

POLLEN-WALL PROTEINS: RELEASE OF THE ALLERGEN ANTIGEN E FROM INTINE AND EXINE SITES IN POLLEN GRAINS OF RAGWEED AND *COSMOS*

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SUMMARY

Cytochemical staining methods and immunofluorescence techniques have been used to follow the release of proteins, including the allergen Antigen E, from whole pollen of ragweeds (*Ambrosia* spp.) and *Cosmos bipinnatus* (Compositae). These proteins have been detected in 2 sites in the wall: in the inner cellulosic intine layer especially at the germinal apertures, and in the cavea and the cavities of the outer exine layer, the sexine. It was found that the release of exine-associated proteins, including the allergenic fraction, begins within 2 s of contact with aqueous media, the loss being mainly through the micropores of the tectum at the bases of the sexine spines. The intine proteins were emitted more slowly, discharge beginning at the colpi after 1 min of moistening. So far as can be judged from the methods used, most of the mobile protein lost from the pollen of *Cosmos* on hydration comes from the exine and is therefore sporophytic in origin, while in the ragweeds the bulk comes from the intine in the neighbourhood of the germinal apertures, and so is gametophytic.

INTRODUCTION

Among the proteins released from moistened pollen grains of *Ambrosia* spp. (ragweeds) are several important as causes of atopic allergy. King & Norman (1962) and King, Norman & Connell (1964) have characterized 2 acidic protein allergens, Antigens E and K, from *Ambrosia* pollen, and since the publication of their results, others have identified also 2 basic protein allergens (Underdown & Goodfriend, 1969; Griffiths & Brunet, 1971). In a previous paper (Knox & Heslop-Harrison, 1971) we have reported that Antigen E is held in the inner cellulosic intine of the pollen grain wall, a layer known to contain several hydrolytic enzymes (Knox & Heslop-Harrison, 1970).

Wall-held materials are released very rapidly from mature pollen grains on moistening, and in work with *Ambrosia* spp. using slurries of mature pollen in gelatin freeze-sectioning media we have found that antigens may be lost even with preparation times as short as 30 s. This fact had hitherto made it difficult to establish what sites in the pollen grain wall of the Compositae may be contributing antigenic materials other than the intine itself. That such materials may be held elsewhere has become clear from the unequivocal demonstration for the large pollen grains of the Malvaceae that

although the intine is the locus of gametophytically-derived proteins other sites exist in the exine which harbour materials from the tapetum, a sporophytic tissue (Heslop-Harrison, Heslop-Harrison, Knox & Howlett, 1973). In the present paper we show that antigenic materials, including the allergen, Antigen E, are carried in both intine and exine of the pollen wall of *Ambrosia* and *Cosmos*, and give some account of the pathways through which these materials are lost from the moistened grain.

MATERIALS AND METHODS

Plant material

Observations were made both on stored and fresh pollen samples of ragweed species. Stored pollen of *Ambrosia elatior* and *A. trifida* was obtained from Greer Laboratories, Lenoir, N.C., U.S.A. Fresh pollen was taken from greenhouse-grown plants of *A. tenuifolia*, *A. psilostachys* and *A. artemisifolia* transplanted from wild populations in Newcastle and Sydney, N.S.W. and Brisbane, Queensland, Australia.

The small size (c. 18 μm diameter) of ragweed pollen grains makes precise observation by optical microscopy difficult, since the low spines that cover the surface are very densely packed. As a closely comparable model system, we have used pollen from *Cosmos bipinnatus* (Tribe Heliantheae), derived from greenhouse-grown plants. Pollen grains of *Cosmos* are about twice the diameter of those of the allergenic ragweeds, and the spines and germinal apertures are readily resolved with the light microscope. We have shown that there is considerable immunological cross-reactivity between at least 2 of the antigens of *Ambrosia* and *Cosmos*, and that an Antigen E-like protein fraction is present in the walls of *Cosmos* pollen (Knox, 1973). With the known structural and other similarities, observations of the pathways of release of wall-held materials made on *Cosmos* can therefore be confidently extrapolated to *Ambrosia*.

Sample preparation

Anthers or mature dry pollen were freeze-sectioned in a gelatin-glycerol medium as in earlier work (Knox, 1970), but improved results were obtained using high-molecular-weight gelatin samples kindly provided by Davis Gelatine (Australia) Pty. Ltd. Sections for immunofluorescence were cut in a IEC Cryostat at 2 μm thickness, and treated as described previously (Knox, 1971).

To stabilize the antigens in their wall sites and minimize diffusion during processing, various fixation methods were tried. Dry pollen was fixed in 40 % formaldehyde vapour for 4 or 24 h, then freeze-sectioned. Alternatively, pollen was fixed in methanol-cyanuric chloride as described by Goland, Grand & Booker (1969), dried and freeze-sectioned.

Freeze-substitution was adopted to achieve completely anhydrous processing. Pollen samples or anthers were frozen rapidly in liquid freon held in liquid nitrogen, then transferred for at least 120 h to several changes of dry ethanol stored in dry ice. The tissue was transferred to *n*-butanol at -70°C before being brought to $+5^\circ\text{C}$ and embedded in paraffin. The material was sectioned at 2 or 4 μm thickness in a cryostat at 10°C , and floated on to gelatin-coated slides in 'Fluorinert' electronic liquid FC-75 kindly supplied by the 3 M Co., St Paul, Minn., using the method of Chen (1972) to flatten the ribbons. The sections were deparaffinized in petroleum ether, dried and processed for immunofluorescence.

The scanning and transmission electron microscopy were carried out using procedures described previously (Heslop-Harrison, 1969; Knox & Heslop-Harrison, 1970).

Pollen-print preparation

The release of materials from moistened pollen grains was followed by preparing pollen prints on moist film media (Heslop-Harrison *et al.* 1973). The thin films were of 1 % agarose made up in Tris-barbital-sodium barbital buffer ($I = 0.05$, pH 8.8) spread on to a microscope slide (3 drops covered an area of 3 cm^2), prepared immediately before use, and kept moist until required in a plastic box. Initially pollen grains were simply sprinkled on to the film, which was

rapidly dried by a stream of warm air. However, this method resulted in excessive diffusion of antigens. Less diffusion was obtained with an alternative method, in which the dry pollen grains were first taken up on single-sided Scotch tape. The tape, bearing the grains, was then pressed gently into contact with the agarose film for periods of 5, 10 or 30 s and 1, 2, 5 or 10 min, after which the tape was removed and the film rapidly air-dried in a stream of warm dry air. The imprinted antigens, fixed by the drying, were then located by the immunofluorescence procedure.

Immunofluorescence methods

Pollen extracts were prepared by incubating 1 g of pollen with 5 ml of 0.2 M NaCl adjusted to pH 7.5 for 16 h at 2 °C. The suspensions were filtered to remove pollen grains, and antisera raised in rabbits by methods previously described (Knox, Heslop-Harrison & Reed, 1970). Anti-ragweed (*A. trifida*) serum and anti-Antigen E serum sample 'AgE' were kindly prepared by Dr C. E. Reed, while anti-Antigen E serum sample '110' was a gift from Dr T. P. King.

For the visualization of antigens in sections or pollen-prints 2 drops of the appropriate antiserum were applied, and the slides incubated in a moist chamber for 40 min at room temperature. Excess antiserum was removed by washing in 3 changes of saline solution over a period of 15 min. Two drops of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG serum (Behringwerke, Marburg Lahn, Germany) were then applied, and the slides incubated for a further 15 min at room temperature. After 3 washes in saline solution for 15 min and a rinse in distilled water, the slides were air-dried and mounted in a non-fluorescent mounting medium for observation by fluorescence microscopy using incident light illumination. Photomicrographs were taken with Kodak Tri-X or High Speed Ektachrome films.

Staining of protein emissions from whole pollen grains

Coomassie Blue has been shown to stain proteins specifically in electrophoretic strips (Fazekas de St Groth, Webster & Datyner, 1963). In the present experiments a 0.25 % solution (w/v) of Coomassie Blue (Brilliant Blue R, Sigma) was dissolved in 45 % methanol containing 10 % acetic acid, and filtered before use. For pollen tests, 2 ml of this solution was added to 4 ml saline or to 10 or 40 % sucrose solutions to give a final dye concentration of 0.09 %.

Fresh pollen was shaken on to a dry microscope slide, a coverslip was applied, and the grains viewed dry under the microscope. The Coomassie Blue solution was then introduced at the edges of the coverslip, and the events as the dye reached the grains were recorded by ciné micrography.

In order to determine the regions of the grains contributing to the protein release, the dye solution was emulsified with immersion oil. A few drops of the emulsion were placed on a microscope slide, pollen was scattered on its surface, a coverslip was gently applied, and the preparation viewed under the microscope immediately. Droplets of the dye solution were suspended in the immersion oil, and with *Cosmos* pollen the droplets were small enough to cover only single spines of the grains so that the contribution of such small regions could be assessed.

RESULTS

Structural features of the pollen wall of the Compositae

The architecture of the exine and intine of *Cosmos* as observed by transmission and scanning electron microscopy has been described and illustrated in earlier papers (Heslop-Harrison, 1968, 1969; Knox & Heslop-Harrison, 1970). It is sufficient here to note certain features of the exine seen in Figs. 1–6. In section, the baculate sexine is drawn away from the nexine in the mesocolpial regions to form the cavea, a feature of common occurrence in the Compositae (Skvarla & Turner, 1966). In material fixed in a late stage of development, the cavea are seen to contain material derived from the plasmodial tapetum (Fig. 5). As in the pollen grains of the Malvaceae (Heslop-Harrison

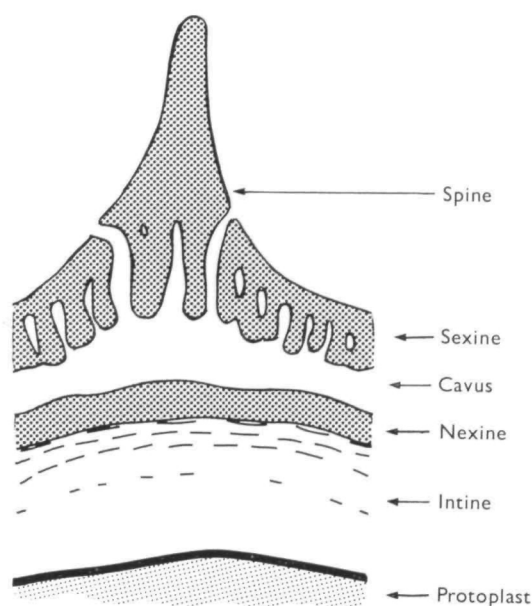


Fig. 1. Diagram of wall stratification in *Cosmos bipinnatus*. The small sexine cavities seen in Fig. 5 have been omitted for simplicity.

et al. 1973) this material is injected through micropores in the tectum, passing into the interbacular spaces and thence into the cavea. The micropores are clustered principally around the bases of the spines, and may be seen conspicuously in scanning electron micrographs of the surface of acetolysed grains (Fig. 6). The importance of these micropores as exit routes for the exine-held materials will be clear from later paragraphs.

While the exine is a repository for sporophytic proteins derived from the tapetum, the intine, particularly at the apertural sites, contains proteins inserted during growth from the cortical cytoplasm of the young spore: proteins, that is, of gametophytic origin. An account has been given of the process of incorporation of this material in the thickening intine (Knox & Heslop-Harrison, 1970).

The pollen-grain walls of *Ambrosia* are in all essentials similar to those of *Cosmos*, except that the spines of the tectum are much shorter – a feature associated with the anemophilous mode of pollination in the genus. Although the spines are less well developed, they are sited in a similar manner, and again the microspores of the tectum are clustered around their bases.

Localization of antigens in mature pollen walls

The earlier work (Knox & Heslop-Harrison, 1971) showed that antigens, including Antigen E, are concentrated in the intine, particularly at the sites of apertures, but as already noted, the mobility of the wall-held materials left some doubt about their presence in other possible sites. Better stabilization has now been obtained by improved

fixation prior to freeze-sectioning, or by freeze-substitution followed by paraffin-embedding and sectioning.

Ragweed pollen fixed for 24 h in the vapour from 40 % formaldehyde before freeze-sectioning still showed considerable antigenic activity on the surface of the grains, suggesting that rapid diffusion had occurred on moistening in the gelatin medium. Partial success in preventing diffusion of the wall proteins during freeze-sectioning was achieved by fixation of the dry pollen by cyanuration in methanol. However, equally good results were obtained when dry pollen of *Cosmos* was rapidly freeze-sectioned in high-molecular-weight gelatin buffered at alkaline pH. Fig. 8 shows that under these conditions antigenic activity was detected in the intine especially at the germinal apertures, but still greater fluorescence was seen in the cavea and cavities of the sexine after the standard immunofluorescence procedure, indicating the presence of antigens in these sites also. This localization has been confirmed in mature anthers and dry pollen of both ragweed and *Cosmos* prepared anhydrously by the freeze-substitution method (Figs. 9, 10). Reference to Fig. 5 will make it evident that the source of the exine antigens is the material injected from the tapetal plasmodium during the final maturation of the pollen.

Sites of release of wall-held proteins

Evidence from freeze-sectioned grains. Antigens are rapidly released from unfixed ragweed pollen grains in the gelatin freeze-sectioning medium (Knox & Heslop-Harrison, 1971), and it is noteworthy that the release appears to occur all around the wall, and not to be associated particularly with the colpi. Similarly in *Cosmos* the emission of antigens takes place in the freeze-sectioning medium within 30 s, but here better localization of the sites of release is possible because of the larger size of the grain. Fig. 7 shows sectioned grains with some antigens held in the intine, but with precipitins also at the surface of the exine, particularly around the bases of the spines. In the surface sliver of Fig. 7B a colpus may be seen with associated antigen localization. In these preparations, wisps of fluorescence can be seen in the gelatin, emanating from the spines. The accompanying freeze-substituted sections clearly show that the specific fluorescence is associated with the micropores (Fig. 9) seen by scanning electron microscopy (Fig. 6).

The emission of antigenic materials from the exine cavities through the micropores of the tectum was unequivocally confirmed from the results of the other techniques.

Protein emission from whole pollen grains. The dry grains of *Cosmos* are ellipsoidal (Fig. 11A), but on hydration they swell and become spherical (Fig. 11B). Within 1 s of contact with the Coomassie Blue medium, material at the base of the spines showed some colouring (Fig. 11B), and in a further second blue-staining proteins were found to pass into the medium from the spine tips (Fig. 11C). The ciné film showed that the material is initially released from the microspores at the bases of the spines, then flows down the length of each before loss into the bathing fluid. In preparations made with dye emulsions, small droplets of dye solution making contact with a spine in a grain otherwise coated with oil quickly accumulated staining protein, providing a very

precise demonstration of the importance of the basal micropores as a pathway of discharge.

The proteins held at the colpi were found to stain after 1 min, indicating a slower release. As with the pollen of the Malvaceae (Heslop-Harrison *et al.* 1973), the presence of surface *Pollenkitt* was found to prevent discharge from any site on the grain by sealing the surface from contact with the medium.

The rate of the early protein discharge and the sites from which it takes place indicate clearly enough that the loss is from the wall and not at all from the vegetative cell of the pollen grain. In preparations made with 40% sucrose, the cell membranes are preserved intact while the release of proteins proceeds, so there can be no question of loss from an autolysing protoplast.

Tests with ragweeds *A. trifida* and *A. elatior* (stored pollen) and *A. tenuifolia* (fresh pollen) gave results similar to those described for *Cosmos*. A halo of purple-stained protein appeared within seconds of wetting, apparently associated with the spines (Fig. 12A), although its origin could not be specified as precisely as in *Cosmos* (Fig. 12B).

Pollenprints. This technique provided further striking evidence of the importance of the micropores around the bases of the sexine spines as routes for the loss of antigens. With contact periods of 5–30 s fluorescence was clearly associated with each of the surface spines of the large *Cosmos* grains, whether localization in the print was with anti-Antigen E or anti-total pollen leachate serum (Fig. 13B, C). After longer periods of contact, the antigens were spread in a general halo around the site of the grain (Fig. 14), and only rarely was it possible to distinguish any concentrations indicative of the colpial sites.

With ragweed grains, whether stored *A. trifida* or fresh *A. tenuifolia* pollen samples were used, release in the neighbourhood of spines was commonly observed (Fig. 15) in 5–10 s prints. However, in 30–60 s pollenprints, the discharged antigens either formed a diffuse halo around the grains or showed concentration at 1, 2 or 3 sites around the imprint (Fig. 16B). Such localized distribution is of course attributable to release from the colpial sites (Fig. 16A), the pattern depending upon the number of colpi making contact with the film surface. The difference between pollen prints obtained from *Cosmos* and those from *Ambrosia* grains is presumably due to the contrast in surface topography; with the smaller spines on the ragweed tectum, the apertures must be much more accessible to the moist film than in *Cosmos*.

DISCUSSION

The results reported above show that in the mature pollen of *Cosmos* and *Ambrosia* antigenic proteins are present in 2 distinct sites in the pollen wall: in the inner cellulosic intine layer, which has also been shown to be the site of enzymic proteins (Knox & Heslop-Harrison, 1970), and in the interbacular cavities and the cavea of the exine. The material in the latter site is derived from the tapetum, as has been demonstrated for the Malvaceae (Heslop-Harrison *et al.* 1973).

Antigenic materials are present in both intine and exine, and the tests with the 2 anti-Antigen E sera leave no doubt that proteins with antigenic determinants similar

to those of Antigen E are present in the 2 sites. Immunodiffusion and immunoelectrophoretic tests have shown that only a single precipitin line is formed when pollen extracts of ragweed are run against anti-Antigen E serum no. 110 (Knox, 1973, and unpublished), but this is inadequate evidence of lack of heterogeneity, and whether the Antigen-E-like fractions of exine and intine are identical in other than immunogenic properties cannot yet be said. The point is under study.

It will be evident that these new findings, while not conflicting with our earlier observations, show that they did not give a comprehensive picture of the protein load of the pollen grain wall of the Compositae. Earlier we were impressed by the association of both enzymes and antigens with the cellulosic intine, and were not fully enough aware of the mobility of the exine-held proteins. The combination of techniques now used leaves no doubt of the importance of the fractions held in the cavea of the exine, which contribute with the intine-held moiety to the outflow of protein from the moistened grain. It may be noted that our experience shows that the procedures used by Hubscher & Eisen (1972) for the antigens of ragweed pollen would be wholly inadequate to give good localization. These authors used aqueous solutions to flatten their paraffin sections of unfixed pollen, and subjected these same sections after the removal of the paraffin to a 15-min incubation in buffer before applying immunofluorescence procedures. It will be obvious that this last wash would be an excellent method of extracting proteins from the wall sites, both exine and intine, and so of inducing diffusion artifacts.

Turning to the pathways of release of the wall-held proteins, we may note that the observations reported now for the Compositae complement those for the Malvaceae (Heslop-Harrison *et al.* 1973), and give further evidence bearing on the functions of the various structural features of the exine that have long been matter for speculation. It is now quite evident that the pollen grain wall plays many roles in the life of the male gametophyte (Heslop-Harrison, 1971). The protective function is evident enough, and the likely importance of surface sculpturing in relation to mode of dispersal has been emphasized by Wodehouse (1935) and others. It is now clear that the *apertures* have other functions than to provide a path of exit for the pollen tube: they are evidently important as sites for the storage and release of gametophytic proteins, including the intine-held enzymes and probably also recognition substances.

These results with ragweed are supported by recent immunofluorescence observations of the fate of the thermostable allergens of *Betula* pollen (Belin & Rowley, 1971). The allergenic proteins were detected all around the exterior surface of whole grains moistened only during the exposure to specific antiserum. However, when whole pollen grains were placed on antiserum-in-agar and the proteins allowed to diffuse out over a 24-h period, precipitin complexes were detected forming haloes around the 3 germinal apertures. Thus, just as in ragweed, the birch allergens are initially released from all over the exine surface but, in the long term, the greatest release is from the germinal apertures.

Perhaps the most important contribution of the present study and that of the malvaceous pollen grain (Heslop-Harrison *et al.* 1973) is the demonstration of the functional importance of the complex internal architecture of the sexine layer of the exine. The

baculate layer – a feature almost universally present in angiosperm pollens – is now seen to be basically a storage site for materials derived from the tapetum. In the tectate exine, the interbacular cavities communicate with the environment through the micropores, which provide both the passage for injection of the stored materials and for their release when the grain is moistened. In non-TECTATE exines, the morphologically homologous sites – the spaces between the pilae or muri – are also concerned with storage of tapetum-derived materials (Heslop-Harrison, 1968; Dickinson & Lewis, 1973). In the Compositae, the exine storage capacity is further enhanced by the development of cavea, which provide a relatively enormous reservoir for products transferred from the sporophyte.

It is entirely probable that these materials themselves have several functions, among them, as proposed earlier (Heslop-Harrison, 1967), a role in inter- and intra-specific incompatibility systems where the genetical control resides with the parental sporophyte. A function for the wall proteins in the control of interspecific incompatibility systems has been demonstrated for the genus *Populus* (Knox, Willing & Pryor, 1972; Knox, Willing & Ashford, 1972), where wall proteins from the pollen of one species have been used to make pollen of an incompatible foreign species acceptable. Direct evidence for their function in the sporophytic intraspecific incompatibility system of *Raphanus sativus* (Cruciferae) is now available from the work of Dickinson & Lewis (1973). In *Cosmos bipinnatus*, which also has an incompatibility system of the sporophytic type (Crowe, 1954), extracts of compatible pollen have been used to make self-pollen acceptable on the stigma (B. J. Howlett, R. B. Knox & J. Heslop-Harrison, in preparation), and there is every reason to suppose that the fraction concerned will prove to be derived from the exine, having been inserted from the tapetum. We have also been able to show that the plasmodial tapetum of *Cosmos* in late developmental stages contains antigens similar to those present in leachates of mature pollen. The allergenic activity of these wall proteins seems to be no more than an unfortunate accident of evolution, their actual role being to act as recognition substances during these pollen-stigma interactions.

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REFERENCES

- BELIN, L. & ROWLEY, J. (1971). Demonstration of birch pollen allergen from isolated pollen grains using immunofluorescence and a single radial immunodiffusion technique. *Int. Archs Allergy appl. Immun.* **40**, 754–769.
- CHEN, V. K. (1972). New method for relaxing paraffin sections in histochemical fluorescence experiments. *Nature, New Biol.* **236**, 15.
- CROWE, L. K. (1954). Incompatibility in *Cosmos bipinnatus*. *Heredity* **8**, 1–11.
- DICKINSON, H. G. & LEWIS, D. (1973). Cytochemical and ultrastructural differences between intraspecific compatible and incompatible pollinations in *Raphanus*. *Proc. R. Soc. B* **183**, 21–38.

- FAZEKAS DE ST GROTH, S., WEBSTER, R. G. & DATYNER, A. (1963). Two new staining procedures for quantitative estimation of proteins in electrophoretic strips. *Biochim. biophys. Acta* **71**, 377-391.
- GOLAND, P., GRAND, N. G. & BOOKER, B. F. (1969). Immunofluorescence microscopy of cyanurated tissues. *Stain Technol.* **44**, 227-233.
- GRIFFITHS, G. W. & BRUNET, R. (1971). Isolation of a basic protein antigen of low ragweed pollen. *Can. J. Biochem.* **49**, 396-400.
- HESLOP-HARRISON, J. (1967). Ribosome sites and S gene action. *Nature, Lond.* **218** 90-91.
- HESLOP-HARRISON, J. (1968). Pollen wall development. *Science, N.Y.* **161**, 230-237.
- HESLOP-HARRISON, J. (1969). Scanning electronmicroscopic observations on the wall of the pollen grain of *Cosmos bipinnatus*, Compositae. *Proc. Engis Stereoscan Colloquium*, pp. 89-96. Illinois: Morton Grove.
- HESLOP-HARRISON, J. (1971). Sporopollenin in the biological context. In *Sporopollenin* (ed. J. Brooks, P. R. Grant, M. Muir, P. van Gijzel & G. Shaw), pp. 1-30. London: Academic Press.
- HESLOP-HARRISON, J., HESLOP-HARRISON, Y., KNOX, R. B. & HOWLETT, B. (1973). Pollen-wall proteins: 'gametophytic' and 'sporophytic' fractions in the pollen walls of the Malvaceae. *Ann. Bot.* **37**, 403-412.
- HUBSCHER, T. & EISEN, A. H. (1972). Localization of ragweed antigens in the intact ragweed pollen grain. *Int. Archs Allergy appl. Immun.* **42**, 466-473.
- KING, T. P. & NORMAN, P. S. (1962). Isolation studies of allergens from ragweed pollen. *Biochemistry, N.Y.* **1**, 709-720.
- KING, T. P., NORMAN, P. S. & CONNELL, J. T. (1964). Isolation and characterization of allergens from ragweed pollen II. *Biochemistry, N.Y.* **3**, 458-468.
- KNOX, R. B. (1970). Freeze-sectioning of plant tissues. *Stain Technol.* **45**, 265-272.
- KNOX, R. B. (1971). Pollen-wall proteins: localization, enzymic and antigenic activity during development in *Gladiolus* (Iridaceae). *J. Cell Sci.* **9**, 209-237.
- KNOX, R. B. (1973). Pollen-wall proteins: cytochemical observations of pollen-stigma interactions in ragweed and *Cosmos* (Compositae). *J. Cell Sci.* **12**, 421-443.
- KNOX, R. B. & HESLOP-HARRISON, J. (1970). Pollen-wall proteins: localization, and enzymic activity. *J. Cell Sci.* **6**, 1-27.
- KNOX, R. B. & HESLOP-HARRISON, J. (1971). Pollen-wall proteins: localization of antigenic and allergenic proteins in the pollen grain walls of *Ambrosia* spp. (ragweeds). *Cytobios* **4**, 49-54.
- KNOX, R. B., HESLOP-HARRISON, J. & REED, C. (1970). Localization of antigens associated with the pollen grain wall by immunofluorescence. *Nature, Lond.* **225**, 1066-1068.
- KNOX, R. B., WILLING, R. R. & ASHFORD, A. E. (1972). Role of pollen-wall proteins as recognition substances in interspecific hybridization in poplars. *Nature, Lond.* **237**, 381-383.
- KNOX, R. B., WILLING, R. R. & PRYOR, L. D. (1972). Interspecific hybridization in poplars using recognition pollen. *Silvae Genet.* **21**, 65-69.
- SKVARLA, J. J. & TURNER, B. L. (1966). Systematic implications from electron microscopic studies of composite pollen - a review. *Ann. Mo. bot. Gdn* **53**, 220-256.
- UNDERDOWN, B. J. & GOODFRIEND, L. (1969). Isolation and characterization of a protein allergen from ragweed pollen. *Biochemistry, N.Y.* **8**, 980-989.
- WODEHOUSE, R. P. (1935). *Pollen Grains*. New York: McGraw-Hill.

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Figs. 2-4. Scanning electron micrographs of whole grains of ragweed, *Ambrosia trifida*. (SEM micrographs by Dr D. F. Cutler.)

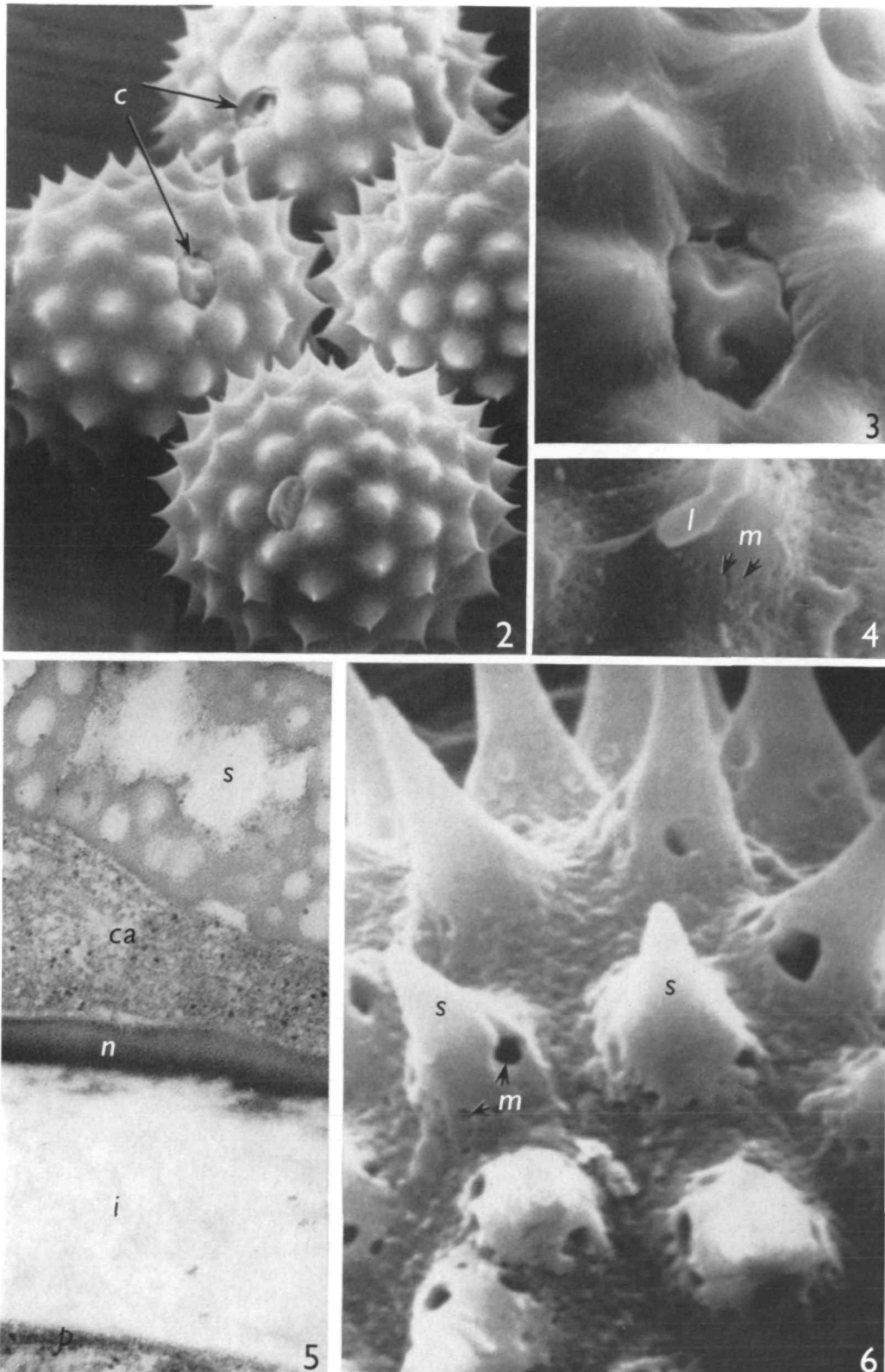
Fig. 2. Ether-washed grains, showing densely packed spines rising from the exine, and germinal apertures or colpi (*c*). $\times 4500$ approx.

Fig. 3. Colpus at higher magnification, prepared as Fig. 2; note prominent furrows on flanks of spines. $\times 15000$ approx.

Fig. 4. Partially-acetolysed grain with some remaining surface lipid (*l*), showing microspores (*m*) at base and on flanks of spines. Each micropore is approx. 20 nm in diameter. $\times 15000$ approx.

Fig. 5. Transmission electron micrograph of radial section of pollen grain wall of *Cosmos bipinnatus* showing sexine (*s*) with cavities, cavus (*ca*) filled with granular-fibrillar proteinaceous material, nexine (*n*), intine (*i*) and plasmalemma (*p*). (TEM photograph by Dr H. G. Dickinson.) $\times 62000$ approx.

Fig. 6. Scanning electron micrograph of surface of pollen grain of *C. bipinnatus* showing spines (*s*) and prominent micropores (*m*) at base of spines. $\times 15000$ approx.



Figs. 7, 8. Pollen grains of *C. bipinnatus* freeze-sectioned after slurry preparation within 30 s, immunofluorescence method.

Fig. 7. Prepared in standard freeze-sectioning medium. Fig. 7A, median sections through grains. Fig. 7B, surface sliver containing colpus region; anti-Antigen E serum no. AgE. Fluorescing precipitins are concentrated on the outer surface of the grains. $\times 1200$ approx.

Fig. 8. Prepared in high-molecular-weight gelatin to reduce wall protein mobility, buffered at pH 7.5 to minimize proteolytic activity; immunofluorescence technique using cross-reacting anti-Ragweed serum. Fig. 8A, fluorescing precipitins are located in 3 sites in the wall: intine (*i*), cavea (*ca*), and on the surface of the sexine (*s*). Fig. 8B shows section treated with normal serum as a control. $\times 1200$ approx.

Fig. 9. Pollen of *C. bipinnatus*, freeze-substituted, embedded in paraffin and sectioned at $2\ \mu\text{m}$ thickness. All processing under anhydrous conditions, to avoid the possibility of diffusion of the mobile wall proteins. Immunofluorescence methods using anti-*Cosmos* serum. In these surface slivers, the intine is absent and fluorescing precipitins are located only in the micropores at the base of the spines, demonstrating unequivocally the presence of antigens in these sites in the mature grain (for geometry, cf. Fig. 6). $\times 1200$ approx.

Fig. 10. Pollen of ragweed, *A. trifida*, prepared as Fig. 9, immunofluorescence method using anti-Antigen E serum no. AgE. Fig. 10A, phase-contrast image; Fig. 10B, fluorescence image; Fig. 10C, parallel section treated with normal serum as a control. The fluorescing precipitins are concentrated in the intine, but some sexine-located activity is also present. $\times 1200$ approx.

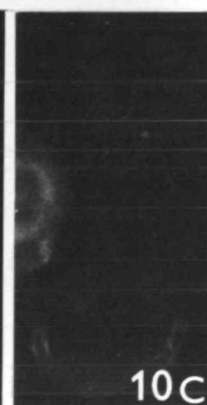
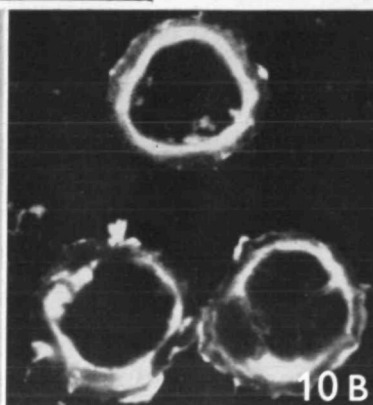
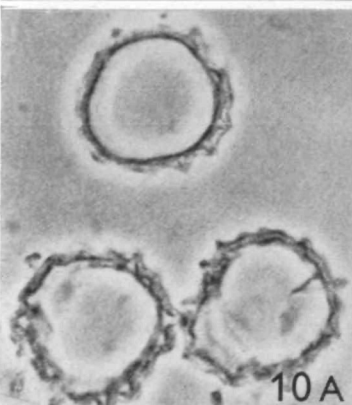
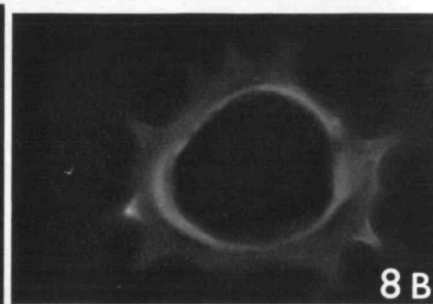
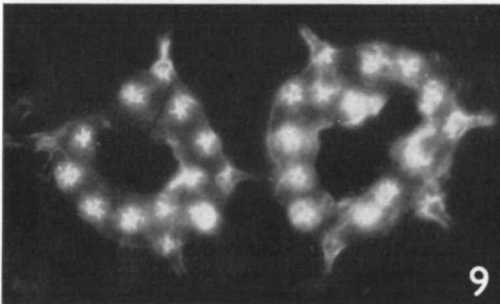
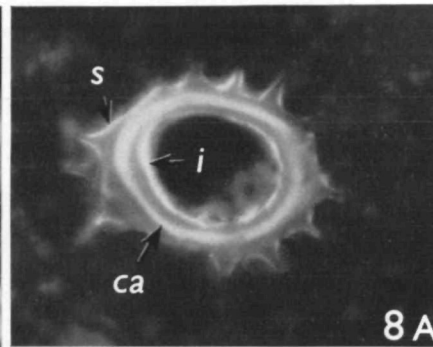
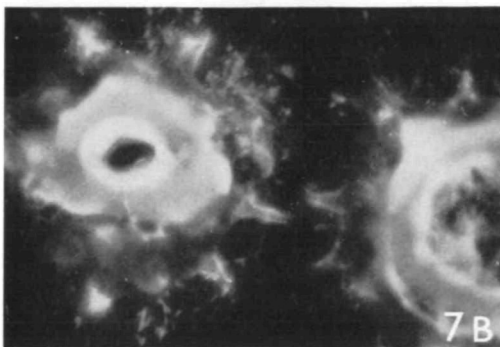
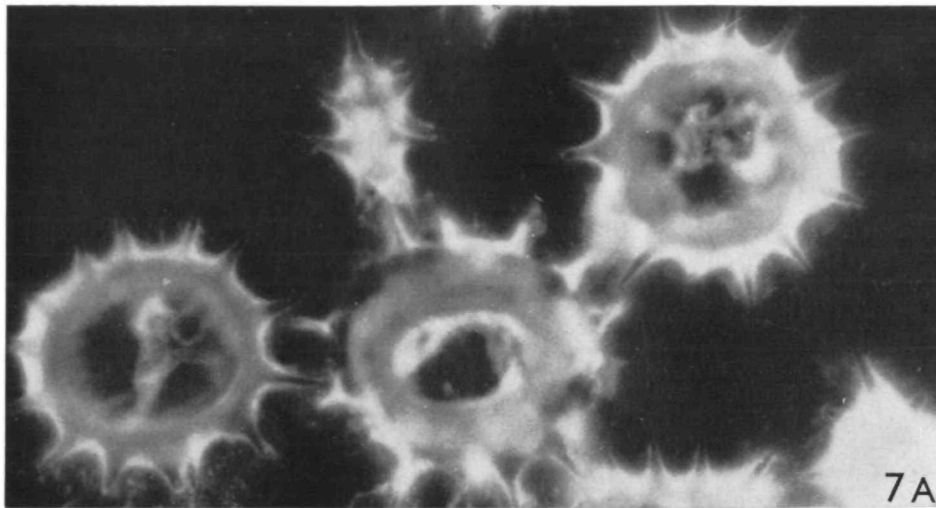


Fig. 11. Sequence of frames from a 16-mm Kodachrome cine film prepared to show the release of proteins from whole grains of *C. bipinnatus*. Fig. 11A, dry grains in the field of view at zero time. Figs. 11B-D show the result of addition of 0.09% Coomassie Blue solution (in 80% saline, 17% methanol and 3% acetic acid, v/v). Fig. 11B, frame exposed 1 s after addition of the dye solution. The grains have swelled and already the bases of the spines (arrows) stain a purple colour. Fig. 11C, frame exposed after 3 s. The proteins are being released and flowing down the surface of the spines to be lost into the medium at the tips. The colpus (c) shows little staining. Fig. 11D, frame exposed 9 s after addition of the dye solution. The stained proteins have aggregated around the spines of the grains (cf. Fig. 12B) and there is still only slight colpus staining $\times 600$ approx.

Fig. 12. Release of Coomassie Blue-stained proteins from pollen grains. Fig. 12A, ragweed, *A. trifida*; Fig. 12B, *C. bipinnatus* after 2 min, as in Fig. 11. In the ragweed, the release is all around the grain surface but is markedly less than in *Cosmos*, where release is clearly associated with the spines. $\times 1200$ approx.

Fig. 13. Pollenprints of rapidly released proteins from whole *Cosmos* pollen, immunofluorescence technique using anti-*Cosmos* serum. Fig. 13A, control print, prepared as in Fig. 13B but treated with normal serum. Fig. 13B, print from grains exposed to the agarose film for only 5 s. The reaction is concentrated around sites of spines, though there is slight antigenic activity diffused around the grains even with this brief exposure. Fig. 13C, print from grains exposed to gel for 30 s. Intense reaction marks the entire site of the grains, with precipitin aggregates around sites of spines (dark circles). Note that antigen release is concentrated beneath sites of grains, with only slight diffusion into surrounding gel. $\times 1200$ approx.

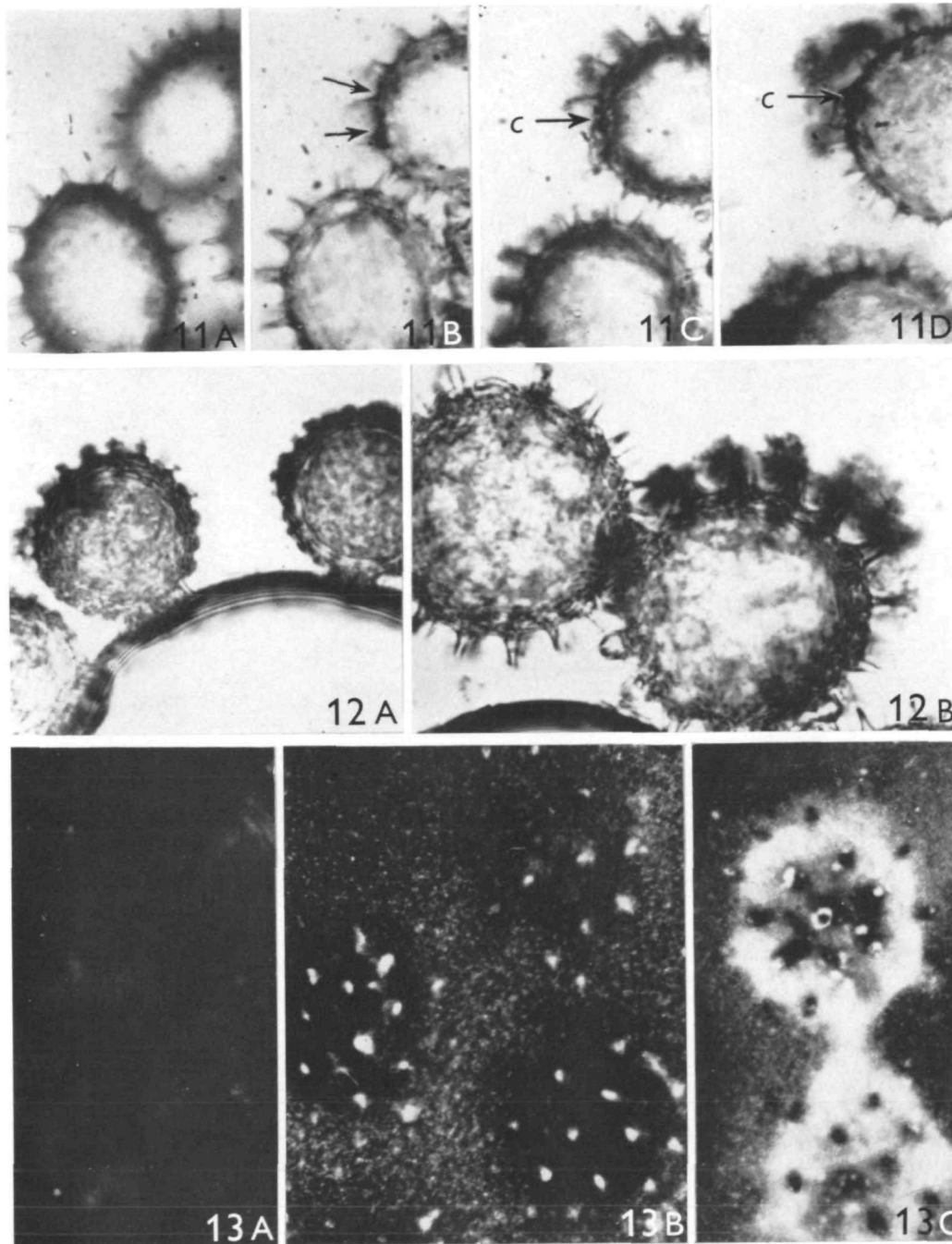


Fig. 14. Pollenprints of rapidly released proteins from *Cosmos* pollen, prepared as in Fig. 13, but exposed to gel for 1 min. Fig. 14A, general view of a field showing the sites of individual grains marked by clustered aggregates of precipitin in the now diffused surrounding gel which shows intense activity. $\times 500$. Fig. 14B, site of one grain at high magnification for comparison with shorter-term prints of Figs. 13B, C. $\times 1500$.

Fig. 15. Pollenprint of ragweed, *A. trifida*, exposed to gel for 5 s, immunofluorescence technique using anti-Antigen E serum no. AgE. Small clustered aggregates of precipitin mark the sites of individual grains. $\times 1200$ approx.

Fig. 16. Pollen of ragweed, *A. trifida*, exposed to agarose gel for 30 s, immunofluorescence technique using anti-Antigen E serum no. AgE. Fig. 16A, whole grain adhering to film, oriented to show fluorescing precipitins associated with the 3 colpi. Fig. 16B, pollenprint from same preparation. Three haloes of precipitin surround the site where the grain had made contact with the film (centre), produced by release of Antigen E from the colpi. $\times 1200$ approx.

