentally from the pollen tube in the flowering plants and higher gymnosperms (the conifers and their allies) (Heslop-Harrison, 1978a; Singh, 1978). This is the basis of the
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distinction between zooidogamous reproduction, represented by Cycas, and siphono-
gamous reproduction, characteristic of the higher gymnosperms and flowering plants.

**Pollen germination in vitro**

Under *in vitro* conditions, a closely coordinated pre-germination developmental sequence can be detected in Cycas pollen. Beginning with a phase of wall deposition accompanied by intense surface activity, by which cytoplasmic inclusions are introduced into the wall, and terminating in a second phase of wall deposition, the sequential process involves only the tube cell of the male gametophyte. That initiation of the sequence depends upon imbibition has been determined by comparing cultured grains with mature pollen fixed without preliminary hydration (author's unpublished observations), and there is no reason to suppose that the particular course of the development was caused by the special experimental conditions. When the grain germinates the two wall layers, exine and intine, are disrupted at the aperture. The tube cell begins to emerge, pushing out the polysaccharide tube cell wall together with the gametophytic inclusions, so forming the pollen tube initial. A tubular growth pattern is then established.

In the cycads (Audran, 1977), as in the flowering plants (Heslop-Harrison, 1972), deposition of the polysaccharide intine layer of the pollen wall occurs at the microspore stage, while the future pollen grain is relatively immature and is still contained within the sporangium. Development of the layer is well advanced by the first microspore mitosis and is complete before the grains are released in the tricellular condition. The polysaccharide material laid down by the tube cell, which forms the wall of the early pollen tube in Cycas is, therefore, in a special category, independent in origin from, and developmentally unrelated to, the intine. The interest here is that this represents an extreme departure from what is normal in spermatophyte pollen. But while exceptional, it may not be unique to Cycas since a similar sequence has been described for a conifer. Duhoux (1972a, b) has found that the ripe pollen of *juniperus communis* has three fibrillar intine strata. Light microscopic observations on living grains germinated *in vitro* and electron-microscopic studies of sectioned specimens revealed that during the hydration stage, before germination commences, a new fibrillar layer with a polysaccharide component is deposited beneath the innermost intine stratum around the entire circumference of the grain, and that the tube wall is continuous with this layer. Formation of the new layer, which Duhoux identifies as

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**Figs. 32–34. Control preparations for Figs. 31, 32.** The germinating grains were incubated in pre-immune rabbit IgG and then in ferritin-conjugated secondary antibody. Ferritin particles can be seen within the fibrillar fabric of the tube wall but very few are attached to the cytoplasmic inclusions. Fig. 32, ×70,000; uranium and lead stained. Fig. 33, ×95,000; bismuth stained. Fig. 34, ×60,000; bismuth stained.

**Fig. 35. Germination.** The exine layer of the pollen wall is ruptured and the intine layer is disrupted as the polysaccharide cap above the tube cell is pushed through the germinal aperture. The radial channels in the fabric of the cap contain proteinaceous cytoplasmic inclusions derived from the tube cell. ×20,000.
additional intine, preceded first microspore mitosis (juniper pollen is unicellular when released) and the pollen tube, formed by the vegetative (or tube) cell, could be observed penetrating the primary intine, if this had not been cast off before the tube emerged.

There is clear evidence from many electron-microscopic studies (Crang & Miles, 1969; Dickinson & Lewis, 1973; Dickinson & Lawson, 1975; Heslop-Harrison, 1975; Rowley & Dahl, 1977; Cresti, Pacini, Ciampolini & Sarfatti, 1977; Heslop-Harrison & Heslop-Harrison, 1981), but not all (Larson, 1965), to show that in flowering plants there is structural continuity between the wall of the early pollen tube and the intine, and this is supported by a most thorough analysis of pollen germination, tube emergence and growth (Heslop-Harrison, 1979a). The altogether different origin of the principal components in Cycas, and possibly in Juniperus, would seem to suggest an absence of strict homology between the wall of the young pollen tube in the gymnosperms and the corresponding structure in the flowering plants. But while morphogenetic evidence can be cited in support of this conclusion, there are perceptible features of structure and composition shared by the two groups. For example, in both gymnosperms, represented by Cycas (Figs. 27–29) and Pinus (Waterkeyn, 1964), and in flowering plants (see Shivanna, Johri & Sastri, 1979, for a review; Dickinson & Lewis, 1973; Heslop-Harrison, 1979a; Heslop-Harrison & Heslop-Harrison, 1981, for cytochemical illustration), callose occurs as a principal constituent of the tube wall.

The cytoplasmic inclusions that occur in the pollen wall intine in the flowering plants and in the pollen tube wall in Cycas are unquestionably of gametophytic origin. Cytochemical and fine-structural studies in the flowering plants have shown that the inclusions are derived from the microspore and that they are concentrated in the sites they will occupy in the mature grain while intine deposition is actively proceeding. Throughout this period, then, the cell is engaged simultaneously in the synthesis and export of structural wall materials and the formation of cytoplasmic evaginations destined for wall incorporation. The inclusions are in place before the end of intine growth, which is usually complete before the first microspore mitosis. The incorporated material is proteinaceous and has enzymic, antigenic, and in some instances allergenic, properties (Knox & Heslop-Harrison, 1970; Knox, 1971; Heslop-Harrison, et al. 1973; Heslop-Harrison, 1975, 1977; Pacini & Juniper, 1979; Vithanage & Knox, 1980). As mentioned above, the considerable mobility of the intine-held proteins has been demonstrated in a number of studies. They begin to be released by diffusion through the germinial apertures of the grain and, where it is permeable, through the exine, within a short time (usually within minutes) of hydration. Emission is independent of tube emergence and can be detected both in vivo and in vitro (Knox & Heslop-Harrison, 1971b; Heslop-Harrison, 1975, 1979a). In some species there is a second period of protein emission after germination from the tip zone of the tube itself (Knox & Heslop-Harrison, 1970; Heslop-Harrison, 1979a).

Even if they are not constitutionally the same, there is a remarkable similarity between the intine inclusions found in the flowering plants and the pollen tube wall inclusions detected in Cycas. The electron-microscopic observations presented above show that the inclusions in the wall of the early tube in Cycas are acquired during the
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pre-germination stage from the tube cell of the male gametophyte. The cytochemical methods reveal the presence of protein, including the enzyme acid phosphatase, in the tube wall. Immunofluorescent staining suggests that one component of the tube wall is immunologically related to the gametophyte and that this is more likely to be the proteinaceous inclusions than the polysaccharide fabric of the tube wall itself. This conclusion is substantiated by the results from immunoelectron microscopy. The immunofluorescent technique was unable to detect any immunological similarity between the antigenic components in the Cycas pollen tube wall and the components of the pollen tube in Lilium, a flowering plant genus known to have protein concentrated in the intine at the germinal site (Knox & Heslop-Harrison, 1970). As stated earlier, the proposition advanced to account for the occurrence of intine-borne proteins in the flowering plants is their utility in post-pollination events, such as germination and stigma penetration. But where, as in the grasses, the genetic control of compatibility is gametophytically determined and interaction is between the pollen tube tip and the stigma surface, the circumstances suggest that they could be directly employed in the regulation of breeding behaviour (Heslop-Harrison, 1976, 1978a; Shivanna 1979; Sastri & Shivanna, 1979). In this connection, it is interesting to find that there is post-germination protein emission from the region of the pollen tube tip in grasses (Knox & Heslop-Harrison, 1970; Heslop-Harrison, 1979a).

Among the cycads, interspecific compatibility has been reliably documented for Zamia, but records of intergeneric compatibility demand more circumspection (Pant & Mehra, 1962). Since the order is uniformly dioecious, the question of self-pollination does not arise. Whether Cycas species are interfertile or intersterile is not known, but if there is some form of surveillance system in the ovule capable of discriminating between compatible and foreign pollen then this investigation suggests a possible mode of operation. By analogy with the situation in the grasses, the gametophytic products held in the pollen tube wall could be envisaged as recognition materials that identify the tube to the nucellus. In an incompatible combination, penetration of the tube is either prevented or severely restricted. Some support for this scheme comes from Sahni’s (1915) discovery of what can be interpreted as effective obstruction of conifer tubes attempting to enter the nucellus in Ginkgo, a zooidogamous gymnosperm. He found that tube growth was influenced by nucellar contact; the response to contact was continued growth, but in a direction away from the tissue.

The distribution of the enzymic moiety in the Cycas tube wall, of which acid phosphatase is presumably representative, can be explained if it is deployed to facilitate penetration and passage of the tube through the nucellus, a function that Willemse & Linskens (1969) have attributed to the enzymes secreted by the tube in Pinus. The cuticle that covers the nucellus in Cycas does not line the pollen chamber (Pant & Nautiyal, 1963). Accordingly, there would be no reason to expect the enzyme system of the Cycas tube to conform to that in flowering plants with regard to a cutinase or cutinase precursors (J. Heslop-Harrison, 1968; Y. Heslop-Harrison, 1977; Heslop-Harrison & Heslop-Harrison, 1981). Consequently, no wall-held esterase activity is found.
Evolutionary significance of the discovery

Claims have been made (Mamay, 1976; Dilcher, 1976) that the flowering plants and cycads have a common origin in the pteridosperms – popularly, seed ferns – an extinct group of spermatophytes that are known to have produced pollen tubes (Rothwell, 1972). It is claimed, too, that among the extant cycads, a group that has been in existence for at least 200 million years, *Cycas* is the most primitive genus (Pant & Mehra, 1962; Sporne, 1965). If the principal features of development described here are general for the cycads, these plants share with the flowering plants the character of gametophytic proteins in the pollen tube wall, and from this arises the probability that this character was present in the ancestral group. Transferance of the time of incorporation of the proteins, from comparatively late in the life of the gametophyte (from the period immediately before germination, exemplified by *Cycas*) to a period earlier in development (during the deposition of the intine, typical of the flowering plants) could have been another, earlier episode in the progressive evolutionary trend towards rapid tube initiation and growth, and hence, rapid fertilization (Heslop-Harrison, 1968, 1979a, c; Hockstra & Bruinsma, 1979). On this view, the pollen of the flowering plants, already furnished with proteins in the fabric of the incipient tube wall at the time of dispersal, need have no extended stage intervening between hydration and germination for synthesis and storage of these components; and none occurs. Pollen germination in flowering plants proceeds rapidly once initiated and tube growth commences within a short time of the grain contacting the stigma in a compatible match; in most of the species investigated the interval is a matter of seconds or minutes. This stands in marked contrast to the situation in the cycads, where germination delay is measurable in days.

It has been suggested that the key to the sudden rise of the flowering plants in geological time was the acquisition of devices that promoted outbreeding through self-incompatibility, that of these the homomorphic system santedate the heteromorphic, and that of the former the system based on the gametophyte is primitive (see de Nettancourt, 1977, for discussion). With the exception of the grasses (Knox & Heslop-Harrison, 1971b; Heslop-Harrison, 1976, 1979a, b), and the possible exception of *Oenothera* (Dickinson & Lawson, 1973), there is no evidence to suggest that effective functioning of gametophytic self-incompatibility in modern flowering plants is dependent upon the presence of those gametophytic factors carried in the pollen wall at the time of dispersal. The wall-borne materials are believed not to be implicated in gametophyte-determined intraspecific compatibility control where, as in the majority of cases, inhibition of tube growth occurs in the style. On the other hand, it seems they may have a part to play in determining some interspecific reactions in flowering plants (Knox, Willing & Ashford, 1972; Ashford & Knox, 1980) and Pandey (1980) regards interspecific specificity as ‘primary’ specificity, primitive in origin and ancient in ancestry, having evolved in the gymnosperms or their antecedents as a protective response against unproductive introgression and ovule sterility that might otherwise result.

Clearly, from the evolutionary point of view, it would be of interest to extend the
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compass of the present study to include the conifers and plants of similar taxonomic alliance.

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