POLLEN-WALL PROTEINS: LOCALIZATION AND ENZYMIC ACTIVITY

R. B. KNOX AND J. HESLOP-HARRISON
Institute of Plant Development, Birge Hall, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

SUMMARY

Cytochemical methods have been used to examine the distribution of acid phosphatase, ribonuclease, esterase, amylase and protease activity and protein in the walls of pollen grains and spores. Enzyme activity was detected in the walls of 50 angiosperm pollens, in pine, and in the spore wall of a species of Equisetum. Activity was absent from the 2 species of ferns examined. The survey covered all major structural pollen types. In all cases enzyme activity was associated principally with the intine, the inner cellulosic part of the wall. In all aperturate grains activity was concentrated in or around the apertural intine; that is, over or near the potential sites of emergence of the pollen tube. Protein concentrations in the intine at these sites could be demonstrated in several pollen types by staining methods and by absorption at 285 nm.

Developmental study showed that the enzymes are incorporated in the intine during the early period of wall growth following the release of the spores from the meiotic tetrads. During this period, stratified ribosomal endoplasmic reticulum lies adjacent to the inner spore wall over the areas of incorporation. In Cosmos bipinnatus, a composite, the material is incorporated as ribbons or leaflets, which interleave with cellulose lamellae. In other species the wall protein may take the form of granules, tubules or vesicles, embedded in the intine cellulose. At maturity the intine is separated from the spore cytoplasm by an intact plasmalemma, so the wall enzymes are to be regarded as being extracellular.

In some species enzyme activity was detected in materials on the surface of the pollen exine derived probably from the surrounding tapetal tissue; however, this was usually insignificant compared with that shown by the intine.

The intine enzymes are very readily leachable, and their function is probably connected with the early nutrition of the pollen tube and the penetration of the stigma. It is likely that the wall proteins make up a very large proportion of mobile protein of the pollen grain, and they may therefore be important in pollen allergenicity.

INTRODUCTION

Tsinger & Petrovskaya-Baranova (1961) have shown that proteins are present in certain strata of the walls of the pollen grains of various flowering-plant species, and that the wall-held proteins possess enzymic properties. These authors concluded that the principal depositions of protein are in the cellulosic part of the wall, especially in the inner stratum, the intine. We have confirmed these findings, and have extended the observations to most of the major structural types of pollen, embracing a sample of more than 50 species. The precise structural location of the pollen wall proteins can now be specified for most of these species, including several noted for the allergenicity of their pollen. Developmental and fine-structural studies have fixed the time of wall-protein synthesis in the course of microsporogenesis and cast
light on the manner of incorporation in the wall. This paper contains a report of this work; a preliminary account has been given elsewhere (Knox & Heslop-Harrison, 1969).

MATERIALS AND METHODS

Pollen sources

Mature anthers were collected from greenhouse- or garden-grown plants, or from wild populations. The anthers were stored in Petri dishes with a few grains of Drierite to hasten dehiscence, and the pollen was then harvested. Tests were also carried out on stored pollen of some of the allergenic species obtained from commercial sources, Allergy Division, Greer Drug & Chemical Co., Lenoir, N.C. and Hollister-Stier Laboratories, Dallas, Texas.

Freeze-sectioning technique

The aim of the study was to localize and characterize some of the properties of the highly mobile pollen proteins, and it became clear early in the course of the work that stabilization could not readily be achieved by the usual processes of chemical fixation. Accordingly, fresh, unfixed pollen was used in most of the tests, freeze-sectioned by a modification of the method used by Knox & Evans (1968) employing a gelatine medium. Petri dishes containing the medium (15% gelatine, w/v, and an antifreeze, either 0.8% dimethyl sulphoxide or 2% glycerol, v/v, gave excellent results) were prepared in advance and stored at 2 °C. The addition of the non-toxic antifreeze solutions to the medium improved the properties of the frozen gelatine during sectioning, preventing the tearing out of tissue. The concentration used is less than one-tenth of that required for cryoprotection (see Ashwood-Smith, 1966), and presumably neither the gelatine nor the antifreeze penetrated into the tissue. For use, a hole was melted in the gelatine plate, and when the melted area had cooled but not set, the pollen sample was added and stirred to make a slurry. Pieces of anther were introduced in the same way. A block containing the sample was then cut out and rapidly frozen on the quick-freeze block of an IEC cryostat. The period between the initial encasement in the gelatine and the actual freezing of the sample varied between 30 and 60 s. Sections were cut at —10 to —15 °C at thicknesses of 2.8 μm, rapidly thawed on clean dry slides (glass or quartz), and air-dried at room temperature. Slides were stored dry at 2-5 °C until required.

In spite of the speed of handling possible with this method and the low temperatures at which most of the processing was carried out, there were protein losses from the walls of some types of pollen, presumably occurring during the few seconds before the freezing of the slurry or while the sections were drying out after thawing. Sometimes loss of proteases resulted in the digestion of the gelatine of the section in a halo around each grain.

Protein localization

Proteins were localized in freeze-sectioned pollen grains and in fresh wall fragments by the natural absorption at 285 nm and by staining methods. The ultraviolet observations were made with a Cooke microscope with quartz optics, using a Zeiss xenon arc and monochromator as a light source. Naphthol yellow S (NYS) was found to be satisfactory as a protein stain (Deitch, 1955). Observations were made using a Wratten no. 98 filter, which has a peak transmission at 430 nm.

The wall-associated proteins tend to diffuse from sectioned grains into the staining solutions even with immersion periods of 1 min or less. Improved stabilization was obtained by pre-soaking the sections for 5 min at room temperature in half-saturated (NH₄)₂SO₄ before rinsing quickly in distilled water and staining for 1 min in 1% NYS in 1% acetic acid. After this treatment, the sections were differentiated by rinsing in 1% acetic acid, and then dehydrated through ethanol and mounted in balsam.

Various chemical fixation methods were tested in attempts to stabilize the wall-held proteins. The most effective fixatives were 3% glutaraldehyde buffered in 0.1 M phosphate buffer at
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pH 7.0 at room temperature for 5–8 h, and acetic-methanol (1:3, v/v) at room temperature for 3 h or at 3–5 °C overnight. Even with these treatments there was reason to believe that considerable losses occurred.

Cellulose and callose

The cellulose of the intine was identified and localized using the periodic acid/Schiff (PAS) procedure. For the more precise localization of the limits of exine and intine, sectioned walls were acetylolyzed to remove the cellulose, leaving the resistant exine (Erdtman, 1960).

The possibility that callose might be present in association with the intine was checked in pollen walls of different structural types using the sensitive aniline blue-fluorescence method. In no case was callose detected in angiosperm pollens. In Pinus, as described by Waterkeyn (1964), a cup of callose is present in the base of the grain, opposite to the thickened area of intine cellulose marking the germination region.

Enzyme localization

Acid phosphatase. The methods of Barka & Anderson (1962) gave consistently reproducible results with incubation periods as short as 1–5 min. The substrate α-naphthyl acid phosphate (Sigma Chemical Co. St Louis) was hydrolysed very rapidly, coupling with hexazonium pararosanilin (Sigma) to give a red-brown reaction product with little evidence of diffusion, in 0.1 M veronal-acetate buffer at pH 6.0. Identical localization of reaction product was obtained when Naphthol AS-BI or AS-TR phosphates (Nutritional Biochemicals Corp., Cleveland, or Sigma) were used as substrates in a similar reaction mixture at pH 5.0, with both hexazonium pararosanilin and Fast Garnet GBC as coupling agents. Ten to 20 min were required for deposition of the bright red or purple-black reaction products, and some diffusion was occasionally evident, particularly in wall fragments. Reactions were stopped by rinsing in water and dehydrating rapidly through absolute ethanol (10 s), cellosolve (10 s), and xylene prior to mounting permanently. No differences in localization were evident if slides were mounted in glycerol-gelatine after rinsing in water (prior to dehydration). The dehydration and clearing provided improved resolution under the light microscope, and no appreciable fading occurred over a period of several months.

The localization obtained by these methods was confirmed for Malvaviscus, Hibiscus, and Crocus using two other techniques. The first was the method of Seligman (1964) and Hanker et al. (1964) in which α-naphthyl thiol phosphate salt was substrate in a simultaneous coupling reaction with Fast Blue BBN in 0.2 M acetate buffer at pH 5.0. The yellow intermediate diazo-thioether was then exposed to OsO4 vapour at 50 °C, giving an intense black reaction product. Unfortunately, the exine layers react with osmium, giving a grey-black colour, so that the high resolution obtainable with this method could not always be exploited, but the method has obvious potential for fine-structure studies, and these are in progress. The second was a modified Gomori (1952) lead salt method (Holt & Hicks, 1961; Essner & Novikoff, 1962; Bitensky, 1963), using sodium β-glycerophosphate as substrate in 0.05 M acetate buffer at pH 5.0.

Controls for all three methods were as follows: (a) reaction mixture with substrate omitted; (b) slides pre-incubated in 0.01M NaF for 5 min prior to incubation in complete reaction mixture containing 0.01M NaF as inhibitor; (c) slides heated at 90 °C for 2 h before incubation in complete reaction mixture. Under these conditions the reaction product was not detectable in pollen walls. Lipetz (1968) has suggested that various coupling agents used for acid phosphatase localization may bind to tannins in plant cells. This possibility is unlikely in pollen grains, since tannins could not be detected histochemically using the ferric sulphate test and nitroso reaction (Jensen, 1962).

Ribonuclease. Modifications of the lead salt method of Enwright, Frye & Atwal (1965) were used to detect sites of ribonuclease activity. Using the original method developed for erythrocytes, results were variable and unpredictable with pollen grains. The high concentration of lead ions in the medium (0.3%) apparently proved toxic, and enzyme activity could not be detected, or only in sections thicker than 8 μm. Accordingly, the lead ion concentration was reduced to 0.12%, the level recommended by Essner & Novikoff (1962) for the Gomori
technique. H$_2$S-saturated water was used to convert the lead phosphate reaction product to black lead sulphide, as this is less harmful to unfixed tissue sections than the ammonium sulphide solution recommended (Bitensky, 1963). A cylinder of H$_2$S (Matheson, Coleman & Bell) was used, the gas being purified through water before use. The schedule finally adopted was as follows: 30 mg RNA (from Torula yeast, Sigma) were dissolved in 25 ml acetate buffer, 0.2 M, pH 5.0, and 10 mg acid phosphatase (wheat germ, Sigma) added. To 60 ml distilled water, 0.8 ml of 0.4 M lead acetate was added, and the mix poured into the RNA solution, agitating throughout. A milky white suspension (apparently a RNA-lead complex) formed and was not filtered off; the reaction mixture was then made up to 100 ml. Slides were incubated in the mixture at room temperature for up to 3 h, rinsed in water, placed for 2 min in H$_2$S water, rinsed thoroughly and dehydrated as described above for acid phosphatase. No fading of the lead sulphide reaction product occurred over several months. Consistent and reproducible results were obtained with this method, independently of section thickness. Controls were as follows: (a) RNA omitted from reaction mixture; (b) slides pre-incubated in 0.01 M NaF for 5 min followed by incubation in the complete reaction mixture containing 0.01 M NaF.

The localization of ribonuclease by this technique has been confirmed for Malvavisais and Hibiscus pollen grains using the RNA-substrate film method of Daoust (1968).

Esterase. The method described by Pearse (1960) with o-naphthyl acetate as substrate was used in a coupling reaction with Fast Blue B Salt in 0.1 M phosphate buffer at pH 6.0-7.4. Controls were run by omitting the substrate.

Amylase and protease. These 2 enzymes were localized using substrate film methods. Amylase proved difficult because the high starch content of the pollen grains prevented use of the PAS reaction or iodine/potassium iodide as stains for the films after incubation. This was obviated using the technique suggested by Shear & Pearse (1963) in which fixed starch films were first treated with periodic acid for 10 min. The films were washed and dried. Sections of pollen grains were thawed on the films, and incubated in a moist atmosphere in a Petri dish for up to 60 min. The PAS reaction was then completed by incubation in Schiff's reagent. The pollen grains were unstained, with sites of amylase activity evident as clear areas in the red-stained film. This method gave improved localization compared with those in which the tissue is washed away before staining. Protease activity was demonstrated using processed colour film by the method of Fratello (1968). Acid protease activity was also demonstrated when slides were stained in NYS, the diffusing enzyme digesting a halo in the gelatine around the grain in the vicinity of the apertures. Controls were run using pollen heated at 90 °C for 1 h prior to encasing in gelatine.

Electron microscopy

Material for transmission electron microscopy was fixed in 3 % glutaraldehyde buffered at pH 7.0 in 0.1 M phosphate buffer at room temperature for 5-8 h, thoroughly washed in buffer, and then transferred to 1 % OsO$_4$ in the same buffer for 3-4 h at 3-5 °C. After fixation, the material was dehydrated through an ethanol series and embedded in Araldite–Epon. Sections were cut with a diamond knife and post-stained in 2 % uranyl acetate followed by lead citrate.

Observations

Pollen wall morphology: general features

The primary subdivision of the pollen wall is into the outer exine and the inner intine. The exine is composed of sporopollenin, a highly resistant polymer most recently interpreted as being produced by carotenoid polymerization (Brooks & Shaw, 1968). Morphologically 2 strata can usually be recognized in the exine, the outer, usually sculptured, sexine, and the inner, non-sculptured, nexine (Erdtman, 1966). The intine may be lamellated, but it does not usually show complex stratification comparable with that seen in the exine. A complication met with in locating the exine/intine interface is that the 2 layers may have no sharp boundary, despite their
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chemical dissimilarity. It has been known from early fine-structural studies such as that of Larson & Lewis (1961) that the nexine may be lamellated over part of its area, and more recently it has become clear that lamellae generated at or near the plasmalemma are concerned in the deposition of this layer (Godwin, Echlin & Chapman, 1967; Rowley, 1966; Rowley & Southworth, 1967; Heslop-Harrison, 1968b), and indeed in the formation of other exine layers (Dickinson & Heslop-Harrison, 1968). The lamellae forming the nexine are apparently passed through the developing intine during the main period of wall growth, and in the mature wall their identity is mostly lost in the compacted nexine (Rowley & Dunbar, 1967). However, in some species nexine and intine lamellae remain interbedded in the mature grain, particularly near apertures (Skvarla & Larson, 1965).

In some species the pollen wall has no clearly demarcated germination site, but in most pollens the wall bears 1 or more apertures, potential locations for the emergence of the pollen tube. These may be circular ('pores') or slit-like ('sulci' or 'colpi'). They are often regions where the sexine is reduced or absent, the exine consisting of a single sporopollenin layer corresponding in derivation to the nexine, although the more elaborate types of apertures have sculptured operculi with full exine stratification. In the regions of apertures, the intine is usually thickened. A common situation in porate species is for this layer to form a plug passing outwards into the aperture, often with a subtending collar or annulus within. The architecture of the tricolporate grain of the Compositae is described further below.

For the purposes of the present survey, the structural minutiae which provide the basis for the detailed classification of apertural types (Erdtman, 1952) are not of great significance. Accordingly, a simplified classification has been adopted using 5 classes: (a) nonaperturate, (b) monoporate, (c) polyporate, (d) monocolpate and (e) tricolpate and triporate. This last class includes 'tricolporate' species where a circular intine aperture underlies each slit-like colpus, as in the example of Cosmos bipinnatus described further below.

Sites of protein deposition in the pollen wall

Attempts were made to localize wall proteins by properties other than enzymic activity in pollens representing 4 of the above structural classes. Proteins have been located in 3 wall sites: (a) in the superficial materials contributed by the tapetum during the later stages of pollen maturation, the tryphine or Pollenkitt (Heslop-Harrison, 1968c); (b) in cavities within the sexine or between sexine and nexine; and (c) in association with the cellulose of the intine. The last site is that given particular prominence in the account of Tsinger & Petrovskaya-Baranova (1961), and our observations confirm the importance they attached to it.

Non-aperturate pollens. Two species were examined, Crocus vernus and Gladiolus gandavensis. In each the exine is relatively thin, and the main thickness of the wall is represented by the intine. In the fresh pollen, some protein was detectable in the superficial Pollenkitt, but the distribution of absorption at 285 nm and NYS stainability shows that the principal protein accumulation is in the intine, a stratum readily defined by its PAS reaction (Figs. 1, 2).
Monoporate pollens. Three species of grasses were observed, *Zea mays*, *Coix lachryma-jobi* and *Alopecurus pratensis*. The single pore in the maize pollen is small relative to the size of the grain, and the intine below it is comparatively thin—0.6 μm from the electron micrograph published by Skvarla & Larson (1966). Wall protein could not be localized by the staining methods so far used, nor did absorption at 285 nm indicate the presence of protein in sectioned grains. In *Coix lachryma-jobi* and *Alopecurus pratensis*, protein was readily detected in the sub-poral intine both by absorption at 285 nm and by NYS stainability (Figs. 6, 7). The thickness of the intine in this region is 1.5–2 μm in these species.

Polyporate pollens. The pollen of *Malvaviscus arboreus* is characteristic of this class. The exine possesses a thick nexine and thin sexine; the pores take the form of funnel-like pits, tapering upwards and capped without by a thin layer of sporopollenin. The intine penetrates the shaft of the pore, and is swollen below in an annulus or collar (Fig. 15). The thick exine of this species absorbs strongly in the ultraviolet, so that wall proteins could not readily be localized by their self-absorption. However, the distribution of NYS stainability clearly defines protein accumulations in the intine at the pore sites (Figs. 16, 17). Again, the superficial Pollenkitt contains some protein.

Tricolpate pollens. The pollen of *Cosmos bipinnatus* was taken as an example. Superficially, the 3 colpi are demarcated as slit-like gashes in the sexine, the tips converging towards each pole. The nexine is thickened around the margin of the colpus, but thinned out over the surface of the slip itself. In the centre of the colpus, the intine is raised in a papilla, elliptical in paradermal section with the long axis at right angles to the long axis of the colpus itself. Some of these features can be discerned in Figs. 19–24.

Over the intine papilla the nexine forms but a thin layer, readily ruptured as the grain is distended on imbibition. Radial sections show that the marginal nexine is cleft in a tangential plane on either side of the intine papilla, the edges being frayed out and in places apparently interbedded with intine cellulose. Protein was detected by staining properties in the superficial Pollenkitt, in the cavities of the sexine, in the cavea between the sexine and nexine (Fig. 35), and most abundantly in the intine of the colpial regions. Further features of the intine deposits are described in the fine-structural account of *Cosmos*.

Enzyme activity in the pollen wall

Taxonomic distribution. The species so far examined for pollen-wall enzymes are listed in Table 1. Initially tests were made on several pollen types for acid phosphatase, ribonuclease, esterase, protease and amylase activity, and then to extend the taxonomic range acid phosphatase and ribonuclease alone were taken as marker enzymes.

Localization. Table 1 contains a brief indication of the main sites of the wall-associated enzymes. In summary, the principal activity is always located in the intine. In non-aperturate pollens activity is distributed in the central zone of this layer over the whole of the grain surface, and in aperturate species it is invariably concentrated in the apertural region, although it may be present in lesser degree in the intine else-
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Table 1. **Taxonomic distribution of pollen-wall enzymes and their relative activities as determined from cytochemical reactions on freeze-sectioned pollen grains**

(酸性磷酸酶被检测使用了Barka & Anderson (1962) 的方法，使用α-萘基磷酸作为底物和5 min的孵育期; 脱氧核糖核酸酶使用了Enwright et al. (1965) 的方法，孵育3 h; 水解酶使用了Pearse (1960) 的方法，使用α-萘基乙酸作为底物，孵育5 min。所有反应都在室温下进行。)

<table>
<thead>
<tr>
<th>Species and pollen type</th>
<th>Location of enzyme activity</th>
<th>Acid phosphatase</th>
<th>Ribonuclease</th>
<th>Esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANGIOSPERMS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Non-aperturate</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Crocus vernus</em> Wulf cv.</td>
<td>thick layer in mid-zone of intine</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td><em>Elodea densa</em> Planch.</td>
<td></td>
<td>+ +</td>
<td>NT</td>
<td>+ +</td>
</tr>
<tr>
<td><em>Populus nigra</em> L.</td>
<td></td>
<td>+ +</td>
<td>+ +</td>
<td>NT</td>
</tr>
<tr>
<td>Monoporate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Festuca elatior</em> L.</td>
<td>concentrated at pore intine</td>
<td>+ + +</td>
<td>+ +</td>
<td>NT</td>
</tr>
<tr>
<td><em>Lolium temulentum</em> L.</td>
<td></td>
<td>+ + +</td>
<td>+ +</td>
<td>NT</td>
</tr>
<tr>
<td><em>Alopecurus pratensis</em> L.</td>
<td></td>
<td>+ + +</td>
<td>+ +</td>
<td>NT</td>
</tr>
<tr>
<td><em>Coix lachryma-jobi</em> L.</td>
<td></td>
<td>+ + +</td>
<td>+ +</td>
<td>NT</td>
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<tr>
<td><em>Zea mays</em> L.</td>
<td></td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Polyporate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Malvaviscus arborescens</em> CAV.</td>
<td>throughout intine but concentrated at pores</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td><em>Hibiscus rosa-sinensis</em> L. cv.</td>
<td></td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
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<tr>
<td><em>Abutilon megapotamicum</em> St. H. &amp; Naud. cv.</td>
<td></td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td><em>Dianthus chinensis</em> L. cv.</td>
<td></td>
<td>+ + +</td>
<td>+ +</td>
<td>NT</td>
</tr>
<tr>
<td><em>Salsola pestifer</em> Nels.</td>
<td></td>
<td>+ + +</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td><em>Plantaago lanceolata</em> L.</td>
<td></td>
<td>+ + +</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td><em>Cobaea scandens</em> CAV.</td>
<td></td>
<td>+ + +</td>
<td>NT</td>
<td>+ +</td>
</tr>
<tr>
<td>Monocolpate</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><em>Magnolia soulangeana</em> Soul.</td>
<td></td>
<td>+ +</td>
<td>+</td>
<td>±</td>
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<tr>
<td><em>Acorus calamus</em> L.</td>
<td></td>
<td>+ +</td>
<td>+ +</td>
<td>NT</td>
</tr>
<tr>
<td><em>Spathiphyllum clevelandii</em></td>
<td></td>
<td>+ +</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td><em>Nymphaea stellata</em> Wild.</td>
<td></td>
<td>+ +</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td><em>Amaryllis vittata</em> Ait. cv.</td>
<td></td>
<td>+ +</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td><em>Lilium longiflorum</em> Thunb.</td>
<td></td>
<td>+ +</td>
<td>±</td>
<td>NT</td>
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<tr>
<td><em>Camassia quamash</em> Greene</td>
<td>concentrated at colpus</td>
<td>+</td>
<td>±</td>
<td>NT</td>
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<td><em>Comsallaria majalis</em> L.</td>
<td></td>
<td>+</td>
<td>NT</td>
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<td>+</td>
<td>NT</td>
<td>NT</td>
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<td><em>Gladiolus gandavensis</em></td>
<td></td>
<td>+ + +</td>
<td>+ +</td>
<td>±</td>
</tr>
<tr>
<td><em>Van Houtte</em> cv.</td>
<td></td>
<td>+ +</td>
<td>±</td>
<td>±</td>
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<tr>
<td><em>Iris xiphium</em> L. cv.</td>
<td></td>
<td>+ + +</td>
<td>NT</td>
<td>±</td>
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<tr>
<td><em>Iris dichotoma</em> Pall.</td>
<td></td>
<td>+ + +</td>
<td>NT</td>
<td>±</td>
</tr>
<tr>
<td><em>Sisyrinchium californicum</em> Dry</td>
<td></td>
<td>+ + +</td>
<td>±</td>
<td>±</td>
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Table 1 (cont.)

<table>
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<tr>
<th>Species and pollen type</th>
<th>Location of enzyme activity</th>
<th>Acid phosphatase</th>
<th>Ribonuclease</th>
<th>Esterase</th>
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<td>Tricolpate and Triporate</td>
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<tr>
<td>Aquilegia caerulea James cv.</td>
<td>throughout intine but concentrated at colpi or pores</td>
<td>+ + + + + + NT</td>
<td></td>
<td></td>
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<td>Paeonia tenuifolia L. cv.</td>
<td></td>
<td>+ + + + + NT</td>
<td></td>
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<tr>
<td>Caltha palustris L.</td>
<td></td>
<td>+ + + NT</td>
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<td>Primula obconica Hance cv.</td>
<td></td>
<td>+ + NT</td>
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<td>Petunia hybrida Vilm. cv.</td>
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<td>+ + NT</td>
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<td>Lycopersicum esculentum Mill</td>
<td></td>
<td>+ + + NT</td>
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<td>Nicotiana tabacum L. cv.</td>
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<td>Antirrhinum majus L. cv.</td>
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<td>+ NT</td>
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<tr>
<td>Anthurium andraeanum Lind.</td>
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<td>Lupinus polyphyllus Lindl. cv.</td>
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<td>Robinia pseudoacacia L.</td>
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<tr>
<td>Begonia tuberhybrida Voss cv.</td>
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<td>Philadelphus lemoinei Lem.</td>
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<td>Cosmos bipinnatus Cav.</td>
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<td>Senecio crus-galli</td>
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<td>Ambrosia artemisiifolia L.</td>
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<td>Ambrosia trifida L.</td>
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<td>Dahlia pinnata Cav. cv.</td>
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<td>+ + + NT</td>
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<td>Tragopogon pratensis L.</td>
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<td>Centaurea montana L.</td>
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<td>+ + + + + NT</td>
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<tr>
<td>Ulmus americana L.</td>
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<tr>
<td>Carex stricta Lam.</td>
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<td>+ + + + + NT</td>
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Pteridophytes

Cyrtomium falcatum Presl. none none none none

Phylitis scolopendrium Newm. none none none none

Equisetum palustre L. throughout intine + + NT

Gymnosperm

Pinus banksiana Lamb in cellulose intine at germinal region + + + NT

Explanation of symbols: + + + = dense deposition of reaction product in pollen walls; + + = moderate deposition of reaction product in pollen walls; + = slight deposition of reaction product in pollen walls; ± = detectable deposition of reaction product at limits of L.M. resolution; — = no detectable deposition of reaction product; NT = not tested. • = Commercial stored pollen used.

where. Examples of several of the enzyme reactions with controls are illustrated in Figs. 2-5, 8-15, 18, 21-26, 29 and 30. Between them these micrographs cover all of the principal classes of pollen wall.

As was to be expected, for those species where wall proteins had been localized by self-absorption or staining properties the highest enzyme activity appeared in parts of the intine showing greatest protein content. Although the data are not given in
Table 1, it may be mentioned that in addition to the enzyme activity associated with the intine many species show some in the superficial Pollenkitt. Because the Pollenkitt is tapetum-derived this property merits more detailed study.

Angiosperms: special cases. A surprising fact is that for all the enzymes tested the cultivar of *Zea mays* used in these studies showed substantially less wall-associated activity than *Coix lachryma-jobi*, a grass of the same tribe, Maydeae, or than *Alopecurus pratensis*, of the tribe Agrostideae. This was associated with a lower content of wall protein, as assessed by absorption at 285 nm and NYS stainability. This observation, which is commented on further below, is being followed up with other maize genotypes to establish whether the deficiency is characteristic of the species.

Many of the species tested were cultivars having some pollen sterility, presumably due to hybridization in their ancestry. This fact offered the opportunity to examine the distribution of enzyme activity in sterile grains. Interestingly enough, many such grains did show activity distributed in the normal locations, again principally in association with the apertural intine (Figs. 29–31). The generalization seems possible that if a spore is capable of developing to the point where exine formation is well advanced and intine synthesis in progress, then the wall enzymes will be incorporated.

Gymnosperms and Pteridophytes. The situation in the pollen grain of *Pinus banksiana* is essentially similar to that found in angiosperm pollens with a single aperture. During germination, the tube emerges at a site between the wings of the *Pinus* grain, a location marked out by a thickened zone of cellulose intine (Fig. 27; compare Waterkeyn, 1964). Enzyme activity is conspicuously concentrated at this site (Fig. 28), with none at all in the walls at the opposite pole.

Of the 3 pteridophytes, *Equisetum palustre* showed distinct enzyme activity associated with an intine giving an intense PAS reaction. No enzyme activity was detected in the walls of the spores of *Phyllitis scolopendrium* or *Cyrtomium falcatum*.

Pollen wall enzymes: developmental aspects

The acquisition of intine enzymes during spore development was followed in *Hibiscus rosa-sinensis* and *Malaviscus arboreus*, and fine-structural features of protein incorporation were examined in the spores of *Cosmos bipinnatus*.

The young spores follow a similar developmental pattern in practically all angiosperms. When released from the meiotic tetrad the spore is non-vacuolate; with the removal of the constraint of the callose tetrad wall it enters a period of rapid expansion, during which it takes up thecal materials (Heslop-Harrison & Mackenzie, 1967) without at first vacuolating. Vacuolation then occurs, and the protoplast comes to form a thin sheath over the inner face of the spore wall. This state persists in most species until after pollen mitosis, when the vacuole is resorbed as reserves are accumulated in the plastids and cytoplasm. The sequence of events in wall formation is quite closely correlated with that in the cytoplasm (Heslop-Harrison, 1968a). The early definition of the principal features of the sexine pattern occurs in the meiotic tetrad, but both sexine and nexine continue to grow after the spores are released from the callose wall. Intine deposition begins before the completion of nexine growth, usually before spore vacuolation. However, the most active thickening occurs in the early
vacuolate period. In aperturate pollens, intine cellulose is generally detectable first at the aperture sites (e.g. Silene pendula; Heslop-Harrison, 1963b), and where these sites are marked by an internal collar or boss of cellulose, they may also be the last where synthesis is active.

It is to be expected that, if the intine-associated enzymes are incorporated during the growth of this layer and not injected in some obscure manner after the completion of growth, activity should be detectable from early in the vacuolated spore phase. That this is so is shown by Fig. 14, of spores of Malvaviscus arboreus in the early vacuolate period. Immediately after release from the tetrad the spores show no wall-associated enzyme activity, but by the period illustrated, when intine growth is actively proceeding, the wall enzymes are already present, concentrated in the sites they will occupy in the mature pollen grains.

This observation indicates that the young spore is engaged simultaneously in the synthesis of structural wall materials and of proteins destined for wall incorporation before and during the vacuolated period. An aspect of the cytoplasmic function in the synthesis of wall precursor materials is to be seen in dictyosome activity during this interval (Heslop-Harrison, 1968b). In Cosmos bipinnatus, fine-structural study has shown what is probably cytoplasmic involvement in wall-protein synthesis. During the vacuolated period when intine growth is most active, the cytoplasm adjacent to the colpi is RNA-rich, and sections normal to the wall show stratified endoplasmic reticulum (Fig. 32). Polysome configurations—presumably membrane-attached—are conspicuous in glancing tangential sections (Fig. 33). In these micrographs it may be seen that the cellulose of the intine marginal to the colpi and in the central papilla is lamellated. The lamellae are separated by zones of finely fibrillar or granular material, and because of the certainty that this is the site of the wall enzymes and cytochemically detectable protein, this material is considered to be the wall protein itself, or rather the residuum surviving glutaraldehyde and osmium tetroxide fixation. In the mesocolpial intine, where enzyme activity is lower, the numbers of protein lamellae are correspondingly fewer (Fig. 34).

At the conclusion of intine growth, activity at the surface of the proplast declines, and the plasmalemma becomes continuous. In Cosmos, as in most of the species studied, a layer of cellulose without included protein separates the surface of the spore cytoplasm from the enzymic zone of the intine (see, for example, Crocus vernus, Fig. 3). This fact indicates that cellulose synthesis continues for a period after the termination of protein synthesis. Thus, if the plasmalemma is taken as defining the limits of the protoplast, there is no doubt that the wall proteins are extracellular.

Although the main mass of protein in the pollen wall of Cosmos is certainly associated with the intine, this is not the only site of deposition. As we have seen, protein can be detected cytochemically on the surface of the grain, and in cavities of the sexine. The source of the extra-intinal protein is, however, quite different from that of the intinal, since it represents the residue of the tapetal plasmidium which comes to invest the developing spores after their release from the tetrad. While the synthesis of the intine protein is in progress, the tapetal plasmidium wholly cloaks the growing sexine, penetrating into the cavities at the base of the spines, and thence into the
Pollen wall proteins

spaces between the bacula and the cavea, which are all interconnected (Heslop-Harrison, 1969). It is remarkable that ribosomes and membrane-invested vesicles may be seen in these spaces (Fig. 35).

DISCUSSION

Significance of the wall-held enzymes

These observations show that several acid hydrolases are present in the pollen wall, specifically associated with the intine. We regard it as likely that the enzymes so far localized are only a sample of several others of a similar character, possibly the majority of the 16 or so hydrolases listed by Mäkinen & Macdonald (1968) as having been recorded in pollen. A striking feature is the rapidity with which the proteins are lost from the wall on moistening in every structural class of grain. That pollen grains do rapidly release enzymes into the medium has been known since the observation of Green (1894), who found that the pollen of 13 tested species liquefied starch paste, and that that of 5 other species readily released invertase into sucrose solution. More recently, Stanley & Linskens (1965) have described the time-course of protein-release from Petunia pollen in the period before germination in sucrose medium, and Mäkinen & Brewbaker (1967) have shown that esterases, catalases, amylases, acid phosphatases and leucine aminopeptidases diffuse from Oenothera pollen within half an hour or so of immersion even in salt media which inhibit germination. It seems probable that in all of these examples, and in others cited in the literature lists of the papers of Stanley & Linskens (1965), Mäkinen & Brewbaker (1967) and Roggen & Stanley (1969), a large part of the enzymic protein emitted by immersed pollen before germination is derived from the intine locations identified by Tsinger & Petrovskaya-Baranova (1961) and described in the present paper. There is no reason to doubt, of course, that during germination and later pollen-tube growth the cytoplasm of the male gametophyte itself becomes the source of secreted enzymes.

As was early recognized by Green (1894), the biological role of the rapidly emitted enzymes is likely to be connected with germination, very early pollen-tube nutrition, and the penetration of the stigma surface. Moistening of the intine must result not only in the emission of enzymes capable of metabolizing substrates encountered on the stigma surface, but in activating others affecting the pollen wall itself, possibly preparing the way for pollen tube emergence (Roggen & Stanley, 1969). It is interesting in this connexion that the newly emergent pollen tube may sometimes carry with it an enzyme-containing cap (Fig. 25), although, as will be reported in a further paper, this is not the only mode of behaviour.

Since all of the angiosperms and the one gymnosperm tested uniformly possess the wall enzymes, a connexion with the heterotrophic mode of nutrition of the male gametophyte may be suspected, particularly since the spores of the two ferns, which germinate to give autotrophic gametophytes, do not. However, Equisetum spores resemble those of the spermatophytes in possessing wall enzymes. Clearly it will be informative to explore correlations between mode of gametophyte nutrition and presence or absence of wall enzymes in more detail.
Wall proteins and pollen allergenicity

There is no doubt that in the mature pollen grain as shed from the anther the principal sites of enzyme activity are in the wall. Moreover, we have the impression that a large proportion of the total protein borne by the pollen grain must be lodged in the wall sites, although this cannot yet be substantiated quantitatively. It is of course possible that, in addition to the enzymes, proteins concerned with the incompatibility reaction are carried in the wall. Since the function of all these proteins would seem to require that they be readily released on the stigma, they are certain to be among the first to be emitted in contact with other biological surfaces. They are therefore strongly suspect as being concerned in pollen allergenicity. An immunological study of this point is currently in progress.

Developmental aspects

The enzymes are incorporated in the wall sites during the very early life of the spore, in the period of wall growth following release from the meiotic tetrad. As we have shown, the process is related to the existence of ribosomal endoplasmic reticulum in the cortical cytoplasm of the spore, and the circumstantial evidence is therefore strong that the synthesis is a de novo one managed by the spore itself; there is seemingly no question of the intine enzymes being derived from surrounding maternal tissue, whatever the source of the later-applied sexine proteins may be.

While the actual synthesis may be carried out within the spore, it remains to be shown whether the process is dependent upon gene action in the haploid spore nucleus, or whether it is programmed by persistent m-RNA bequeathed from the pre-meiotic mother cell. Although there is a radical reorganization of the mother-cell cytoplasm during the meiotic prophase (Mackenzie, Heslop-Harrison & Dickinson, 1967), there is reason to believe that some diplophase-derived 'information' may be carried through to the spores (Heslop-Harrison, 1968a). It is perhaps significant that in the sterile grains of Dianthus synthesis proceeds normally, since this could be taken to imply that this part of spore metabolism does not depend upon a functional and balanced haplophase genome. If it can be shown that even in severely aneuploid grains the full range of enzymes is present, the case for diplophase control would be essentially proved, since it is inconceivable that the loci concerned would invariably be located in the chromosomes that do happen to be included in each spore nucleus, whatever the pattern of meiotic segregation.

The actual process of protein incorporation in the intine is of considerable interest since it accompanies the deposition of cellulose. The inclusion of cytoplasmic material in the course of intine growth was first observed in Silene pendula, a species with a polyporate grain very similar to that of Dianthus (Heslop-Harrison, 1963a, b). In Silene granular material and membrane-bounded vesicles were noted at the plasma-lemma, particularly at the pore regions, and it was concluded that these were incorporated in the lamellated intine. Similar observations were made by Erdtman & Dunbar (1966) in Armeria species. There are now many published accounts of intine inclusions in mature pollens considered to be derived from the spore cytoplasm, and
it is invariably true that the greatest concentrations are observed at aperture sites, where these are present. Among papers commenting upon, or at least illustrating, such intine inclusions are those of Rowley, Mühlethaler & Frey-Wyssling (1959); Larson & Lewis (1961); Rowley (1963, 1966); Barth (1965, 1966); Skvarla & Larson (1966); Godwin et al. (1967); Guinet & Barth (1967); Rowley & Erdtman (1967); Rowley & Dunbar (1967); and Roland (1968).

The incorporation of the wall enzymes is concluded before the end of intine growth, and so precedes pollen mitosis and the final accumulation of reserves in the pollen cytoplasm. Neat confirmation that the principal syntheses of certain enzymes are concluded during the early life of the spore is provided by the work of Linskens (1966) on the pollen enzymes of *Lilium* using gel electrophoresis. This study showed that acid phosphatase, thiamine pyrophosphatase and peroxidase, absent or at low levels in the mother cells during the later stages of meiosis, appeared around the tetrad period, increasing rapidly in the young spores to a maximum which was maintained through to the mature pollen. For acid phosphatase, our observations are in close agreement with those of Linskens for the post-tetrad period.

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R. B. K. is on leave from the Botany Department, Australian National University, Canberra.

REFERENCES


Pollen wall proteins


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Fig. 1. *Gladiolus gandavensis*, freeze-sectioned pollen. A, PAS localization of intine cellulose; B, approximately the same field before staining, showing natural absorption at 285 nm attributable to intine proteins; C, same photographed at 540 nm; and D, same, phase-contrast. × 100 approx.

Fig. 2. *Gladiolus gandavensis*, freeze-sectioned pollen. A, NYS localization of intine protein, photographed at 430 nm; B, PAS localization of intine cellulose; C, RNase reaction; D, RNase control + 0.01 M NaF as inhibitor; E, RNase reaction, intact pollen electrophoresed before testing. × 1000 approx.

Fig. 3. *Crocus vernus*, freeze-sectioned pollen. A, Acid phosphatase reaction; B, acid phosphatase control less substrate. Naphthol AS-BI phosphate-pararosanilin reaction. × 1000 approx.

Fig. 4. *Lilium longiflorum*, freeze-sectioned young spore, RNase reaction. The activity is practically restricted to the area of the colpus. × 900 approx.

Fig. 5. *Lilium longiflorum*. As Fig. 4, but showing the area of activity associated with the colpus in longitudinal view. × 900 approx.
Pollen wall proteins

1A

1B

1C

1D

2A

2B

2C

2D

2E

3A

3B

4

5
Fig. 6. *Alopecurus pratensis*, freeze-sectioned pollen. NYS localization of poral protein photographed at 430 nm. There has been some loss of protease from the grain with resultant digestion of the encasing gelatine in the vicinity prior to fixation in methanol/acetic acid. × 1000 approx.

Fig. 7. *Alopecurus pratensis*, fresh pollen, absorption at 285 nm. Most pores show an absorbing annulus, probably associated with the poral intine. × 400 approx.

Fig. 8. *Alopecurus pratensis*, freeze-sectioned pollen, acid phosphatase in poral intine, α-naphthyl acid phosphate-pararosanilin reaction. × 1000 approx.

Fig. 9. *Coix lacryma-jobi*, freeze-sectioned pollen, acid phosphatase in poral intine, α-naphthyl acid phosphate-pararosanilin reaction. × 1000 approx.

Fig. 10. *Lolium temulentum*, freeze-sectioned pollen. A, RNase reaction in poral intine; B, RNase control without substrate. × 1000 approx.

Fig. 11. *Hibiscus rosa-sinensis*, freeze-sectioned pollen. A, Pores in a surface sliver, RNase reaction; B, pores in a surface sliver, RNase control + 0.01 M NaF as inhibitor. × 1000 approx.

Fig. 12. *Dianthus chinensis*, freeze-sectioned pollen, RNase reaction, showing localization in the poral intine with slight activity in the inter-poral zones. × 1000 approx.
Fig. 13. *Hibiscus rosa-sinensis*, freeze-sectioned pollen, showing localization of acid phosphatase. Naphthol AS-BI phosphate-pararosanilin reaction. $\times 250$ approx.

Fig. 14. *Malvaviscus arboreus*, freeze-sectioned young spores at the early vacuolate stage, showing concentration of phosphatase activity in the poral intine. Naphthol AS-BI phosphate-pararosanilin reaction. $\times 250$ approx.

Fig. 15. *Malvaviscus arboreus*, freeze-sectioned mature pollen wall. A, Naphthol AS-BI phosphate substrate for acid phosphatase, with Fast Garnet GBC as coupling agent; b, control without substrate. $\times 1000$ approx.

Fig. 16. *Malvaviscus arboreus*, freeze-sectioned pollen, NYS protein localization after treatment with ammonium sulphate. A, Photographed at 430 nm showing concentration in the pore; b, same preparation, photographed at 540 nm. $\times 1000$ approx.

Fig. 17. *Malvaviscus arboreus*, surface sliver of mature pollen grain, NYS protein localization after fixation in methanol/acetic acid for 3 h at room temperature. Proteases leaking from the wall fragment have digested the surrounding gelatine. $\times 400$ approx.

Fig. 18. *Malvaviscus arboreus*, freeze-sectioned pollen. A, RNase reaction; b, RNase control + 0.01 M NaF as inhibitor. $\times 1000$ approx.

Fig. 19. PAS, localization of polysaccharides. The interior of the grain is darkened due to stored starch, but the intine plugs in 2 colpi can be discerned. × 1000 approx.

Fig. 20. Grain in approximately equatorial section, NYS protein localization photographed at 430 nm, after fixation in methanol/acetic acid for 3 h at room temperature. Proteases diffusing from the colpi have digested the encasing gelatine in 3 arcs. × 1000 approx.

Fig. 21. A, Grain in approximately equatorial section, RNase reaction; B, RNase control + 0.01 M NaF as inhibitor. × 1000 approx.

Fig. 22. Acid phosphatase localization in colpial intine, α-naphthyl acid phosphate-pararosanilin reaction. × 1000 approx.

Fig. 23. Colpus in surface view, RNase reaction. × 1000 approx.

Fig. 24. Grain in equatorial view, showing the intine papillae at each colpus, somewhat exserted. RNase reaction. × 1000 approx.

Fig. 25. Pollen tube emerging from 1 colpus, RNase reaction. Enzyme activity is concentrated mainly in the plug of intine carried out at the tip of the extending tube. × 1000 approx.

Fig. 26. *Tragopogon pratensis*, sliver from grain surface showing the colpus in face view, acid phosphatase, α-naphthyl acid phosphate-pararosanilin reaction. Activity is concentrated in the intine papilla, in the centre of the colpus and at the colpial margins. × 1000 approx.

Fig. 27. *Pinus banksiana*, freeze-sectioned pollen. PAS localization of intine cellulose. × 1000 approx.

Fig. 28. *Pinus banksiana*, freeze-sectioned pollen. Localization of acid phosphatase in the intine near the germination zone between the alae. α-Naphthyl acid phosphate-pararosanilin reaction. × 1000 approx.

Fig. 29. *Dianthus chinensis*, surface sliver of a sterile pollen grain, blocked in development at the vacuolated stage, showing poral localization of RNase. × 1000 approx.

Fig. 30. *Dianthus chinensis*, sterile grain in section showing poral RNase. × 1000 approx.

Fig. 31. *Dianthus chinensis*, sterile grain in section showing PAS localization of intine cellulose. × 1000 approx.
Pollen wall proteins
Figs. 32-35. Electron micrographs of spores of *Cosmos bipinnatus* in the vacuolate stage during active intine growth. (*c*, cavus between sexine and nexine; *i*, intine; *n*, nexine; *pl*, protein lamellae; *pm*, plasmalemma; *s*, sexine; *sc*, sexine cavities; *tp*, tapetal plasmodium.)

Fig. 32. Intine and adjacent cytoplasm at the margin of a colpus in radial section. × 90000 approx.

Fig. 33. Intine and adjacent cytoplasm in the central region of a colpus in tangential section. Polysome configurations are seen in the underlying cytoplasm and surfaces of the protein lamellae in the cytoplasm itself. × 90000 approx.
Pollen wall proteins
Fig. 34. Intine and adjacent cytoplasm in the mesocolpial region, showing the incorporation of protein lamellae. \( \times 62,000 \) approx.

Fig. 35. Spine base. The tapetal plasmodium has invaded the cavities in the sexine and is present also in the cavus between sexine and nexine, where ribosomes and membrane-bounded vesicles may be seen. \( \times 48,000 \) approx.
Pollen wall proteins