

Ultrastructural Morphology and Allergen Detection in Birch Pollen After Aqueous, Anhydrous-Liquid, and Vapor Fixation Techniques

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ABSTRACT In order to find a good compromise between preservation of ultrastructural morphology and retention of antigenicity, birch pollen grains were chemically fixed in aqueous p-formaldehyde or glutaraldehyde, in p-formaldehyde or glutaraldehyde dissolved in anhydrous glycerol, and in p-formaldehyde or glutaraldehyde vapor. Representative cytoplasmic areas were inspected for the preservation of ultrastructural morphology and for their capacity to bind a monoclonal antibody against Bet v I, the major birch pollen allergen. The experiments showed that cytoplasmic morphology was best preserved after vapor fixation in p-formaldehyde. This fixation also led to the highest degree of specific antibody binding. © 1992 Wiley-Liss, Inc.

INTRODUCTION

In biological electron microscopy, the fixation of dry cells or tissues (seeds, spores, lichens) and of soluble proteins (e.g., steroid receptor proteins, pollen proteins) presents a difficult task for the methodologist. Rehydration must strictly be prevented for this would give a distorted and therefore false image of the specimen. In the case of soluble proteins, rehydration would possibly involve a migration and perhaps complete loss of such components.

There are many hints that conventional aqueous fixation of dry specimens for electron microscopy leads to a certain rehydration of the cells before the effect of fixation sets on. Soluble proteins are displaced and eventually lost (Hallam, 1976). Therefore, alternative fixation strategies have been invented: 1) non-aqueous fixation in anhydrous fluids such as dimethylsulfoxide or glycerol (Hallam, 1976); 2) vapor fixation using fixatives with a sufficiently high vapor pressure such as osmium tetroxide (Hallam, 1976; Heslop-Harrison, 1979), p-formaldehyde (Eränkö, 1964, 1967; Frederik et al., 1984; Grote, 1991; Hayat and Giaquinta, 1970; Pearse and Polak, 1975; Pekki and Tuohimaa, 1989), benzoquinone (Pearse and Polak, 1974; Pekki and Tuohimaa, 1989), and glutaraldehyde (Staff et al., 1990); 3) aqueous fixation in the presence of precipitating compounds such as the detergent cetylpyridinium chloride (Grote et al., 1983; Grote, 1989) or the phthalocyanine dye cuproline blue (Grote, 1989; Völker et al., 1986); and 4) cryotechniques such as freeze-drying (Vithanage et al., 1980, 1982) or freeze-substitution (Howlett et al., 1973; Martinez and Wick, 1991).

Since we had already tested aqueous fixation with the addition of precipitating agents (Grote et al., 1983; Grote, 1989), the aim of the present study was to compare the effect of aqueous fixation with that of fixation in anhydrous liquids and fixation in vapor using an object characterized not only by its desiccated state, but additionally by its possession of highly water-soluble

proteins, i.e., a pollen grain. We chose pollen of *Betula verrucosa* (L), the birch tree, for these investigations because of its importance for the elicitation of pollinosis in Europe (Horak and Jäger, 1979). In evaluating the experiments, first we looked for the preservation of ultrastructural morphology (criteria, e.g., given by Hayat, 1981) and second for the preservation of antibody binding capacity using a monoclonal antibody directed against the major birch pollen allergen Bet v I (Jarolim et al., 1989).

As these experiments aim at future investigations tracing up the possible sites of synthesis and/or storage of allergens within the pollen grain, two pollen compartments were chosen for a separate analysis: 1) the pollen wall and the germination pores, and 2) a representative area of the cytoplasm showing the ordinary assembly of cell organelles such as mitochondria, ribosomes, endoplasmic reticulum, dictyosomes, and reserve materials but letting apart the organization of the two pollen grain nuclei. The results of the first analysis will be presented in a later publication; the present paper deals with the effect of various fixation methods on the cytoplasm.

MATERIALS AND METHODS

Pollen Material

Pollen grains from *Betula verrucosa* (L) were collected from the flowering catkins and stored in a refrigerator at -18°C until use. Usually there is no protection against freeze-damage carried out in dehydrated plant pollen showing no central vacuole.

Aqueous Fixations

For fixation in p-formaldehyde, pollen grains were fixed in 4% freshly prepared p-formaldehyde (Riedel-de

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Haen, Seelze, Germany) in 0.1 M cacodylate buffer, pH 7.4, for 4 h at room temperature. For fixation in glutaraldehyde, pollen grains were fixed in 2.5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M Sörensen phosphate buffer, pH 7.4, for 2 h at room temperature. For the matter of comparison of the three different ways of fixation, no aldehyde mixtures were used, although this would have been easily possible in the case of the aqueous and anhydrous-liquid fixation and the use of aldehyde mixtures has shown to improve results in many specimens (Plattner and Zingsheim, 1987).

Fixation in Anhydrous Liquids

For fixation in p-formaldehyde, a 30% solution of p-formaldehyde in distilled water was diluted with anhydrous glycerol until a final p-formaldehyde concentration of 6% was reached. The water introduced by the stock solution was removed by treating the fixing solution with molecular sieve (aluminum sodium silicate, type 13X, particle size 3 mm, pellets 1/8 inch; Serva, Heidelberg, Germany). Pollen grains were fixed in this solution for 24 h at 30°C. This fixing temperature was chosen to improve infiltration of the formaldehyde/glycerol mixture.

For fixation in glutaraldehyde, a stock solution of 50% glutaraldehyde was diluted with anhydrous glycerol until a desired glutaraldehyde concentration of 6% was reached. The water introduced by the glutaraldehyde was removed by treating the fixing solution with molecular sieve (see above). Pollen grains were fixed in this solution for 24 h at 30°C.

Vapor Fixations

Pollen grains were fixed in p-formaldehyde vapor or glutaraldehyde vapor for 24 h at room temperature. This was done by placing the pollen samples in small desiccator vessels together with a large quantity of p-formaldehyde powder or concentrated glutaraldehyde solution.

Embedding, Ultramicrotomy, and Electron Microscopy

Specimens fixed in aqueous aldehyde solutions were dehydrated and embedded into Lowicryl K4M according to Grote and Fromme (1984). Pollen samples anhydrously fixed in aldehyde/glycerol were washed three times in anhydrous glycerol at 30°C (total washing time 4 h). Then the pollen grains were treated with dimethoxypropane (DMP) overnight at room temperature in order to remove chemically any residual water in the specimens. This comparatively long period of DMP treatment was chosen in view of our laboratory experience, which had shown desiccated pollen grains to be a specimen very difficult to infiltrate with dehydrating agents and embedding resins (see below). So the danger of extraction of pollen material was considered to be inferior to that of getting not fully infiltrated and perhaps not sectionable preparations. They were carried through a series of mixtures between DMP and ethanol (2:1, 1:1, 1:2) at room temperature with 2 h for each step. They stayed overnight in absolute ethanol and were then put into a 1:1 mixture between ethanol

and Lowicryl K4M for 8 h (both steps at room temperature for a still better infiltration of the pollen samples with the resin). The pollen grains were put into the pure resin overnight at room temperature, then transferred into another charge of the pure resin, which was placed into the Lowicryl embedding apparatus. There they were slowly (over a period of 4 h) cooled down to -35°C, at which temperature polymerization was carried out.

Vapor fixed pollen specimens were dehydrated in DMP for 24 h, taken through a graded series of DMP: ethanol (2:1, 1:1, 1:2, with 8 h for each step), stayed in absolute ethanol for 12 h, and transferred to a 1:1 mixture of ethanol:Lowicryl K4M for 8 h. Infiltration with the pure resin was for 24 h with one exchange of the medium (all previous steps at room temperature). Pollen grains were put into fresh Lowicryl K4M and transferred into the embedding apparatus where they were slowly cooled down to -35°C over a period of 4 h. Polymerization by irradiation with ultraviolet light took place at -35°C.

Ultrathin sections were conventionally cut on a Reichert OM U3 ultramicrotome and placed on pioloform-coated copper grids when being destined for morphological inspection. Sections used for immunocytochemical labeling were placed on pioloform-coated nickel grids.

Sections were double-stained with saturated aqueous solution of uranyl acetate (Merck) for 3 min and with lead citrate (Merck) for 5 s.

Sections were examined in a Hitachi H500 transmission electron microscope operated at 75 kV. Photographs were taken using Agfa Gevaert 23D56 films.

Immunocytochemistry

Immunochemicals. A monoclonal antibody (cell culture supernatant) against the major birch pollen allergen Bet v I was obtained from Dr. Dietrich Kraft (Institute of General and Experimental Pathology, University of Vienna, Austria). In Western blots, this antibody clearly recognized a 17 kD glycoprotein in birch pollen extract which was proved to represent the major birch pollen allergen Bet v I. For control purposes, we received another monoclonal antibody (cell culture supernatant) of different specificity. This control antibody showed no reaction with birch pollen proteins but a sufficiently strong binding to its target protein. Both antibodies belong to subclass IgG1. The concentration of specific IgG in both cell culture supernatants was not determined.

Labeling Protocol. Sections were incubated at room temperature in a moist chamber on drops of the following solutions:

1. 5% bovine serum albumin (BSA; Serva, No. 11923) in PBS buffer, pH 7.4, 15 min.
2. Monoclonal antibody against Bet v I, undiluted, 2 h.
3. Washings: once PBS buffer, pH 7.4, 10 min; twice Tris-BSA buffer, pH 8.2, 10 min each.
4. Goat-anti-mouse IgG antibodies coupled to 10 nm gold particles (GAM IgG 10), diluted 1:20 in Tris-BSA buffer, pH 8.2, 2 h.

5. Washings: once Tris-BSA buffer, pH 8.2, 10 min; twice distilled water, 10 min each.

Controls.

1. Incubation of the sections with a monoclonal antibody of irrelevant specificity instead of the specific antibody against Bet v I.

2. Omission of the primary antibody and incubation of the sections with the secondary antibody (goat-anti-mouse IgG) alone.

RESULTS

Aqueous Fixations

Fixation in aqueous p-formaldehyde leads to a rather unsatisfactory preservation of the cytoplasmic architecture. Mitochondria cannot be recognized. Very rarely dictyosomes are (poorly) preserved. However, some strands of the endoplasmic reticulum and ribosomes can be identified. Apart from the presence of numerous vesicles (polysaccharide storage?), the cytoplasmic matrix shows many diffusely delineated, more or less empty spaces (Fig. 1). Starch grains, lipid bodies, and the pollen nuclei are present.

Fixation in aqueous glutaraldehyde preserves pollen grain morphology slightly better than fixation in p-formaldehyde. Mitochondria and dictyosomes can be recognized, although the ultrastructure of these organelles is poorly preserved. As after fixation in p-formaldehyde, the rough endoplasmic reticulum and the numerous ribosomes are satisfactorily maintained. The number of empty spaces in the cytoplasmic ground substance is decreased. Vesicles are well preserved, partially showing an amorphous or fibrillar interior (Fig. 2). Starch inclusions, lipid bodies, and the nuclei together with their membranous envelopes can be identified.

Anhydrous-Liquid Fixations

Anhydrous fixation of birch pollen by p-formaldehyde in glycerol preserves the morphology of pollen cytoplasm better than aqueous fixation. Now the outer and inner membranous structures of the mitochondria are present. Individual dictyosomes with cisternae and vesicles can be quite easily identified, as is the case with the endoplasmic reticulum. Ribosomes, however, can hardly be recognized as individual bodies along the endoplasmic reticulum and between the other cell organelles, but rather they form electron-dense patches and accumulations. The matrix contains many vesicles most of which are rather clearly delineated (Fig. 3). Starch grains, lipid bodies, and nuclei are rather well preserved.

The morphology of birch pollen after anhydrous fixation by glutaraldehyde in glycerol looks very much like that obtained after fixation in p-formaldehyde. The cisternae of the rough endoplasmic reticulum, however, appear enlarged compared with fixation in p-formaldehyde/glycerol. Ribosomes can faintly be recognized as individual particles (Fig. 4).

Vapor Fixations

Fixation in p-formaldehyde vapor well preserves the ultrastructural morphology of birch pollen. Mitochondria, dictyosomes, and endoplasmic reticulum can be identified without difficulties by their typical structural elements. Ribosomes are present as electron-dense patches rather than as clearly recognizable individual particles. Starch grains, lipid bodies, and vesicles are also present, the latter being in most cases clearly defined in their shapes and dimensions. There are almost no diffusely demarcated empty spaces in the cytoplasmic matrix (Fig. 5). The pollen grain nuclei are also well preserved, as are the nuclear membranes.

Pollen morphology after fixation in glutaraldehyde vapor much resembles that observed after vapor fixation in p-formaldehyde. However, the cisternae of the endoplasmic reticulum and many vesicles appear enlarged and deformed. Individual ribosomes can faintly be recognized (Fig. 6).

Immunocytochemistry

Binding of monoclonal antibodies against the major allergen Bet v I to ultrathin sections of birch pollen grains could not be observed in all specimens fixed in glutaraldehyde (aqueous, in anhydrous glycerol, or as vapor). Aqueous fixation in p-formaldehyde equally did not give positive labeling results. However, after non-aqueous fixation, the binding of gold particles could be observed in the sections. There was a low degree of labeling after fixation in the p-formaldehyde/glycerol mixture (Fig. 7), but strong labeling appeared after fixation in p-formaldehyde vapor (Fig. 8). Most gold particles were observed between the cisternae of the rough endoplasmic reticulum and between the membrane-bound vesicles in the cytoplasm. No special accumulation of the label in the cisternae of the rough endoplasmic reticulum or the Golgi apparatus could be observed. All control experiments showed a very low degree of non-specific binding of gold particles to the pollen sections (micrographs not shown).

DISCUSSION

As regards the ultrastructural morphology of the birch pollen grain, the preservation of pollen cytoplasm

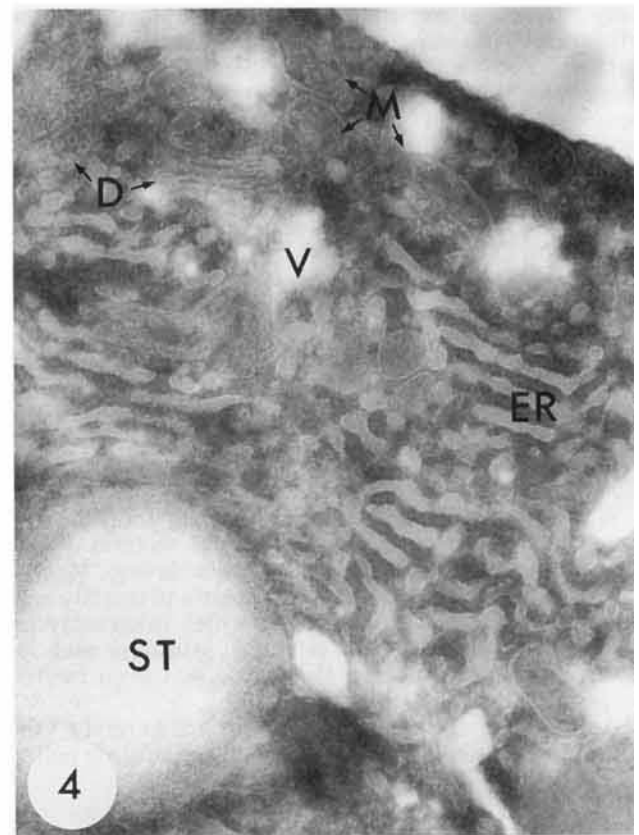
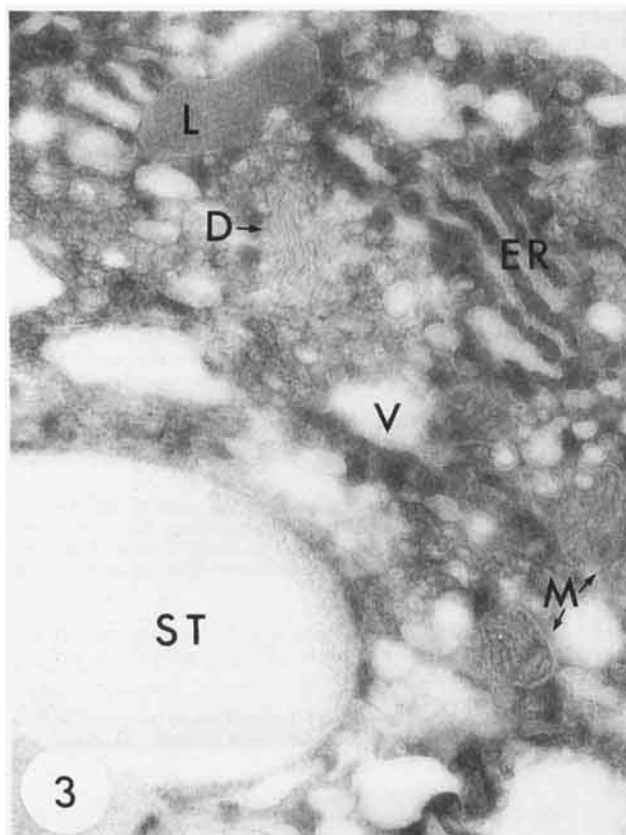
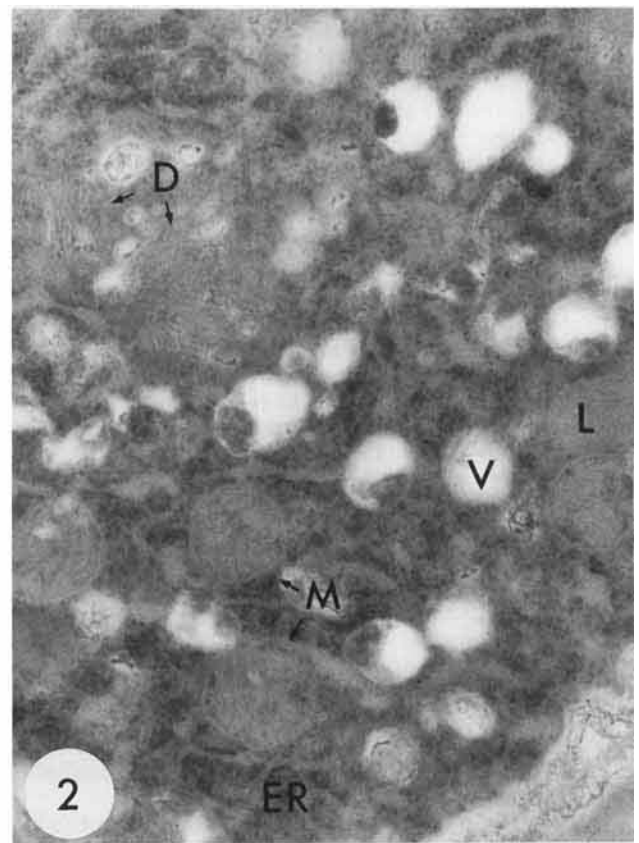
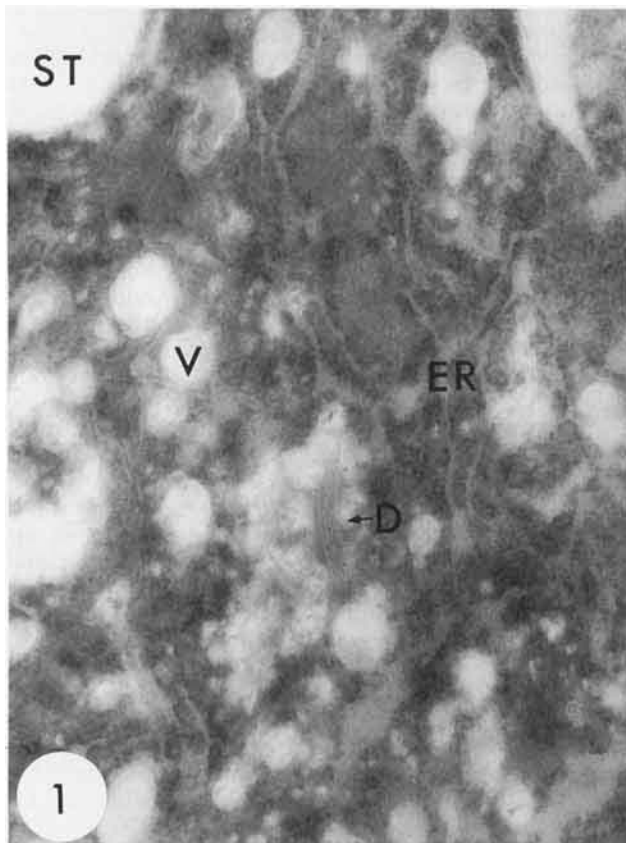
Figs. 1–6. Ultrathin sections of birch pollen grains showing part of the cytoplasm after different chemical fixation procedures. $\times 48,750$.

Fig. 1. Aqueous fixation in p-formaldehyde. D, dictyosome; ER, endoplasmic reticulum; ST, starch grain; V, vesicle.

Fig. 2. Aqueous fixation in glutaraldehyde. D, dictyosome; ER, endoplasmic reticulum; M, mitochondrion; L, lipid; V, vesicle.

Fig. 3. Non-aqueous fixation in anhydrous p-formaldehyde/glycerol. D, dictyosome; ER, endoplasmic reticulum; M, mitochondrion; L, lipid; ST, starch grain; V, vesicle.

Fig. 4. Non-aqueous fixation in anhydrous glutaraldehyde/glycerol. D, dictyosome; ER, endoplasmic reticulum; M, mitochondrion; ST, starch grain; V, vesicle.



Figs. 1-4.

after aqueous fixation is very poor. This was the case with p-formaldehyde (as expected), but also with glutaraldehyde. Whether this must be attributed to the slow penetration rate of this fixative, which might be even more unfavorable in the case of dense specimens (seeds, spores, etc.), must be questioned. Surely, birch pollen grains possess a durable wall (exine) around a closely packed, desiccated cytoplasmic core, but their diameter does not exceed 15 μm .

Perhaps some co-parameters of fixation (e.g., osmolarity, pH value) were not adequately chosen for an object that continually changes its internal conditions during fixation, for increasing rehydration may mean, e.g., increasing solubilization of cellular compounds. The observation that conventional chemical fixation does not automatically lead to a good preservation of ultrastructure in pollen grains was also reported by Martinez and Wick (1991), who found that the cytoplasmic and organellar matrix of ryegrass pollen tended to appear as a heterogeneous mixture of clumped material and electron-translucent areas.

Non-aqueous fixation generally improved the ultrastructural preservation of the pollen grain. Fixation in anhydrous glycerol gave moderately better results than aqueous fixation, provided the glycerol/aldehyde mixture was warmed up to at least 30°C to facilitate infiltration (Hallam, 1976). Anhydrous fixation using dimethylsulfoxide/glutaraldehyde (Hallam, 1976) was also tested in our laboratory, but the results were found inferior to glycerol/glutaraldehyde (Grote, unpublished observation).

The best fixation results were obtained when the two fixatives under examination were applied in the vapor form. It is especially p-formaldehyde that leads to a very attractive picture of pollen cytoplasm, whereas glutaraldehyde vapor, at least in our hands, seemed to introduce some artifactual phenomena (cf. e.g., the morphology of the endoplasmic reticulum). This evaluation is based on the criteria commonly set up for a satisfactory specimen preservation (cf. Hayat, 1981), but since there are no independent methods to determine the morphology of cell organelles in the living state this conclusion may not necessarily be valid (Sjöstrand and Barajas, 1968; Sjöstrand, 1989).

Fixation in p-formaldehyde vapor was also preferred by Frederik et al. (1984) for stabilizing the ultrastructure of freeze-dried cryosections. Pearse and Polak (1975) and Pekki and Tuohimaa (1989), however, found fixation results after p-formaldehyde vapor inferior to those using benzoquinone vapor. This latter vapor fixative was also tested in our laboratory with rather negative results concerning structural morphology and antibody binding (Grote, 1991).

A logical continuation of anhydrous fixation and preparation techniques would be to cut sections on dry knife or to float them on anhydrous fluids (e.g., Morris, 1968). However, since our experiments primarily aim at immunocytochemical labeling which inherently involves the application of aqueous solutions and reagents, we did not follow this route, not even for the inspection of morphology.

As regards the preservation of antigenicity, the present results clearly show that the major birch pollen

allergen Bet v I, like many other antigens, is sensitive to glutaraldehyde.

For obtaining optimal preservation of ultrastructure and antigenicity very often a "mixed" fixation is used, based upon glutaraldehyde in low concentration (morphology) and p-formaldehyde in higher concentration (antigenicity). In the present study, however, this was not looked at as a practicable alternative. Our results clearly show that, from the different chemical fixation techniques tested, vapor fixation should be used in birch pollen in order to keep the highly soluble antigens "in situ." So a "mixed" fixation would mean to apply a mixture of p-formaldehyde vapor and glutaraldehyde vapor in different concentrations. From the technical point this seems rather difficult to carry out.

However, the observed "fading" of morphological information after immunocytochemical treatment in the specimens fixed in p-formaldehyde vapor (cf. Fig. 8, which no longer shows ribosomes) very probably points to the known instability of the chemical bonds formed by this fixative so that cytoplasmic structures do not seem to be sufficiently stabilized against the influence of several hours of aqueous treatment during the incubation with antibodies and gold probes. This might be overcome by the action of glutaraldehyde which is thought to produce stable linkage.

As regards the preservation of antigenicity after different fixation techniques, it is not surprising that there is no antibody binding after aqueous fixation since pollen proteins may rapidly diffuse away from the pollen grain upon aqueous contact (Howlett et al., 1973; Knox and Heslop-Harrison, 1969, 1970; Knox et al., 1970). Loss of antibody binding after fixation in anhydrous p-formaldehyde/glycerol must not be attributed to the action of the fixative since specimens fixed in p-formaldehyde vapor show positive antibody binding. Possible explanations are: 1) Anhydrous glycerol as a highly hygroscopic chemical might have extracted the residual water out of the pollen grain (mature pollen grains have a water content between 20 and 50% according to Heslop-Harrison, 1979; Stanley and Linskens, 1974) and the allergens might have gone with the water. 2) Glycerol itself or the resulting glycerol/

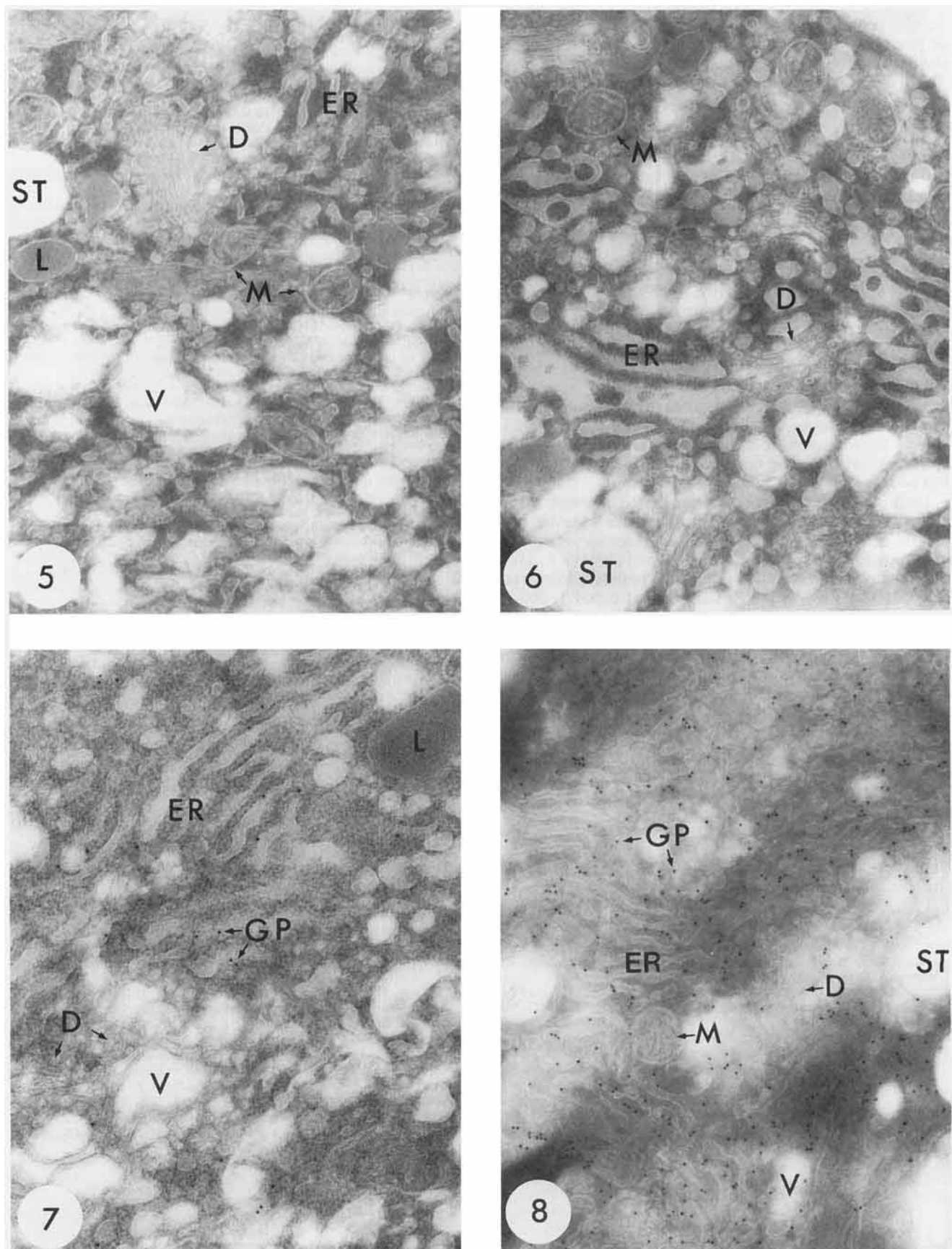
Fig. 5. Non-aqueous fixation in p-formaldehyde vapor. D, dictyosome; ER, endoplasmic reticulum; M, mitochondrion; L, lipid; ST, starch grain; V, vesicle.

Fig. 6. Non-aqueous fixation in glutaraldehyde vapor. D, dictyosome; ER, endoplasmic reticulum; M, mitochondrion; ST, starch grain; V, vesicle.

Figs. 7, 8. Ultrathin sections of birch pollen grain showing part of the cytoplasmic after different fixation procedures and immunocytochemical labeling with a monoclonal antibody against the major birch pollen allergen Bet v I, followed by a secondary antibody coupled to 10 nm gold particles. $\times 56,250$.

Fig. 7. Non-aqueous fixation in p-formaldehyde/glycerol. D, dictyosome; ER, endoplasmic reticulum; GP, gold particle; L, lipid; V, vesicle.

Fig. 8. Non-aqueous fixation in p-formaldehyde vapor. D, dictyosome; ER, endoplasmic reticulum; GP, gold particle; M, mitochondrion; ST, starch grain; V, vesicle.



Figs. 5-8.

water mixture (cf. 1) might function as a sort of solvent for the proteinaceous allergen. 3) Glycerol might bind to proteins and mask epitopes. Due to its trivalency, changes of conformation might occur.

As regards our immunocytochemical results, there is reason to consider the observed binding of antibodies as specific although only one monoclonal antibody has been used so far and although tests with antibodies preabsorbed with the Bet v I allergen have not so far been carried out. The observed labeling shows that most gold particles are located either between cytoplasmic vesicles or between the cisternae of the rough endoplasmic reticulum. In both cases this might mean an association of the antibody binding sites with ribosomes which, however, can no longer be discerned in Figure 8, but whose presence may be deduced from a comparison between, e.g., Figure 5 and Figure 8. Staff et al. (1990) observed *Lolium* pollen allergen Lol p I also in sites of (free) ribosomes, but not in the endoplasmic reticulum and the Golgi apparatus. They concluded from their observations that Lol p I synthesis may take a route different from that of many other plant cell proteins. However, it seems rather speculative to draw any definitive conclusion as to the site of synthesis, storage, or transport of Bet v I in birch pollen at the present state of investigation.

Deep temperature methods seem to present an attractive alternative to chemical fixation techniques in pollen grains as regards structural preservation and preservation of antigenicity (Martinez and Wick, 1991) but they are time-consuming and expensive. Moreover, they do not always solve the sophisticated technical problems connected with the localization of soluble antigens such as pollen allergens. If these antigens are not sufficiently stabilized during the preparation procedures they will be mobilized during incubation of the section with the aqueous antibody solution, which in any case will mean a rehydration of the specimen. However, experiments using a modified freeze-substitution technique in the preparation of birch pollen are ongoing.

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