INTERSECTIONAL INCOMPATIBILITY IN *POPULUS*

by

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This thesis embodies the results of my own original investigations. To the best of my knowledge and belief it contains no material previously published, or the result of work by another person, except where due reference is made in the text.

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To Peter, Louisa, Hilry,
Rodd and Kirsten
... with thanks
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Erratum Plate 5.1

The total protein ordinate is incorrectly labelled, the existing label applies to the enzyme assays only.

Total protein is measured as mg. protein per gram of pollen.
INTRODUCTION

*Populus* (Salicaceae) is a genus of considerable economic importance and the breeding of new varieties better able to cope with particular environments, and/or fulfil special requirements, extends the potential of the genus. A good example of such a contribution is the production of the *P. euramerica* (Dode) Guinier hybrids, which group of hybrids has played a central role in poplar production since their initial inception. These, and numerous other *Populus* hybrids, have been produced by crossing geographically or seasonally remote species within the limits permitted by intersectional incompatibility barriers. The recent findings that such barriers may be mutable in *Populus* using a variety of treatments, and resulting in the production of completely new hybrids, raises exciting breeding prospects. Such an approach has already yielded new varieties of considerable promise (Willing and Pryor pers. comm. 1975).

The particular stimulus for the initiation of this project was the earlier finding (1972), since reported (Whitecross and Willing, 1975), that the intersectional incompatibility barrier of *Populus* could be overcome by treating the stigma with certain low polarity solvents prior to making an 'incompatible' cross pollination.

The effectiveness of this apparent subtraction of stigma material in overcoming the *Populus* incompatibility barrier was contrasted with the results of Knox, *et al.*, (1972a,b) who showed that the incompatibility barrier of the same species (and in some cases, clones) could be overcome by the addition of 'compatible' pollen, or its extracts, to normally 'incompatible' pollen. Knox, *et al.*, (1972a) proposed that the active component of such material should be termed 'recognition protein', and further, that this 'protein' was necessary for a compatible pollination.
Taken together the success of each of the stigma 'subtraction' and pollen 'addition' treatments provides a basis for the elucidation of the incompatibility mechanism in *Populus* and each helped to determine the method of approach to the problem.

In Chapter 1 the *Populus* pollen and stigmata will be examined, largely using microscopic techniques, and their behaviour and appearance following compatible or incompatible pollination determined.

Chapter 2 will report on the behaviour of pollen in compatible and incompatible situations after various artificial treatments, some of which are already known to overcome incompatibility in *Populus* or other genera.

As Chapter 2 will demonstrate the effectiveness of solvent pollen treatments in overcoming *Populus* incompatibility, Chapter 3 will concentrate on determining the effect that certain organic solvents have on the pollen, and whether each of the solvents enter the pollen, or not, during such treatments.

Chapter 4 will focus on the composition of both pollen and stigma surface materials, with particular emphasis on the lipid components of each, following the earlier suggestion by Whitecross (pers. comm. 1972) that surface lipids of the stigma, at least, may be involved in the incompatibility process in *Populus*.

Chapters 5 and 6 will focus on the materials involved by Knox, *et al.*, (1972a) in the *Populus* 'recognition' process, that is, the materials which diffuse from pollen into buffered aqueous media. Chapter 5 will be concerned with quantitative and qualitative determinations of the enzymes contained in such diffusates, whilst Chapter 6 will examine their antigenicity. In both the latter chapters particular attention will be paid to the similarity in isozyme pattern, and the
antigenicity of *Populus* pollen diffusates, as well as the timing of their release, and the effect of solvent pretreatments on both diffusate release, and on the composition of the diffusate following such pretreatments.

The results of each of the Chapters 3, 4, 5 and 6 will present evidence which brings into question whether proteins are located in the wall of *Populus* pollen, prior to its hydration, or not.

The final chapter (number 7) will draw general conclusions from the study as a whole, and will provide an hypothetical explanation for the nature of the *Populus* incompatibility mechanism from the results obtained in these, and earlier, *Populus* studies.

A review of the pertinent literature will precede each chapter.
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The genus *Populus* of the family Salicaceae has been divided into five sections including *Populus*, *Eurameris*, *Alaschkae*, *Salix*, and *A通俗* (white poplars) and *Tuphidae* (asians) (FAS 1964).

The results of numerous studies have established the existence of the breeding relations within and between sections of the genus (for example: Steen and Schjoerring 1973; Petersson 1948; Oppe and Olmg 1969; Carrion and Catalan 1963; Catalan 1963; Knaup et al. 1972b; Willing and Fryer pers. commun. 1973).

Following Nogdeman's (1934) report that inter sectional grafting successes were related according to the series: *arbus* - *silver poplars*; *rotundifolia* - *britain poplars*, Heimbueger (1940) saw a strong correlation between these affinities and those evidenced by the breeding results of Nottstoga (1932) and Steen and Schjoerring (1933). This series may also reflect the degree of genetic affinity between sections. Johnson (1940) saw little limitation to species hybridization within the genus *Populus* as for an artificial hybridization was concerned.

That Johnson's proposal should not be accepted as a generalization is evident from a number of studies, including those of Willing and Fryer (pers. comm. 1973), who have made extensive intra- and inter-sectional breeding trials and whose conclusions as to the breeding affinities of *Populus* are presented in diagrammatic form (diagram 1.1).
CHAPTER I

POPULUS POLLEN-STIGMA INTERACTIONS

INTRODUCTION

The genus *Populus* of the family Salicaceae has been divided into five sections including *Turanga* Bge.; *Leucoides* Spach; *Algeiros* Duby (cottonwoods and black poplars); *Tacamahaca* Spach. (balsam poplars); and *Leuce* Duby. The section *Leuce* is further divided into the subsections *Albidae* (white poplars) and *Trepidae* (aspen) (FAO 1965).

The results of numerous studies have enabled the elucidation of the breeding relations within and between sections of the genus (for example Stout and Schreiner 1933; Heimburger 1940; Hyun and Hong 1959; Carrion and Catalan 1963; Catalan 1963; Knox et al. 1972b; Willing and Pryor pers. comm. 1975).

Following Bogdanov's (1934) report that intersectional grafting successes were related according to the series: aspens - silver poplars - cottonwoods - balsam poplars, Heimburger (1940) saw some correlation between these affinities and those evidenced by the breeding results of Wettstein (1933) and Stout and Schreiner (1933); this series may also reflect the degree of genetic affinity between sections. Johnson (1939) saw little limitation to species hybridization within the genus *Populus* as far as artificial hybridization was concerned.

That Johnson's proposal should not be accepted as a generalization is evident from a number of studies, including those of Willing and Pryor (pers. comm. 1975), who have made extensive intra- and inter-sectional breeding trials and whose conclusions as to the breeding affinities of *Populus* are presented in diagrammatic form (diagram 1.1).
The attention of this thesis is largely focussed on the *Aigeiros* (or *Tacamahaca*) X *Leuce* intersectional incompatability barrier. As Willing and Pryor (1975) report, "intersectional hybrids between *Aigeiros* and *Tacamahaca ... are widely known", as are hybrids between various pairs of species within the sections *Leuce*, *Aigeiros* and *Tacamahaca*, whether produced spontaneously or following artificial manipulation. (Stout and Schreiner 1934; Syrach-Larsen 1956; Willing and Pryor pers. comm. 1975). Among the many reports of poplar breeding trials there have
been few records of success in attempts at intersectional crosses involving *Leuce* and either *Aigeiros* or *Tacamahaca* species. Exceptions include the following: Stout and Schreiner, 1933, 1934 (*P. balsamifera virginiana* X *P. grandidentata*); Hyun and Hong, 1959 (some crosses involving *P. alba*, *P. tremuloides* and *P. davidiana* as female parents with several *Aigeiros* or *Tacamahaca* species as pollen parents) and Catalan, 1962; Carrion and Catalan, 1963; Catalan, 1963 (*P. tremula* X *P. deltoides* var. 'carolinensis'), and Joennez and Vallée, 1972, (*P. tremuloides* X *P. X jackii*, *P. alba* X *P. X jackii*, and *P. tremuloides* X *P. X euramericana*). In each of the above cases pollination was achieved by simply dusting the pollen onto the stigma. With few exceptions the seed set resulting from the above crosses was poor and the resulting progeny was weak and variable. The *Aigeiros* (*Tacamahaca*) X *Leuce* incompatibility barrier has been overcome by several authors using a variety of techniques (Stettler 1968; Stettler and Bawa 1971; Knox et al. 1972a,b; Whitecross and Willing 1975; Willing and Pryor 1975), yet has not been recorded for wild populations to my knowledge. The nature of the intersectional incompatibility mechanism in *Populus* is unknown, although several factors have been implicated including a possible stimulatory function of specific enzymes and hormones (Stettler 1968); proteins apparently located in the pollen wall and described as recognition proteins (Knox et al. 1972a) and required for a successful pollination (Knox et al. 1972a,b), and the (apparently lipoidal) stigma surface materials (Whitecross and Willing 1975).

A comprehensive description of floral morphology and development of both black (*P. deltoides*) and white (*P. tremuloides*) poplars has been presented by Nagaraj (1952). More recently Sattler (1973) has made a light microscopic study of floral development of *P. tremuloides*, as
also has Fechner (1972), who studied the development of the pistillate flower of this species following controlled pollination, with emphasis on events involving the megagametophyte. Smith (1943) made a comprehensive review of the cytology of *Populus*, noting that in all species studied the normal diploid chromosome number of the genus is 38. Triploid (Johnsson, 1942 for example) and haploid (Stettler and Bawa, 1971) progenies of *Populus* have also been found and are not uncommon in the genus. It has been suggested (Lawrence, 1931) that plant families (such as Salicaceae) with high chromosome numbers may be found to be secondary polyploids.

A number of authors have described the pollen of *Populus* as being binucleate (Chamberlain, 1897; Smith, 1943; Nagaraj, 1952; Brewbaker, 1967; Kirby and Smith, 1974) although from the results of work arising from this study (Hamilton and Langridge, 1975) it has been demonstrated that, although binucleate grains may be found, the trinucleate state has been more commonly observed in a number of species. The significance of this latter finding will be discussed later.

*Populus* is a dioecious genus, with the exception of the occasional tree which may be hermaphroditic (at least in some seasons), bearing hermaphrodite catkins, or even flowers; this has often been reported (for example Stettler, 1971). Several such clones of *P. deltoides* have been found in the experimental garden used for this study.

In Canberra the flowering season of *Populus* extends from late August to early December with some overlaps of the flowering periods of several clones, both male and female, of *Leuce* on the one hand, and *Aigeiros* and *Tacamahaca* poplars on the other. Nagaraj (1952) reported the number of flowers in the pistillate catkins to be 40-60 in *P. deltoides* and 90-100 in *P. tremuloides*. The stigma was described as deeply lobed
in each case, there being 2 to 4 stigma segments in *P. deltoides* and 2 in *P. tremuloides*. Pistillate catkins were reported to begin their development towards the end of summer, remaining as buds over winter, and completing their development the following spring; staminate catkins start their development at the beginning of summer, overwintering at the spore - mother - cell stage (Nagaraj, 1952).

*Populus* pollen is shed from the male catkins following their rapid elongation, the precise timing of which is determined largely by short-term environmental factors including temperature and humidity. *Populus*, being an anemophilous genus, produces abundant pollen, Snyder and Clausen (1973) found that 100 catkins of *Populus* could produce 75cc of pollen. The pollen of many, but not all *Populus* species, is known to be allergenic. (Wodehouse, 1959; Stanley and Linskens, 1974). The pollen of *Populus* is reported to be between 24 and 37 µm in diameter (in its hydrated state) (Wodehouse, 1959). Manzhos (1960) has reported that size of the pollen of *P. balsamifera* can be related to the vigour of its progeny.

The water content of *Populus* pollen is not recorded, yet Stanley and Linskens (1974) note that grass pollens have a water content of greater than 50% at anthesis, whilst in *Typha*, *Pinus*, "and longer surviving pollen the water content is usually 20% or less".

The development of the pollen grain of *Populus* has been followed by Rowley and Erdtman (1967) who found that maturation of the grain could be markedly accelerated under "rapid growth conditions". The pollen wall is without colpi and apparently uniform over the whole surface and, as interpreted by Rowley and Flynn (1971): "the entire pollen wall is similar to the apertural region of distinctly aperturate pollen types". The uniformity of the *Populus* pollen wall was also indirectly demonstrated
by Müller-Stoll's (1956) finding, that on the hydration and germination of the grain, the exine split in an irregular manner. Rowley and Erdtman (1967) found the nexine-2 wall layer to be the only uninterrupted part of the exine, (albeit very thin) whilst a nexine-1 layer was absent and the tectum was of 'weak continuity' and irregular. In the earlier stages of development the nexine was described as being "not a sheet but a lattice composed of tapes" (Rowley, 1970). During the plasmodial tapetal stage the exine changed little, despite rapid increase in size of the grain, yet considerable material was deposited into the nexine-2, the pollen wall, and/or the pollen protoplast. Rowley later (1973 and 1975a,b), in reference to pollen generally, determined that the "tapes and p-channels apparently contain lipopolysaccharide while the irregular channels although very transitory, contain mucopolysaccharide when open" (Rowley, 1973). He also held that such tapes and channels were marked by uptake of lipids (Rowley, 1973).

Rowley and Erdtman, (1967) and later, Rowley (1970) reported that the dense materials deposited in the nexine-2 layer in Populus are almost entirely lost at maturity and "may even be replaced by areas of extremely low density that take no stain" (Rowley and Erdtman, 1967). Rowley (1970) believed that this was not simply due to surface area increase but rather to autolysis of the exine. He had previously held that in this respect "Populus is a special, although probably not unique, case" (Rowley and Erdtman 1967).

Rowley and Dunbar (1970) allowed for the possibility that during, and subsequent to, the tetrad stage of Populus pollen development the pollen was able to take up certain materials by a process similar to pinocytosis. They were able to locate tracers in vesicles or channels in the grain, apparently following such uptake, and suggested that "the focal infolding of the plasma membrane to form invaginations seems to us
strongly to suggest pinocytosis". Rowley (1970) held the view that, in general, there was room to doubt the impervious nature of the exine and that "substances can be considered as available for uptake by the pollen cytoplasm everywhere over its surface", and not, as in aperturate pollens, only at the aperture. This conclusion would logically hold for *Populus* pollen. Rowley and Erdtman (1967) found that few, if any, osmiophilic vesicles were apparent in the exine region of *Populus* pollen until near maturity, when, with tapetal breakdown, the exine became enveloped with osmiophilic material, which was also apparent within the cytoplasm and just outside the plasmalemma. They also observed a 'membrane' outside the exine of *Populus* pollen which they took to be covered by a thin layer of sporopollenin. In their preliminary studies they had found the corresponding surface membrane of *Hakea* pollen to be undigested by proteases. As a 'working hypothesis', they proposed that "it is tempting to suggest an immunological role for membranes at the surface of pollen grain exines" which could also be responsible for some of the specific pollen allergens (Rowley and Erdtman, 1967).

*In vitro* germination of *Populus* pollen has been reported by a number of authors (Smith, 1943; Nagaraj, 1952; Catalan, 1962, for example). In each of the above cases pollen was sown on an agar medium whose osmolarity was adjusted to a relatively high level (15-20%) by the addition of sucrose. Both Smith (1943) and Catalan (1962) used gelatine in their germination media. Only Catalan (1962) has reported the germination rate of *Populus* pollen following *in vitro* pollination on such media. Using sugar concentrations ranging from 5-40%, and three *Populus* species (*P. alba*, *P. euphratica* and *P. tremula*), Catalan (1962) found the optimum sucrose level for germination to be 20%. The most efficient germination (56.4%) was obtained from *P. alba* pollen within two weeks of collection, after which germination rapidly tailed off to zero. There was
no evidence of germination of the *Populus* pollen 8 weeks after anthesis. Catalan (1962) was unable to germinate *P. tremula* pollen *in vitro*. Nagaraj (1952), referring to *Populus*, reported that "no germination was obtained from any pollen which had been stored, even when stored for only 3 days". Although Nagaraj (1952) is not clear on this point, it seems that one should interpret his comment as referring to the *in vitro*, and not the *in vivo* situation.

In a study of the development of the pistillate flower following controlled pollination, Fechner (1972) concluded that fertilization of *P. tremuloides* probably occurred between 72 and 120 hours after pollination of this species. Neither Nagaraj (1952) nor Fechner (1972) have described the events which occur in the pistillate flower of *Populus* between the time of its initial pollination and that of the entry of the pollen tube into the megagametophyte tissue.

Knox *et al.* (1972b), referring to *Populus* pollen, proposed that "germination occurred within hours" having observed pollen tubes in abundance, penetrating between stigma cells 24 hours after a compatible (*P. deltoides* X *P. yunnanensis*) cross. In another paper, Knox *et al.* (1972a) reported that following a compatible *Populus* cross, "most grains germinated, the tubes penetrating and growing through the thalloid stigmatic tissue, polarized towards the style, many reaching it within 90 min. after pollination". Following an incompatibility pollination Knox *et al.* (1972a) found the rate of germination and penetration to be less, the tubes taking 7 to 20 hours to reach the style after pollination. On an incompatible stigma, as observed 24 hours after pollination, *Populus* pollen was seen to have germinated, yet the tubes were unable to penetrate the stigma surface (Knox *et al.* 1972b).
It is apparent that none of the above studies have examined the events following pollination of *Populus* pollen with either compatible or incompatible pollen in any great detail, as should be done if we are to obtain a better understanding of the incompatibility mechanism of the genus. In this chapter the state of the pollen and stigma of *Populus* are examined before, and at different times after pollination with comparisons made of events following both compatible and incompatible pollinations. The study is not intended to be comprehensive and focuses on pollen stigma interactions at the stigma surface.

**MATERIALS AND METHODS**

Mature (receptive) female catkins of grafted (see Appendix II) *P. deltoides* Marsh. or *P. deltoides* ssp. *monilifera* material were lightly dusted with pollen of *P. nigra* L. var. 'italica' or *P. bolliana* Lauche. using a small sable hair brush. Other crosses were made using *P. alba* L. as the female parent.

At each of a number of predetermined times ranging from 5 minutes to 48 hours after pollination, 6 to 8 flowers were removed from the catkins and immediately placed into fixative solution. Wherever possible pollination was timed so that all the flowers of a particular experiment could be fixed at the same time. Material to be examined by fluorescence microscopy was placed directly in fixative whereas that destined for embedding in resin, prior to light or electron microscopic examination, was cut (within the fixative) into portions, ranging from 1 mm$^2$ to several mm$^2$ in surface area, the larger pieces being satisfactory for examination of pollen-stigma surface interactions. Material was left in fixative overnight at 4°C. The fixative used consisted of 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1M. cacodylate buffer, pH 7.3, containing
calcium chloride, the tonicity being adjusted for this tissue by the addition of 10% pure filtered sea water.

**Fluorescence Microscopy**

Whole, fixed stigmata were removed from the fixative, washed three times in tap water for a total of 30 minutes duration, then left for 4 hours in 10 N sodium hydroxide at room temperature to soften the tissue. After washing several times in tap water over a one hour period, the tissue was stained in 0.1% water-soluble aniline blue (BDH) in 0.1 N potassium phosphate for 4 hours. The method used was based on that of Martin (1959). Where stained material was to be stored, this was accomplished by placing vials containing the specimens in stain solution in a refrigerator freezing compartment. Controls included, firstly, those employing the above procedure, but without the staining, and secondly, the direct staining (without fixation) of fresh material immediately prior to its examination. By these means it was determined that the fluorescence recorded was largely due to the (callose) staining rather than auto-fluorescence. Softened stained material was gently squashed between slide and cover slip and examined with a Zeiss Ultraphot Photomicroscope using an HBO 200 light source and appropriate exciter and barrier filters. Photographs were taken on Tri-X film (Kodak) rated at 2400 ASA and developed in 'Diafine' fine grain developer.

**Transmission Electron Microscopy**

The fixed stigma portions were subsequently treated as follows:

1. The tissue was washed in 0.1 M cacodylate buffer, pH 7.3, containing calcium chloride and 10% sea water, for 30 minutes with 3 changes.

2. Post-fixation was effected in 2% osmium tetroxide dissolved in the above buffer, generally overnight although occasionally for 2 hours only with no detectable differences. In some cases this post-fixation was omitted.
3. After washing 4 times in distilled water over a period of one hour, the tissue was then dehydrated through a graded methanol/water series to pure methanol (previously dried with the aid of molecular sieve pellets (BDH)). Each dehydration stage was of 15 minutes duration and included methanol : water percentages of 25, 50, 75, 90 and 95%. Material was then washed 3 times in pure methanol over a period of one hour then transferred to pure acetone, similarly dried.

4. After dehydration, the tissue was infiltrated with Spurr's resin (Spurr 1969) - acetone mixes containing increasing proportions of resin in 3 stages (1:3, 1:1, 3:1) of one hour each. The standard (Polysciences) proportions of resin components, (E.R.L. 10g, D.E.R. 6g, N.S.A. 26g and catalyst 0.4g), were used for all embedments. To ensure the thorough infiltration of tissues by Spurr's resin in each of the latter stages of the schedule, tissue was placed in glass vials on a slowly rotating inclined tray driven by a small electric motor.

5. Polymerization was effected in either silicone rubber or disposable polythene moulds, at 70°C overnight.

6. Blocks were trimmed, then 0.5 µm and 1 µm sections cut with glass knives using a Reichert OM U2 ultramicrotome. These were examined under the light microscope after staining with toluidine blue, pH 4.4, areas of particular interest selected and the blocks retrimmed and resectioned using a diamond knife. Sections with gold to grey interference colours (90-75 µ) were collected on parlodion coated 200 mesh copper grids.

7. Grid mounted sections were post-stained, first with a saturated solution of uranyl acetate in 70% methanol for 40 minutes, rinsed thoroughly in distilled water, then stained with Reynold's lead citrate (Reynolds, 1963) for 10 minutes, washed again with distilled water, and dried prior to examination. Some sections, including those which had
not been post-fixed with osmium tetroxide, were stained with lead citrate only, whilst others were stained with 1% phosphotungstic acid in 50% ethanol for 30 minutes.

8. Sections were examined in a JOEL 100 B or 100 C transmission electron microscope at 60 or 80 Kv and recorded on Ilford sheet film.

Where pollen alone was prepared for light- or electron-microscopy (as in both this chapter and chapter 3) the whole process was carried out in disposable polythene centrifuge tubes, rather than glass vials. Prior to each new treatment the pollen was spun down to form a pellet and the supernatant decanted prior to the addition of the next reagent, with which the pellet was then blended by gentle stirring using either a toothpick or an orange stick.

**Light Microscopy - general**

The appearance of the fresh stigma, prior to its pollination, as well as that of pollen on *in vitro* germination media, was monitored using the stereomicroscope; in addition, sections (0.5 to 1.0 µm) taken from resin-embedded material, as described above, were mounted on glass slides and examined and photographed with a Zeiss 'Ultraphot' photomicroscope. The rapid sequence of events following the initial hydration of pollen was also followed using the light microscope to observe the changes in the appearance of pollen which had been placed beneath a cover-slip on a slide, then irrigated with water. In some cases 1 ml of a 0.25% solution of Coomassie blue dye, dissolved in 45% methanol in 10% acetic acid, was added to 4 ml of 1% saline (after Howlett, 1973) and this protein stain (Fazekas *et al*. 1963) used to follow pollen protein release. The latter technique was used with caution, however as the final solution has a methanol content of about 8% and cannot be guaranteed to indicate the normal pattern of protein release from pollen. Cinematography was used
to record the events which occurred at the microscopic level following Coomassie blue staining.

The appearance of Spurr's resin-embedded pollen was determined in each case by polymerizing a drop of the pollen-containing resin on a slide beneath the coverslip - the result being a permanent mount. This process is used with particular effect in chapter 4.

**Scanning Electron Microscopy**

After early trials using the processes of freeze-drying and freeze-substitution and vacuum coating with metal, it was concluded that these offered little short term advantage and some disadvantages, compared with the direct examination of fresh, uncoated material. Fresh material was generally in better condition with little evidence of collapse, provided that delays in mounting tissue on stubs, evacuation and examination prior to photography, were minimized. For most studies a Cambridge 'Stereoscan' 180 scanning electron microscope was used at preferred accelerating voltage (for fresh material) of 10 Kv. Photographic records were made on Polaroid film.

**RESULTS**

(i) The *Populus* stigma.

The stigmata of *P. alba* and *P. deltoides* were those examined in most detail in this study. The stigmata of the poplars studied are pale green in colour, with the exception of *P. tremuloides* where these are claret red. The stigma lobes of *P. deltoides*, *P. monilifera* and *P. sp* (Tacamahaca) are generally 3 in number and broadly triangular, whilst the *P. tremuloides* stigma is bi-lobed, again broadly triangular and 2-3 mm wide; those of *P. alba* are filiform, 1-2 mm long and 0.2 - 0.4 mm wide. In each case the margins of the stigma lobes are recurved so that the receptive
papillae are on the convex surface (figs. 1.I(a), (b), (c)).

Stomata are present on the non-receptive stigma surfaces, as also on the ovary walls of the flowers examined. The receptive surfaces of the stigmata of *P. deltoidea* and *P. alba* are formed of slightly raised papillae (figs. 1.II(a) and (c)). Using the classification of Heslop-Harrison *et al.* (1975), based on stigma characteristics, *Populus* might be placed in their 'group IV', in which papillae are described as being "no more than bullate or barely raised at all". None of the *Populus* stigmata observed, however, is "inundated by secretion fluid in the receptive state" (Heslop-Harrison *et al.* 1975), regarded by these authors as another characteristic of group IV stigmas. The *Populus* stigma conforms more to the 'dry' type described by them.

The individual papillae of the *P. deltoidea* stigma are elongated, being 20 to 50 µm broad and 70 to 100 µm long. Those of *P. alba* are slightly more raised yet shorter, being 34 to 50 µm in length (fig. 1.II (c)). In each species the receptive and non-receptive stigma surfaces are prominently different, the latter being smooth whilst the former is covered by slight bumps or protrusions, approximately 1 to 3 µm in diameter and raised about 1 µm above the surface (figs. 1.II(a), (c)). These appear to correspond in location and appearance to the 'waxy substances' or 'wax layer' observed by Roggen (1972) on *Brassica* papillae. They shall hereafter be referred to as 'wax structures'; some basis for their description as waxy being provided in chapter 5. Sectional views of the wax structures by transmission electron microscopy reveals that they are largely outside the cuticle, although the possibility remains that they may develop within the cuticle itself (figs. 1.III(a), (b) and 1.IV (b)).
Accumulating beneath the cuticle, particularly beneath the wax structures, are numerous spheroidal bodies containing material of similar electron density to that of the wax structures themselves (figs. 1.III(a), (b) and 1.IV(b)). These bodies may occasionally be discerned within the wax structures and may be closely related to them in composition (fig. 1.VII(b)). They appear to be bounded by membranous material, or at least material with similar staining properties, for which reason it is tempting to describe them as 'vesicles'. In some sections blebbing of the plasmalemma is apparent, and at maturity collapsed vesicles can be seen between the plasmalemma and the outer tangential wall of the papilla cells (fig. 1.III(a)). Material of similar electron density to the wax structures may be seen within the outer pecto-cellulosic wall of the papilla cells and may represent precursors of the vesicles or wax structures (fig. 1.IV(b)). A proper understanding of the events leading to the formation of *Populus* stigma surface features awaits a more detailed developmental study. The cuticles of the receptive and non-receptive stigma surface cells are distinctly different (figs. 1.III(b), (c)), and the transition from one type to the other is abrupt, taking place at the junction between the two types of cell, as also evidenced in surface view where the wax structures are present on receptive cells only (fig. 1.II(c)). The non-receptive cuticle is thicker (0.05-0.1 µ) than that at the receptive surface (0.01 to 0.02 µ) (fig. 1.III(c)). The non-receptive cuticle has a prominent 'cuticular' layer as evidenced by the reticular network of pectocellulosic material, whilst the receptive cuticle apparently abuts directly against the cell wall, is lamellate and possibly more akin to the 'cuticle' proper (figs. 1.III(b), (c)).

The outer tangential cell wall of the non-receptive surface is thinner (approx. 0.2 to 0.4 µm) than that of the receptive papillae.
which is 1 to 2 µm thick. The receptive surface of the *Populus* stigma is covered by a thin (approximately 0.025 µm) diffuse, electron-dense layer of irregular thicknesses which is evident over the wax structures (fig. 1.III(a)) which is equated with the 'pellicle' of Maltson *et al.* (1974). The receptive surface of the *Populus* stigma stains positively for esterases, a reported characteristic of the 'pellicle', the intensity being pronounced in the region of the wax structures (as determined in chapter 6), a feature not previously reported. There is no sign of a 'pellicle' on the non-receptive stigma surface.

The most prominent difference between receptive and non-receptive stigma surface cells, in sectional view, is the presence of large vacuoles containing osmiophilic material, possibly tannin (figs. 1.II(b) and 1.IV(a)). Such vacuoles are absent from the non-receptive epidermal cells. There is no evidence in *Populus* of the crystalline protein bodies which were observed in the papilla vacuoles of *Raphanus* by Dickinson and Lewis (1973). Nuclei are found towards the base of the papillae and rough endoplasmic reticulum is accumulated particularly under the papillae, yet otherwise there is no obvious polarity of papilla organelles (fig. 1.II(b), 1.IV(b)). The papilla cytoplasm contains: numerous mitochondria; plastids with, or without, starch and with few grana; vacuoles (1 to 4 µ diameter) of unknown function; extensive rough endoplasmic reticulum; polyribosomes and organelles resembling microbodies by their size (0.5 - 1 µm), single bounding membrane and granular cytoplasm. The latter organelles, for instance, closely resemble the microbodies of *Pisum* cotyledons as described by Clandenin *et al.* (1972). Dictyosomes may be seen occasionally, but are not common in mature papillae. The vesicles found outside between the plasmalemma and cell wall could have been formed by dictyosomes, but if so then their formation may have taken place prior to anthesis.
The 'stigmatoid' (Esau, 1960) or conducting tissue, of Populus is traversed by vascular tissue but is otherwise uniform in appearance (fig. 1.IV(a)). There are no intercellular spaces in the tissue, these regions containing pectocellulose materials as evidenced by toluidine blue and periodic acid-Schiff staining. The mature stigmatoid cells are characterized by their possession of large vacuoles which displace the cytoplasm to the periphery of the cells. The cytoplasmic organelles present include plastids which are generally larger than those of papilla cells, and contain what appear to be lipid droplets, rather than the starch which is evident in the papilla plastids. Rough endoplasmic reticulum is prominent in the stigmatoid cells and microbody-like vesicles are often present (figs. 1.X(c)).

(ii) *Populus* pollen.

(a) Nuclei

The pollen of each *Populus* species studied at anthesis included bin- and trinucleate grains, the proportion of trinucleate grains being greater in most cases (see Hamilton and Langridge 1975, Appendix I and fig. 1.V(a)). In some species, and particularly in *P. balsamita*, the pollen included those whose generative cells were in the process of division, (fig. 1.V(b)) indicating that in *Populus*, generative cell division occurs at, or close to, the time of anthesis. In feulgin stained mature pollen the vegetative nucleus appears to be larger (approximately 3 to 5 \( \mu \)), and its staining more diffuse, than either of the generative nuclei which are 1.5 to 2.5 \( \mu \) in diameter, densely stained and granular in appearance. The vegetative nucleus usually contains a prominent nucleolus. There is no obvious polarity in the position of either type of nucleus within the grain (fig. 1.V(a)).

The generative nucleus is contained within the generative cell, the cytoplasm of which contains rough endoplasmic reticulum, mitochondria,
vesicles, lipid inclusions, dictyosomes, polyribosomes and possible microbodies. The generative cell is bounded by an electron lucent layer which may consist largely of callose (Gorska-Brylass 1967; Kroh and Munting 1967) (fig. 1.V(c)).

(b) Pollen Anthesis

Sections made of anther material fixed between the time of opening of the bud and anthesis have shown that tapetal breakdown precedes catkin extension. In the early stages of catkin elongation the pollen contains numerous prominent starch grains (fig. 1.VI(a)). The intine contains material of similar staining properties to that of the stigma surface wax and of the material which ultimately fills the exine spaces in the mature grain (fig.1.VI(b)). It seems reasonable to propose that this is the same material which ultimately fills the exine cavities in the mature grain.

At this, earlier, stage and following phosphotungstic acid staining, the cavities of the sexine region contain crystalline bodies which are apparent by their negative staining (fig. 1.VI(a)). These crystals are 50 to 100 nm long and 10 to 30 nm broad. In places the plasmalemma (as visualized by phosphotungstic acid staining) extends inwards to follow the contours of electron transparent regions which are irregular in shape and continuous with the intine (fig. 1.VI(b)). There is no indication that these inward extensions are continuous with the generative cell wall.

Just prior to anthesis the starch content of the grain has decreased and the exine has the more typical appearance of that of the pollen wall at anthesis. The crystals seen earlier are no longer evident and the interstices of the exine are filled with homogeneous material of medium electron density which is taken to be largely lipidic (fig.1.VI(c)).

*Populus* pollen, as shed, is collapsed as is apparent by its light - and scanning electron - microscopic appearance and by its sectional appearance following fixation in methanol (rather than aqueous fixatives
(figs. 3.1(b) and 3.11(c)). The size of the collapsed grains is approximately 15-20 μm in diameter. The water content of the *Populus* pollen at the time of its release is about 30% and drops rapidly within 10 hours to 8%, a level maintained thereafter. The rate of water loss is demonstrated in diagram 1.2, the same curve being invariably produced by each of the *Populus* species examined in this way, including *P. nigra* var. *italica*, *P. deltoides* var. *angulata* and *P. bolleana*.

(iii) Events following pollination.

I. Compatible pollinations.

Within a few minutes of contact of *Populus* pollen with the stigma surface most grains have become hydrated as evidenced by their swollen state, and have produced a yellow exudate apparent at the exine surface. This exudate can appear at any part of the pollen surface, and causes the pollen grains to stick to the stigma surface and/or to each other (figs. 1.VII(a) and 1.VIII(a), (b)). *In vitro* hydration of *Populus* pollen grains in Coomassie blue stain has shown that protein may be released within minutes of moistening of the grain under these conditions.

Invariably the 'sticking reaction', which follows pollen-stigma contact involves at least one of the wax structures (figs. 1.VII(b), (c)) though whether this is due to the preferred adherence of the grain to such regions, or is simply unavoidable by virtue of their distribution cannot be ascertained. Within 3 hours of this contact the pollen grain can be seen to have visibly eroded the wax structures and liberated the vesicular structures within and below them (fig. 1.IX(a)).

In the *in vitro* experiment where pollen was sown onto dried hexane washings (wax) of the stigma in a moist chamber, little germination was observed yet, particularly with 'compatible' pollen-wax combinations, the pollen had obviously degraded the wax, presumably by enzyme action (fig. 1.IX(b)).

There is no sign, in *Populus*, of the sporopollenin degradation
Showing the rate of loss of water from pollen *P. nigra* var. *italica* from the time of its release from the anther.

Similar results were obtained with pollen of *P. deltoides* var. *angulata* and *P. bolleana*. 
Rate of water loss from time of shedding of pollen of *P. nigra* var. *italica* at 20°C.
reported by Gherardin and Healey (1969) in *Pharbitis*, and Roggen (1972) in *Brassica*. By 4 hours after pollination the compatible pollen tube has begun to emerge (fig. 1.IX(c)) and there is evidence of some callose production within the grain. Between 6 and 8 hours after pollination many pollen tubes have made contact with the stigma surface (fig. 1.XI(a)).

The cuticle is apparently degraded and the pollen tube then penetrates beneath the cuticle and largely outside the cellulose wall of the papilla cell (figs. 1.X(a) and 1.X(b)). The process is most likely one of cell wall breakdown at the pollen tube tip. It is difficult to determine the precise boundary between the pollen tube and the papilla wall. The pollen tube penetrates between the papilla cells in the cuticular flange region then, keeping within the middle lamella region, enters the stigmatoid tissue and grows in the direction of the ovary (fig. 1.X(c)). The pollen tube cytoplasm is notable for its high dictyosomal and mitochondrial content, and has prolific quantities of rough endoplasmic reticulum, much of which is closely associated with the tonoplast of vacuoles within the tube (figs. 1.X(c) and 1.XII(a), (b)). Some vesicles are present as are occasional plastids and lipid bodies, the latter being characteristically electron dense. The pollen tube wall has two discernable layers including a thin inner, electron transparent layer, and an outer layer of medium electron density (following normal staining), somewhat fibrous in appearance (figs. 1.X(c) and 1.XIV(a)). It is proposed that the inner electron-transparent layer consists largely of callose, as evidenced by its electron transparency and by the callose-positive staining of the inner part of the pollen tube wall using the method of Martin 1959 (fig. 1.XI(a)).

Following the pollen tube penetration of the stigma surface, the pollen grain and the regions of the tube remaining above the surface show prominent callose staining (fig. 1.XI(a), (b)) and electron micrographic sections show that these regions are packed with large vesicles with electron transparent contents, apparently including callose.
Occasionally the vegetative nucleus has been observed to remain isolated within the callose filled grain, the generative cells having moved down the pollen tube.

The generative cells can be observed within the pollen tubes in the stigmatoid tissue. Their amoeboid movement is feasibly achieved with the aid of the prominent microtubules apparent within the generative cell cytoplasm, and aligned in the direction of their movement (fig. 1.XII (a)).

II Incompatible pollinations.

The initial events following incompatible pollination (P. deltoides X P. bolleana) are indistinguishable from those resulting from the compatible situation, there being no evidence, in particular, of any difference in the sticking reaction.

Without a more extensive and detailed investigation it would be difficult to determine whether subsequent degradation of the wax structures by the pollen is less efficient in the incompatible, than in the compatible, situation although this does appear to be the case.

Within 15 minutes of pollination it is obvious that most of the incompatible grains contain granular callose deposits, not evident in compatible grains after a corresponding period of time (fig. 1.XIII(b) versus fig. 1.XIII(a)). There is no evidence of a callose reaction in the stigma papillae (figs. 1.XIII(b), 1.XV(b) and 1.XVI(a)).

By 2 hours after pollination, the incompatible pollen tube starts to emerge from the grain (fig. 1.XIII(c)), in contrast with compatible (P. nigra var. italic) pollen tubes which are slightly slower in emerging. In most cases the incompatible pollen tube makes contact with the stigma
surface yet usually fails to penetrate the cuticle (fig. 1.XIV(c) and fig. 1.XI(b)). It wanders across the stigma surface in an erratic manner, though often in fairly close contact with the stigma surface (figs. 1.XV(a),(b), (c) and 1.XVI (a)). The pollen tube, at this stage, often appears irregularly swollen and contorted possibly due, in part, to its (usually) thinner wall (fig. 1.XV(a) and fig. 1.XVI(b).

Where the pollen tubes do manage to penetrate the stigma surface and reach the stigmatoid tissue their growth thereafter is directed, as in the compatible situation.

The cytological appearance of the incompatible grain 3 hours after pollination is one of great activity and apparent disorder (figs. 1.XIV (a), (b)). In particular the contents are highly vesicular, possibly attributable largely to the numerous dictyosomes present. Plastids and mitochondria are in evidence but do not have their normal appearance. Incompatible pollen tubes are often more vesicular, than compatible grains at the same stages but not as pronouncedly as in the pollen cytoplasm. Apart from this feature the appearance of the cytoplasm of incompatible pollen tubes which are penetrating the stigma is not obviously different to that of corresponding compatible tubes. Concentric membrane structures are occasionally seen (fig. 1.X(b)) but do not characterize the incompatible tubes, unlike those observed by de Nettancourt et al. (1974).

In wall structure, incompatible tubes have a thicker electron transparent inner layer (following staining), particularly in the earlier stages of their growth. This may be correlated with the more intense callose staining of incompatible pollen tubes of Populus. Vesicles within the pollen grain and tube also contain callose as evidenced by granular callose deposits within their cytoplasm.
DISCUSSION

The preliminary observations made in this study seem to indicate that the lipidic material held within the sexine cavities of *Populus* pollen may include material derived from the gametophyte, rather than solely from components of the plasmodial tapetum. Such a suggestion would be in accord with the observations of Mepham and Lane (1969), that in *Tradescantia* the exine lipids are in part, at least, derived from within the grain. On the other hand, Dickinson and Lewis (1973b) regarded the sexine-held lipids as being derived from breakdown products of the plasmodial tapetum in *Raphanus*. Echlin noted in 1971: "the full significance of the extrusion of lipid material from within the pollen grains is not at present fully understood". The situation in *Populus* might be explicable in terms of such an extrusion at a late stage of development (within days of anthesis). This extrusion could feasibly cause the displacement of earlier deposited tapetal materials from the sexine cavities to the surface, where on dehydration they may form the surface 'membrane' noted by Rowley and Erdtman (1967). Such a mechanism could reasonably explain Rowley's (1970) observation that dense staining nexine-held materials are replaced, during development of the pollen grain, by other material of uniformly low density staining properties in *Populus*. In a later chapter the latter material is identified as being largely lipidic in composition (see chapter 5). The 'extrusion-displacement' explanation for wall lipid derivation must remain speculative until a more detailed developmental and histochemical study of *Populus* pollen is completed. Such a study is currently in progress (Ashford 1973 pers.comm.).

The low water content of *Populus* pollen after anthesis (less than 10%), is apparently not typical of such short-lived pollens (Stanley and
Linskens 1974). The rate of dehydration does not appear to have been recorded for any species, let alone *Populus*. The dehydration of *Populus* pollen is probably initiated shortly after, or upon, anther dehiscence and, thereafter, proceeds rapidly until the water content has reached a certain level, after which this is maintained, under normal conditions, for as long as the pollen remains viable.

The importance of the dehydrated state to the pollen *in vivo* is uncertain, although it may be that, in most cases, pollen will germinate more effectively when it is in a dehydrated state on reaching the stigma; the observed rapidity of dehydration would thus be a reasonable way of making sure that the pollen is in this state by the time it reaches the stigma. This might ensure, for example, uptake of materials from the stigma surface during the normally observed swelling of the grain. The results of the present study (Chapters 5, 6 and 7) demonstrate the rapidity with which protein is released from *Populus* pollen on moistening, a phenomenon widely observed (for example see Mäkinen and Lewis, 1962; Mäkinen and Brewbaker, 1967; Belin and Rowley, 1971; Knox, 1973, and the recent review by Heslop-Harrison, 1975). In addition, this present study has included a cinematographic examination of the events following hydration of *Populus* pollen, using the technique established by Howlett (1973), which involves filming the behaviour of pollen after its contact with an isotonic buffered solution containing Coomassie blue as a protein stain (Fazekas *et al.* 1963).

The results (not presented) showed release of protein from *Populus* pollen within 30 seconds and, of particular interest to this study, no obvious quantitative or qualitative difference in the release of protein
between intact, and hexane pre-washed pollens. Results obtained by this
technique should be treated with caution however, as the final dye
solution contains a 7% concentration of methanol, a solvent known,
in its pure state, to render pollen inviable (see chapters 3 and 4 and
Iwanami, 1972). In his recent review, Heslop-Harrison (1975) commented
that: "proteins pass out freely from moistened pollen, even from intact
cells where free passage through the plasmalemma seems improbable" and
further, that "the mobile fractions are in fact those housed in the wall
layers" (Heslop-Harrison 1975). The latter observation is based on his
detection of protein within the pollen wall after freeze sectioning
(involving transitory hydration of the grain), after aqueous fixation,
or in intact hydrated grains. As will be stressed in subsequent chapters
(see chapters 5, 6 and 7), the appearance of such proteins in the cell
wall following hydration need not, by itself, indicate that these proteins
are present in pollen wall sites prior to hydration; considering the
dynamic nature of events which follow pollen hydration, one should not
assume that proteins cannot traverse the plasmalemma with the rapidity
necessary to explain their appearance in the pollen wall shortly after
hydration. The apparent intactness of the grain during in vitro hydration
need not, by itself, discount this proposal. Stanley and Linskens (1965)
observed a similarly rapid loss of proteins from Petunia pollen on
hydration and suggested that this may indicate that at low osmotic
pressures "pollen behaves as a leaky membrane" (Stanley and Linskens 1965).
The latter proposal is seen as particularly reasonable if one considers
the rapid increase in size effected by its hydration, with, in some cases, as
Watanabe (1955) observed, an initial brief overshoot in size. Commented
Watanabe (1955): "Generally it takes about thirty to sixty seconds, for
the phenomenon of emerging and disappearing of bulgings, and the
phenomenon is hardly seen two minutes after pollination". Speculatively, a second process whereby protein might be rapidly released from the grain upon its hydration, would be for protein-containing invaginations of the plasmalemma into the pollen cytoplasm to become evaginated, on swelling of the grain, with release of their contents into the pollen wall or beyond, as a consequence. There is certainly evidence that the plasmalemma of *Populus* pollen is occasionally seen to be invaginated (fig.1.VI(b) & 3.II(a)). Such invaginations could be found to be part of a continuum between plasmalemma, endoplasmic reticulum, vacuoles formed as enlargements of endoplasmic reticulum and the nuclear membrane - such a continuum being originally proposed by Buvat (1963).

Such a continuum would permit the presence of materials (including protein) to be present within the grain, yet still allow their rapid release from the grain without the need for membrane disruption. In either case, following hydration, one would expect to find at least some of the protein released to be present within the intine matrix and/or the exine cavities. Obviously the mechanism of protein release may vary with the pollen species, with pollen wall proteins being present in some species, but absent in others. The difficulties in attempting to determine the location of materials in pollen, prior to hydration, are stressed by Dickinson and Lewis (1974) who comment that "as the pollen grains swell when immersed in an aqueous medium it is clear that material prepared using an aqueous fixative cannot give an accurate picture of the dry pollen as it is released from the anther". There is no firm evidence, from this study, which demonstrates the presence of proteins within the pollen grain wall of *Populus*, prior to hydration.

Several authors have proposed that the generative 'cell wall' incorporates pectins (Dexheimer 1965; Marayama *et al.* 1965; Horvat 1969
and Roland 1971) and possibly cellulose (Roland 1971). The appearance of this region in *Populus* pollen suggests rather, the presence of callose, as observed in other pollen species by Gosska-Brylass (1967); Kroh and Munting (1967) and Sassen and Kroh (1974). Lutz and Sjolund (1973) relate the activity of endoplasmic reticulum to generative cell wall formation in *Monotropa* yet make no mention of callose. This raises the possibility that the development of callose in pollen may similarly be related to endoplasmic reticulum activity.

The presence of microtubules within the cytoplasm of the generative cell of *Populus*, during its passage down the pollen tube, lends weight to Steffen's (1963a) proposal that generative cells have amoeboid movements of their own and are not, as suggested by Navashin et al. (1959), transported simply by cytoplasmic streaming of the pollen tube alone. There is no previous record of microtubules within generative cells in transit and yet this evidence for their amoeboid movement corroborates that of Polunina and Sveshnikov (1959), who filmed the passage of such cells along pollen tubes of species of Amaryllidaceae.

One outcome of this study, as reported by Hamilton and Langridge (1975, see appendix I), was the finding that *Populus* pollen from a number of species was predominantly trinucleate, rather than simply binucleate, as previously reported (Nagaraj, 1952; Smith, 1943; Brewbaker, 1967; Kirby and Smith, 1974). Brewbaker (1967) made the general comment that: "anthers are observed only rarely with both binucleate and trinucleate grains, and in these few instances one type clearly predominates", and further that, "with almost surprising regularity binucleate and trinucleate pollen types are mutually exclusive in angiosperm species and genera" (Brewbaker 1967). Previously, in most cases where bi- and trinucleate grains have been found in the same species this has been
ascribed to a precocious second mitosis (Wulff and Maheshwasi, 1938), and has been found particularly in old anthers (Wunderlich, 1936), and where there are changes in water availability (Poddubnaja - Arnoldi, 1936). Even allowing for the possibility that the trinucleate pollen phenomenon may be a consistent artefact, which seems most unlikely in view of the range of samples taken, the tendency of at least some of the grains to undergo the second mitotic division prior to anthesis is in agreement with the other features of Populus pollen, according to the correlations recognized by Brewbaker (1957; 1959, 1967) and Heslop-Harrison (1975). Brewbaker (1959) observed that many plants with exposed anthers have pollen which fails to germinate in vitro, with trinucleate forms prevailing. Populus pollen, which is shed from exposed anthers, is difficult to germinate in other than an in vivo situation at any time beyond the first week or two after shedding. Although many attempts were made to obtain good germination of stored pollen, no method was found to be reliable enough to enable its use in bioassays; this, in turn, meant that effects of various treatments could only be tested on the stigmata themselves. This provided restraints on the experimental time available for bioassays, as they required the use of fresh female flowers, little success being obtained with flowers which had been either freeze dried or stored in liquid nitrogen. Also little success was obtained in attempts to obtain germination on floral extracts including surface wax alone. This behaviour of Populus pollen is not uncommon, as was noted by Rangaswamy (1963) who reported that: "stored pollen, which fails to germinate in vitro, may nevertheless cause a satisfactory seed set", which he explained by the suggestion that "stigmatic and stylar tissues help to make up some of the deficiencies imminent to storage and provide a natural environment for the germination of the pollen and the growth of the pollen tube".

Linskens (1973) recalled that stigmatic incompatibility inhibition was
typical of trinucleate pollens and held that: "the first cause should be sought in the supply of reserve substances", which might be expected to have become depleted as a consequence of the second mitotic division. He further proposed that as trinucleate pollen germinates with greater difficulty, and has a higher respiratory activity than binucleate pollen, then "rapid decrease of germination strength is caused by such high respiration of dormant pollen" (Linskens 1973). Ascher and Peloquin (1968) held that the self incompatible system of *Lilium* might function to inhibit pollen tube growth by constraining the pollen tube to grow to a limited extent, because of "a low growth rate metabolism depending primarily on pollen reserves"; they further held that in the case of stilar incompatibility, "certain stilar substances must be present and available for the switch of pollen-tube metabolism to occur" (Ascher and Peloquin 1968). As poplar pollen shows a lower germination *in vitro* yet germinates readily *in vivo*, this indicates that the stigma surface provides the closely defined medium necessary for germination of *Populus* pollen and/or some stimulus which could also be involved in the incompatible mechanism.

The first contact the *Populus* pollen makes with the stigma is with the 'pellicle', whose presence on the *Populus* stigma is reported here for the first time. The pellicle was originally described by Mattson *et al.* (1974), who noted its widespread occurrence on stigmata of the 'dry' type (Heslop-Harrison *et al.* 1975) and proposed that it was involved in the rapid hydration of the pollen by virtue of its hydrophilic nature. If so, then it should not be surprising that emitted pollen proteins "bind to the pellicles of the stigma papillae very quickly after the initial contact" (Mattson *et al.* 1974), and need not indicate by itself that this is the primary recognition site, as they suggest.
As an addition to the correlation noted earlier by Brewbaker (1957), including that between the possession of trinucleate pollen and a sporophytic incompatible control, Heslop-Harrison et al. (1974) added a further correlate: "namely that in incompatibility systems where the pollen response is controlled sporophytically, the receptive surface of the stigma is of the 'dry' type, with little secretory exudate" (Heslop-Harrison et al. 1975). As already mentioned, (Hamilton and Langridge, 1975), such a correlation accords well with the Populus observations including the finding that most of the grains were trinucleate in the species examined. As pointed out by Knox et al. (1972b), the fact that pollen grains of Populus may interact to overcome incompatibility in a 'recognition pollen' situation, indirectly indicates they are less likely to have a gametophytic incompatibility control in which each grain would be expected to act independently of its neighbour. Thus although, to my knowledge, no previous worker has either found or stated that the incompatibility control of Populus is sporophytic it seems almost certain that this is so, and accords well with other features of the pollen and stigma according to the Brewbaker (1957, 1959 and 1967) and Heslop-Harrison (1975) correlations.

The Populus stigma does not conform completely to any of the four stigma groups of Heslop-Harrison et al. (1975) as it has the 'dry' surface characteristics of both groups 'I' and 'II', yet also the 'group IV' characteristic of barely raised papillae. It might be better placed in 'group II', considering the observation of Heslop-Harrison et al. (1975) that of the genera studied, this was the only group to contain plants with sporophytic incompatibility.

Although Heslop-Harrison et al. disputed Roggen's (1972) description of the lipidic patches or droplets on the papillae surfaces of Brassica as a 'waxy coating', the corresponding structures on Populus papillae are justifiably thus described, as is demonstrated in chapter 5. The
observed early association between the wax structures and the pollen of *Populus* agrees well with similar observations by Roggen (1972), with *Brassica*, and more recently by Lecocq and Dumas (1975), who referred to structures of similar appearance on the (dry) stigma papillae of *Begonia* as "gentellettes de sécrétion" (exudate droplets) and similarly observed their early association with pollen following pollen-stigma contact.

Roggen (1972) placed some emphasis on the 'sticking reaction' which he observed to occur between compatible pollen and stigma combinations in *Brassica*, but could not detect following incompatible pollination. That the 'sticking reaction' occurs on the *Populus* stigma following pollination by either compatible or incompatible pollination indicates that this reaction *per se* is not the factor determining incompatibility, although events associated with it may well be. It is held that the degree or rate of degradation of the wax structures may well play a central role in the incompatibility mechanism of *Populus*, its degradation following pollen contact being demonstrated in this study (fig. 1.IX(a)).

Brewbaker (1957) noted that in species with trinucleate pollen the incompatibility reaction occurred at the time of germination, that is, at the stigma surface. That this is true of *Populus* is indicated in several ways. Firstly the build up of callose in incompatible grains within 15 minutes in *Populus* appears to be a consequence of an incompatible reaction, although need not comprise the reaction itself. This accords well with the conclusion of Heslop-Harrison *et al.* (1975) that in the sporophytic (surface) incompatible genus *Brassica*, the decision between acceptance and rejection takes place within 10 minutes of pollen-stigma contact. Secondly the difference between a compatible and an incompatible response is evident in pollen tubes at the stigma.
surface, and indeed, there is little difference in their behaviour once complete penetration is effected. Thirdly the success obtained by Whitecross and Willing (1975), using purely surface treatments, again reinforces the argument that the stigma surface is the site of the incompatibility barrier in *Populus*.

There is no sign, in *Populus* of the 'stigma reaction' of Konar and Linskens (1966a) following incompatible pollination. Dickinson and Lawson (1975) similarly found an absence of this reaction after incompatible pollinations of the *Oenothera* stigma and took this to indicate that the stigma surface was preprogrammed.

The evidence in *Populus* points to an initiation of the incompatibility reaction with pollen-stigma contact, rather than at the time when the pollen tube first makes contact with the surface. It is proposed that the difference between compatible and incompatible grains is in the better ability of compatible grains to degrade the surface lipid with which they make their initial contact, and that this degradation is achieved enzymatically. This suggestion is supported by the observation that pollen grains are able to degrade the isolated stigma wax alone, shortly after their hydration. Such enzymes could be equated with the 'recognition substances' of Knox et al. (1972a). It seems that if a metabolic switch is necessary for proper pollen tube metabolism, as suggested by Ascher and Peloquin (1968), then this should operate shortly after the grain has made intimate contact with the surface - in the case of *Populus* by degradation of wax structures. At this point the contents of the 'vesicles' may play some part, particularly as they are concentrated beneath the wax structures. On the other hand, 'vesicles' may simply contain wax structure precursors, and the breakdown products of the wax structures themselves may play some part in the subsequent behaviour of the grain: the feasibility of
this proposal is evidenced by the recent findings of Koshimizu et al. (1972), that solvent extracts of flowers of *Tulipa* stimulated the germination of the pollen of *Chrysanthemum*, and that the stimulatory substances were aliphatic esters. As will be shown in chapter 4, the 'wax structures' of *Populus* stigmata are largely composed of aliphatic esters.

In this study stigma 'vesicles' are reported for the first time in *Populus*, and do not appear to have been recorded for other genera. Results obtained by Kroh (1966) showed that, in the cases of *Arabis* and *Brassica*, even contacts with the stigma within 5 minutes of pollination, resulted in the commitment of the behaviour of the grain, depending on the initial 'compatibility' or 'incompatibility' of the contact, a result she interpreted in terms of activation, or blockage of activation, of 'cutinase enzyme'; 'cutinase' was regarded as essential for later penetration of the stigma surface by the pollen tube. Similarly, Heslop-Harrison et al. (1975) have proposed that, following pollen-stigma surface contact, "an enzyme activation ensues that ultimately leads to the degradation of the cuticle and the entry of the pollen tube into the papilla wall", a process they regarded as probably involving esterase. It is evident that following an incompatible pollination of *Populus*, many of the pollen grains and tubes have a markedly different ultrastructural appearance to those of compatible pollen, including changes in wall structure, callose content and a higher degree of vesiculation. This would seem to reflect a more general metabolic disturbance which, in turn, could be expected to affect the production of not just one, but possibly a number of enzymes including esterases, 'cutinases', pectinases and cellulases all of which may be required for efficient pollen tube penetration of the stigma surface. That the presence of callose may
reflect a metabolic disturbance, and particularly of carbohydrate metabolism, has been suggested before (for example Tupy, 1959; Townsend, 1971; Dickinson and Lawson, 1975). It is proposed that, in Populus, this disturbance in incompatible grains may be brought about by the lack of a metabolic switch in the sense of Ascher and Peloquin (1968) rather than by inhibition; thus incompatible grains, being less capable of efficiently degrading the stigma surface ('wax structures'), either fails to receive, or receives less of, the 'switch' substance, or receives it at a less appropriate time, that is, after some delay. The determination of the presence, function, or nature of such a substance be it a co-factor, nutrient, hormone, or whatever, is beyond the scope of this study.

A more general metabolic disfunction of incompatible pollen tubes would be quite in keeping with the Populus results, as it would help to explain the erratic growth and conformation of the pollen tubes, yet allow for a restoration of normal behaviour on the chance occasions where they are able to penetrate the surface.

Owing to the thinness of the receptive cuticle of the stigma of Populus, 'cutinase' might be less important for the early stages in the penetration of the pollen tube than pectinase, which is obviously required for penetration of the cell wall, and subsequently the middle lamella, by the pollen tube. The observation that pollen of Populus is apparently inhibited at different stages of development (and to different degrees) in an incompatible situation enhances the argument for a more general and non-uniform metabolic disfunction and thus it would be difficult to cite, for instance, the cutin (Linskens and Hein 1962), or the cellulose-pectic layer of the papilla cell wall (Kanno and Hinata 1969), as the 'incompatible barrier'. The apparent normalcy of incompatible pollen tubes which have penetrated to the stigmatoid tissue may either indicate a restoration of the normal metabolism, or simply
that some grains, less affected than others, behave in a similar fashion
to compatible grains. Control incompatible crosses, however (see
chapter 2) have shown that few such grains must reach the ovules, if at
all.

The observation that incompatible pollen tubes of *Populus* have
thinner walls parallels similar observations made by de Nettancourt *et al.*
(1974), who similarly found the pollen tube walls of *Lycopersicon* to be
thinner following incompatible interspecific crosses, whereas self
incompatible tubes of the same genus were markedly thicker than normal;
Schlösser (1961) similarly observed the self incompatible pollen tubes of
*Petunia* to be thicker.

The close association between pollen tube vacuoles and rough endo-
plasmic reticulum in *Populus* recalls similar vacuole - endoplasmic
reticulum associations evident in the stigmatic cytoplasm of *Forsythia*
during the lipidic secretory phase (Dumas 1974a). He correlated this
association with "a phase of intense active transport of material to
(and or from) the vacuole" (Dumas 1974a). The vacuole - endoplasmic
reticulum association in *Populus* may also indicate active transport,
though in this case, not of lipid; as mentioned earlier, it may also
be related to callose build-up.

Unlike *Lycopersicon*, in which concentric smooth endoplasmic
reticulum was observed to be a constant feature of incompatible pollen
tubes (de Nettancourt *et al.* 1974), those of *Populus* occasionally contained
such bodies but these were by no means typical of, or universal in, the
incompatible situation. de Nettancourt *et al.* (1974) saw this concentric
endoplasmic reticulum-incompatibility pollen tube association as indicative
of "a general cessation of protein synthesis". The occasional
appearance of concentric endoplasmic reticulum in *Populus* pollen tubes may similarly reflect such a metabolic disturbance. There is no evidence that the interspecific incompatibility mechanism need be other than a passive process resulting from a lack of growth stimulation, as opposed to, for example, the conclusions by de Nettancourt *et al.* (1973) that the self-incompatibility of *Lycopersicon* results in the bursting of the pollen tube due to lytic action, which they regard as an active incompatibility event.

The genetic basis of the interspecific (intersectional) incompatibility of *Populus* has not been determined. Hogenboom (1975) held that in general "the influence of the S-gene has been greatly overestimated" and forwarded the proposal that other principles may be involved in the non-functioning of interpopulation pistil-pollen relationships; he held that the latter should be termed 'incongruity' rather than incompatibility. 'Incongruity' was described by Hogenboom (1975) as "non-functioning as a consequence of non-maturing of the partners of the genetic information which regulates interaction and coordination" and further proposed that "pollen may, for example, lack genetic information on the pistils osmotic pressure, cutin or waxy layers on the epidermis, length, and on one or more of its many other physiological, biochemical or structural characters, depending on the direction and degree of evolutionary divergence". He held 'incongruity' to be the rule and 'congruity' the exception. Hogenboom further recalls the results of his own experiments which showed that, in several cases, interspecific pollen tube growth inhibition resulted from the action of "a number of independent dominant genes". Mather (1975) commenting on Hogenboom's (1975) paper, concurred with his thesis, noting that: "bars to crossing may be built on the incompatibility reaction where it is available, but they do not require the incompatibility
reaction as a basis; and, one supposes, in the great majority of cases they arise independently of this incompatibility reaction". (Mather 1975).

On this basis and considering the labile nature of the intersectional 'incompatibility' barrier of *Populus*, this situation might better be described as intersectional incongruity.*

Although the results presented in this chapter do not alone demonstrate the nature of the incongruity barrier, they are consistent with the conclusion (Chapter 7), based on both these results and those of later chapters, that the effective incongruity barrier is, by its composition, the wax coating at the stigma surface.

* To avoid confusion the term 'incompatibility' will be used throughout the thesis, bearing in mind that this should possibly be more correctly termed 'incongruity' in *Populus*. 
fig (a)  Macrophotograph of mature female flowers of *P. deltoides*.

fig (b)  Scanning electron micrograph (S.E.M.) of stigma lobes of *P. deltoides*.

fig (c)  S.E.M. of stigma lobes of *P. alba*.
Plate 1.11

fig (a) S.E.M. of the receptive surface of the stigma of *P. deltoidea*. The papillae (P) are bullate, and, each covered with 'wax structures'.

fig (b) Transmission electron micrograph (T.E.M.) of a section of the stigma epidermis of *P. deltoidea*. Each papilla (P) contains a prominent tannin vacuole. (Os/Ur/Pb staining).*

fig (c) S.E.M. of a lobe of the stigma of *P. alba*. The receptive surface (R) is characterized by the presence of wax structures, not evident on cells at the non-receptive (NR) surface. (*Osmium tetroxide/Uranyl acetate/Lead citrate - see Materials and Methods).
Plate 1.III

T.E.M. of a section of the surface of a receptive papilla of 
P. deltoides. (Os/Pb/Ur staining).

fig (a) Shows a sectional view of a 'wax structure' (w.s.) over lain by a 'pellicle' (pe). Vesicles (arrow) are apparent just outside the plasmalemma (p) and also (v) beneath the 'wax structure'. The most prominent feature in the papilla cytoplasm are vacuoles (va) [and plentiful endoplasmic reticulum (e.r.)].

fig (b) A close view of the surface showing the spatial relationship between the vesicles (v), cuticle (c), 'wax structure' (w.s.) and 'pellicle' (pe).

fig (c) (inset) the cuticle (c) of the non-receptive epidermis differs in structure and thickness from that of the receptive surface (compare with (a)).
Sections of the stigma of *P. deltoides*.

**fig (a)** Light micrograph of a lobe of the stigma showing that tannin vacuoles are prominent in receptive surface epidermal cells, but not in either the non-receptive (NR), epidermis or stigmatoid (ST) cells. (toluidine blue stained pH 4.4).

**fig (b)** T.E.M. of section at the junction between two receptive papillae. 'Wax structures' are evident at the surface.

**fig (c)** T.E.M. of section of the cytoplasm of two adjacent papillae. As evident from (b) and (c), the papilla cytoplasm contains prominent vacuoles (va), plastids (p) (containing starch (s)) and the mitochondria (M), rough endoplasmic reticulum and the nucleus (n).
fig (a)  Light micrograph (L.M.) of whole pollen of *P. nigra* var. *italica* (stained with Feulgen) showing the presence of a single vegetative nucleus (V.N.) with prominent nucleolus, and two denser staining generative nuclei (G.N.)

fig (b)  L.M. of whole pollen of *P. bolleana* showing several pollen grains at various stages of pollen grain mitosis (Feulgen stained).

fig (c)  T.E.M. of a section of the pollen grain of *P. nigra* var. *italica* in a plane through the generative cell (G.C.) and the vegetative nucleus (V.N.). The generative nucleus (G.N.) is apparent within the generative cell, whose cytoplasm also contains dictyosomes (d), mitochondria (m), endoplasmic reticulum and lipid vesicles (lv). The generative cell has its own electron transparent 'cell wall' (g.c.w.) which apparently consists of callose (ca). A nucleolus (nu) is prominent within the vegetative nucleus. (Os/Ur/Pb stained).
Sections of pollen of *P. deltoidea* var. *angulata* at stages of development prior to anthesis.

**fig (a) and (b)** T.E.M.'s of sections of pollen at a stage just before the catkin has started to elongate (both stained with phosphotungstic acid (P.T.A.). Starch granules (s) are numerous and prominent within the cytoplasm. Both micrographs show the presence of electron transparent 'crystalline' bodies (arrowed in (a)), between the exine baculae, a prominently stained plasmalemma (p) and the presence of material of medium electron density within the intine (i) possibly in transit. Fig (b) shows that the plasmalemma extends into the cytoplasm in some regions (arrowed in this case.)

**fig (c)** T.E.M. of a section of pollen in catkins which have fully elongated, but whose anthers have not opened. The starch (s) content is less than before and what appear to be lipid vesicles (l.v.) are now apparent. The cytoplasm is packed with mitochondria (m) and ribosomes (mainly associated with rough endoplasmic reticulum) indicating a high level of activity. The appearance of both exine (e) and intine (i) differs from that at earlier stages in development. The vegetative nucleus (V.N.) is also evident in this section.
fig (a) S.E.M. of two pollen grains of *P. bolleana* showing that the grains may stick to each other (arrowed) as well as to the stigma surface. The exudate, apparently partly responsible for such sticking, is apparent at the surfaces of the walls of each grain.

fig's (b) and (c) T.E.M.'s of sections through the point of contact between the pollen grain and the papilla surface. In (b) this clearly involves a wax structure (w.s.) Vesicles (v) are apparent beneath the cuticle covering the cell wall (c.w.) their contents in (b) having similar electron density to that of the 'wax structure'. Entities which may represent vesicles within the 'wax structure' in (b), are arrowed.
fig (a)  T.E.M. of contact region of *P. nigra* var. *italica* pollen with the *P. deltoides* stigma surface, three hours after pollination. The 'wax structure' is obviously degraded and vesicles (v) liberated into the region between the 'wax structure'(w.s.) and the pollen exine (e).

fig (b)  S.E.M. of whole pollen of *P. nigra* var. *italica* on wax extracted from the stigma surface of *P. deltoides* by hexane washings. After remaining (*in vitro*) on the wax for three hours in a humid environment, the grains have sunk into the wax to some degree (for example see arrowed region).

fig (c)  S.E.M. pollen of *P. bolleana* on the stigma surface of *P. alba*. The grains have begun to germinate and the pollen tubes are just beginning to emerge (arrow).
fig (a)  S.E.M. of *P. nigra* var. *italica* pollen on the *P. deltoides* stigma 12 hours after pollination. Note the 'crazing' of the surface of the exine. The apparent site of pollen tube penetration of the papilla surface is arrowed.

fig (b)  T.E.M. of section in the region of the junction between two papilla cells (P) showing the position of the pollen tube (P.T.) following a compatible cross and after 12 hours. The metabolic activity of the pollen tube cytoplasm is suggested by its endoplasmic reticulum and mitochondrial content. An electron transparent region, (following Os/Ur/Pb staining), possibly callose (ca), is apparent in the tube wall. Note the presence of a concentric membranous body (arrow) in the cytoplasm.

fig (c)  T.E.M. of cross section through a ('compatible') pollen tube (P.T.) between the base of the papilla cell (P) and the stigmatoid cells (ST). The cytoplasm has similar contents to that of (b).
Plate 1.XI

Fluorescence micrographs of pollen on the stigma 8 hours after pollination (stained for callose).

fig (a)  *P. nigra* var. *italica* pollen on the *P. deltoides* stigma.

fig (b)  *P. bolleana* pollen on the *P. deltoides* stigma.
Plate 1.XII

T.E.M.'s of sections of *P. nigra* var. *italica* pollen tubes within the stigmatoid tissues of *P. deltoides*. (Staining Os/Ur/Pb.)

**fig (a)** Section in the plane of the generative cell (G.C.) which is moving through the pollen tube cytoplasm. The pollen tube wall (c.w.) has an inner electron-transparent region (ca), possibly largely callosic, and an outer fibrous region. The generative cell contains its own nucleus (G.N.) and those structures already described for this cell when in the pollen grain. Microtubules (mt) are clearly visible in the generative cell cytoplasm.

**fig (b)** Oblique section of the pollen tube. Dictyosomes (d) and endoplasmic reticulum (e.r.) are particularly prominent in this section.
Plate 1.XIII

Fluorescence micrographs of pollen on the stigma *P. deltoides*.
Pollination was performed *in vivo*, then fixed at the times stated.
(Stained for callose.)

**fig (a)**  *P. nigra* var. *italica* ('compatible') pollen after 15 minutes on the stigma.

**fig (b)**  *P. bolleana* ('incompatible') pollen after 15 minutes on the stigma. Note the more intense callose staining of the pollen.

**fig (c)**  *P. bolleana* ('incompatible') pollen after 2 hours on the stigma.
figs (a) and (b) T.E.M.'s of sections of 'incompatible' (*P. bolleana*) pollen grains 3 hours after their first contact with the stigma. The cytoplasm has prominent vacuoles (va) and numerous vesicles (v) apparently produced by the dictyosomes (d) present.

fig (c) S.E.M. of an 'incompatible' pollen grain (*P. bolleana*) on the stigma showing the presence of a sticking reaction between the grain and the stigma (arrowed). The second arrow points to the contact region between the pollen tube and papilla.
Plate 1.XV

Incompatible pollen stigma combinations 24 hours after pollination.

figs (a) and (b) *P. bolleana* pollen on *P. deltoides* stigma.

As observed by both S.E.M. (a) and fluorescence microscopy (b).

fig (c) S.E.M. of *P. nigra* var. *italica* pollen on the stigma of *P. alba.*
Micrographs of *P. bolleana* pollen on a *P. deltoides* stigma 18 hours after pollination.

**fig (a)** Fluorescence micrograph (after callose staining) showing callose (ca) to be prominent both within the grain and the irregularly swollen pollen tubes.

**fig (b)** T.E.M. of a section through the pollen tube at the stigma surface. The thin tube wall (c.w.) and the thick callose (ca) wall lining are apparent. The cytoplasm contains swollen endoplasmic reticulum (e.r.) much of it smooth.
CHAPTER 2

METHODS USED TO OVERCOME THE INCOMPATIBILITY BARRIER IN POPULUS

INTRODUCTION

The approach in this study has been largely tempered by the expectation that the nature of the incompatibility barrier should be clarified not only by a determination of the ways by which it may be circumvented, but also by an understanding of how these treatments may function. Linskens (1973) recognized the potential of such an approach, commenting: "experiments on overcoming the reaction of incompatibility offer ... precise corroboration of ideas about the nature of the inhibition reactions gained from analytical biochemical investigation". In the following review of the methods known to overcome incompatibility in a variety of plants it will be seen that only a few have been attempted previously using Populus, most having been shown to be effective with self incompatible plants (including those whose compatibilities are both gametophytically and sporophytically controlled). Many of the same methods will be used in this study either in attempts to overcome the intersectional incompatible barrier in Populus, or simply to observe the resulting pollen and stigma behaviour at the microscopic level. Their effectiveness, or otherwise with Populus will be discussed.

Methods Used In Attempts to Overcome Incompatibility

1. Breeding

Genetic methods are reviewed by de Nettancourt (1972) and include haploidization, tetraploidization, haploidization-diploidization, and the induction of permanent self incompatibility by S-locus mutation and polygenic modification.
2. Timing of Pollination

The (in)compatibility of *Lilium longiflorum*, for example, was shown by Ascher and Peloquin (1966) to be a function of physiological age, and thus by delaying pollination until 6 to 9 days after anthesis, these authors were able to circumvent the incompatibility. Similar results were obtained by Kakizaki (1930) by delayed selfing of *Brassica*, yet could not be obtained with other genera by Stout and Chandler (1933); Yasuda (1934) or Shivanna and Rangaswamy (1969).

At the other end of the scale, bud pollination has been used in many genera following initial success with this technique by Pearson (1929), using *Brassica*. The technique consists of pollinating the stigma prior to antheses and, apparently, prior to the full development of the incompatible barrier. Bud pollination has been employed with success by a number of authors (Yasuda 1934; Attia 1950; Williams 1951; Lewis 1951; Pandey 1959; Sampson 1962; Shivanna and Rangaswamy 1969; Johnson et al 1970), using a number of genera including *Trifolium*, *Petunia* and a number of the Cruciferae. Shivanna and Rangaswamy (1969) found that bud pollination of *Petunia axillaris* was more effective where the stigma was first precoated with exudate from an open flower. The technique of bud pollination has been reviewed by Wiering (1958).

3. Emasculation

Linskens (1964) reported that emasculation of the flower of *Petunia* limited the stylar elongation resulting in growth of pollen tubes closer to the ovaries, yet still insufficiently for fertilization.

4. Environmental Factors

That environmental factors could affect the incompatibility of plants was determined as early as 1876 by Darwin who noted that the self incompatible *Escholtzia* from Brazil could be selfed in the cooler...
climate of Britain. Lewis (1942) found that increased temperatures magnified the existing pollen tube growth response, the incompatible pollen tubes growing slower and compatible tubes faster in *Primula*, *Prunus* and *Oenothera*. Hagman (1963) was able to self *Betula* by lowering the temperature, by contrast with most studies which have concentrated on the effects of increased temperature on incompatibility. Decreased incompatibility on raising the temperature has been demonstrated by, for example, Lewis (1942); Modlibowska (1945); Lewis (1954); Bali and Hecht (1965); Kwack (1965); Hopper *et al* (1967); Ascher and Peloquin (1968); Townsend and Danielson (1968); Kendall and Taylor (1969) and Gonai and Hinata (1971). Ascher and Peloquin (1968) found that incubation of stigmata of *Lilium longiflorum* at 39°C, would remove self incompatibility but had no effect on interspecific incompatibility.

Townsend (1971), in a review of the literature reported:

"in general, only relatively high temperatures have been found to inactivate the incompatibility mechanisms and the site of inactivation has been the style and not the pollen". Commenting on the extensive research into the effect of temperature on the self incompatibility of *Trifolium*, Heslop Harrison (1975) asserted that these studies have been "so far yielding little of importance in relation to the biochemistry of incompatibility".

Yasuda (1928) found that plants growing in soils of different moisture level had different levels of incompatibility, which may be a result of their vigour, as Yasuda (1929) also showed that self incompatible plants were better able to express their incompatible character when growing vigorously. Linskens (1961), reporting the observation that selfing of Cruciferae was often enhanced under conditions of high humidity, held that this could be due to the lowered stress on water recovery from the stigma by the pollen.
5. Nutrients

Kwack (1965) found that "the presence of calcium ions at certain levels in either cultural medium or presoaked stylar sections partially overcame the incompatibility reaction". He also noted a population effect, which may have been due to the increase in available calcium. Kendall (1968) found that although di- and tri-saccharides, boric acid and calcium all stimulated pollen tube growth in *Trifolium*, none of these overcame its incompatibility barrier.

Of possible relevance in this context is the result of Eklundh-Ehrenberg *et al.* (1946) who found that a single seed of *P. tremuloides* contained radioactivity derived from eight pollen parents, indicating that each seed may develop under the influence of a number of separate grains; the nature of this influence was not determined by Ehrenberg *et al.*, but could be hormonal or of nutrient value. Also Kopecky (1960) found that *Populus* pollen, kept moist in a stoppered test tube for several days, was able to stimulate, but not fertilize ovules in a compatible cross.

6. Hormones

Many of the hormone treatments successful in overcoming the incompatibility barrier have involved the use of either α-naphthalene-, or indole-acetic, acids (Crane and Marks, 1952; Davies and Wall, 1961; Pandey, 1968; de Nettancourt *et al.* 1971) and 2,4 di-chlorophenoxy acetic acid (Charles *et al.* 1974). It has been proposed that such hormones function by preventing floral abscission and thus facilitate fertilization by the slower growing pseudo-compatible pollen (de Nettancourt, 1972).
7. **Stigma and Stylar Extracts**

Michurin (1949) reported his use of a technique, involving transfer of "a particle of the cellular tissue of the stigma and its secretion (of an acid reaction) from the flower of the male parent to the stigma of the pistil of the female parent", to make interspecific and intergeneric crosses. He claimed that it was, "the specific odour of the secretion that plays the specific role", and cited Timiryazev's (1914) findings that the role of such stigma portions was not affected by boiling.

Bali (1963) had no success in overcoming the incompatibility barrier of *Oenothera* using extracts of compatible stigmas, yet Pandey (1963) gained some improvement thus, with *Nicotiana*, but only when transfer of exudate was followed by bud pollination.

8. **Radiation**

Ionizing radiation of pollen or styles has been used by a number of authors in attempts to overcome incompatibility (Linskens *et al.*, 1960; Davies and Wall, 1961; Hopper and Peloquin, 1968; de Nettancourt and Ecochard, 1968; Pandey, 1974). Hopper and Peloquin (1968) attributed their success with *Lilium* to enzyme inactivation. de Nettancourt and Ecochard (1968) held that their results with *Lycopersicon*, using chronic radiation (7.5 rad/hr), were obtained by inhibition of floral abscission and thus the radiation was similar in effect to hormone treatments; this proposal was supported by their observation that increased seed production was due to the increased number of fruits set, rather than the number of seeds produced by each fruit. Pandey's (1974) results with *Nicotiana* showed that radiation could effectively overcome incompatibility in self incompatible species, but not in those which are self compatible; he thus proposed that there was a gametophytic control of the former, the latter being sporophytically controlled, and that where plants have both forms of control, the two alleles may be expressed without interaction.
9. RNA and Protein Inhibitors

As both proteins and RNA have been implicated in many theories of incompatibility, the use of inhibitors affecting their production should provide some indication of the nature and timing of their involvement.

Cycloheximide, which inhibits the synthesis of protein, was found by Ascher and Drewlow (1970) to retard the growth of both compatible and incompatible pollen tubes in *Lilium* styles; the RNA inhibitor 6 methylpurine diminished the level of compatible pollen tube growth to the level of that of incompatible tubes - a result they interpreted as indicating that, "only compatible pollen tube growth requires RNA synthesis".

Ascher (1971) then showed that if RNA inhibitors were introduced into the style of *Lilium* 6 to 12 hours prior to incompatible pollination, then the incompatibility barrier would be overcome; he took this to indicate that RNA synthesis in the style is necessary for the self incompatibility reaction. He also showed that RNA synthesis is necessary for pollen tube growth of *Lilium*, using RNA inhibitors.

10. Carbon Dioxide

Following his observation that pollen tubes of (self incompatible) *Brassica* would penetrate the stigma when placed in a dessicator containing apples, Nakanishi *et al* (1969) found that the effect was due to carbon dioxide, and not ethylene vapour.

11. Electric Aided Pollination

Michurin (1949) facilitated difficult crosses by, "use of the influence of discharges of static electricity upon the pollen", yet held that "the cause of success could hardly be attributed to the action of the electricity alone, which in these experiments was inseparably connected with the inevitable ozonization of the pollen." Roggen *et al* (1972)
reported that the incompatibility barrier in *Brassica* could be overcome by using an electric potential of 100 V between pollen and stigma at the time of pollination.

12. **Grafting**

Several methods involving the use of grafts have been used to overcome incompatibility. These may be placed in two main categories:

(i) as early as 1849, Gartner is reported as having overcome the self incompatibility of *Passiflora* by grafting material bearing the flowers to be pollinated onto another plant (Yasuda 1934). Similar results were obtained by Denward (1963) with self incompatible *Trifolium*. Evans (1959) found that heterografts of *Trifolium* provided better seed set than did homografts. Denward (1963) described plants which could be successfully treated in this way as "the labile group". In making "obstinate" interspecific crosses, mainly of fruit trees, Michurin (1949) reported successes by "first producing vegetative unions and only then crossing the plants sexually". The male graft in this preliminary vegetative union was described as the 'mentor' by Michurin.

(ii) incompatibility barriers have been overcome either by grafting compatible stigma and/or stylar tissues onto previously decapitated pistils, as has been done in *Petunia* (Yasuda 1931) and *Oenothera* (Emerson, 1940; Hecht, 1960, 1964; Bali and Hecht, 1965); also by shortening the style by removing a portion then regrafting the stigma onto the remaining stump (Gardella 1950).

13. **Removal of, or Damage to, the Stigma Surface by Physical Means**

Success using the above treatment has been recorded in the following studies:

(i) by abrasion of the stigma surface, as achieved by Oelke (1957) with *Raphanus*, Singh (1958) with *Brassica* and Holden and Bond (1960) with *Vicia*. 
(ii) by decapitation of the stigma, pollen being applied to the cut end, this method being successful with *Solanum* (Swaminathan, 1955), *Raphanus* (Kroh, 1956); *Brassica* (Singh 1958) and more recently with *Ipomoea* by Charles *et al* (1974).

(iii) Kroh (1956) bypassed the stigmatic barrier of *Raphanus* by inserting pollen directly into a slit made in the style, an experiment successfully repeated by Tatebe (1959). Cheng and Mattson (1972) attempted to overcome incompatibility by intrastylar pollination of *Lilium*, yet found that crosses made by this method were less effective than normal, and that corresponding self pollination gave marginally different results.

14. Modification of the Stigma Surface by Chemical Means

Immersion of the stigma of *Oenothera* in water at 50°C for 5 minutes, was shown by Hecht (1964) and Kumar and Hecht (1965) to diminish the incompatibility reaction, a result interpreted by Kwack (1965) as indicating: "the inactivation of a certain protein, possibly enzymic in nature, involved in the incompatibility reaction."

Linskens and Heinen (1961, 1962) noted the 'cuticular' nature of the incompatibility barrier in *Brassica* and *Tropaeolum* and determined that 'cutinase' enzymes were present in pollens of the species whose stigmata were covered with cuticle (for example *Brassica*) and not in those without (for example *Petunia*). That this barrier could be eliminated by other means than 'cutinase' was demonstrated by Tatebe (1968) who found that, by treating the stigma surface of *Raphanus* with very small quantities of either 10% potassium hydroxide or diethyl ether, self incompatibility could be overcome. Tatebe concluded that "ether treatments cannot dissolve the stigmatic cuticle, but make it permeable to water" (Tatebe 1968), a proposal in agreement with Linskens (1973) later
statement that the cuticle is "characterized by a high degree of resistance to all lipid solvents."

Whitecross and Willing (1975) used hexane and diethyl ether pretreatments of the stigma surface, (using techniques similar to those of Tatebe (1968), and thus obtained intersectional hybrids of \textit{Populus}. They proposed that their results lent support to the notion that the incompatibility barrier "might be in the form of emulsifiable lipids on the surface of the stigma." (Whitecross and Willing 1975).

15. Chemical Treatment of the Pollen

\textit{Knox et al} (1972a) found that if, prior to an incompatible cross with \textit{Populus}, the incompatible pollen was mixed with ammonium sulphate precipitated, and lyophilized extract of 2 hour saline diffusates of compatible pollen, the intersectional incompatibility barrier could be overcome. The extract was regarded by these authors as proteinaceous and to be derived from the pollen wall, and was described as 'recognition protein' (Knox \textit{et al}, 1972a).

\textit{Iwanami} (1973) was apparently the first to use the organic solvent treatment of pollen in an attempt to overcome the incompatibility barrier, obtaining a marginal benefit by thus pretreating pollen prior to the selfing of \textit{Petunia hybrida}.

Better success was obtained by Roggen (1974) who overcame the incompatibility barrier in \textit{Brassica} by use of acetone and acetone-water mixes. He found chloroform to be ineffective, and since this solvent was observed to remove the pollen coat ('PC') of \textit{Brassica} pollen, took this to indicate that "the PC plays an important role in successful pollination."
16. **Conditioning of the Pollen**

Results obtained by Kroh (1966) with *Brassica* are particularly interesting. She demonstrated that by placing pollen on a non-self stigma, then transferring it back within 4 to 30 minutes to a 'self stigma', the self incompatibility barrier could be overcome. She interpreted this as evidence of an early activation of a pollen 'cutinase' on the compatible stigma, the activation being apparently irreversible. Kroh commented that: "The fact that under favourable conditions only a short contact of the pollen grain with the stigma is necessary for the activation of the pollen cutinase suggests that the reaction inducing the activity of the cutinase occurs between specific structures present in the wall of the pollen grain and papilla."

17. **'Mentor', 'Recognition' and Pollen Mix Effects**

The mentor technique, as described by Michurin (1949) was performed by establishing a vegetative union (graft) between two individuals prior to crossing them.

Besides his 'mentor graft' technique Michurin also found that "in dealing with interspecific crosses definitely known to be difficult, I have often achieved success by adding a very small amount of pollen from the maternal parent to the pollen of the paternal parent."

Pollen mixes were also used with success by Michaj'lova (1950) and Polyakov (1950) to overcome interspecific incompatibility in *Nicotiana*, the effect of the so-called 'mentor pollen' being believed, by Polyakov (1964), to be at a biochemical level involving cytoplasmic interactions.

Polyakov (1965) furthered this proposal by suggesting that "physiology of fertilization has a direct effect on heredity, variability and vitability of progeny." This "effect of polyfatherhood" as Polyakov describes it, is claimed to be expressed in physiological participation
in the fertilization yet "without participating in double fertilization". It must be allowed that the 'hereditary effects' noted by Polyakov in pollen mix effects may be due, in part at least, to occasional double fertilization. Gorshkov (1958) also used the pollen mix technique to facilitate the 'wide hybridization' of a number of fruit trees. Stanley (1964) proposed that such mixed pollinations may delay abscission of the style and thus allow subsequent fertilization by otherwise incompatible pollen - an effect similar to that produced by hormone treatments.

Pfahler (1967) used pollen mixes in an attempt to determine the relative independent fertilization ability of each pollen component of the mix, an approach which apparently fails to recognize that interaction may occur between the two which could produce misleading results.

That irradiated 'mentor' pollen could still be effective in pollen mixes was demonstrated by Stettler (1968), who used this method to overcome the *Aigeiros* (Tacamahaca) X *Leuce* incompatibility barrier in *Populus*. Stettler irradiated his *Populus* 'mentor' pollen with 100 Krad. and observed that it was still capable of germination when observed 24 hours after pollination. Stettler and Bawa (1971) repeated Stettler's (1968) earlier success in overcoming the *Aigeiros* X *Leuce* incompatibility barrier in *Populus* using irradiated mentor pollen, but in this case the purpose of the experiment was to induce haploid parthenogenesis, an aim which was also successful. Knox *et al.* (1972b) similarly achieved success in overcoming the intersectional incompatibility barrier in *Populus* using the (100 Krad.) irradiated mentor pollen. Their mentor pollen, which they preferred to describe as 'recognition pollen', was prepared in several ways, including prior radiation, and also alternate freezing and thawing, and methanol or ether prewashing ('chemical methods'); in each case the pollen was reported to have either very low viability, or to be killed, by the treatment. More recently Dayton (1974) reported
his ability to overcome the self incompatibility of *Malus* using killed compatible pollen mixed with 'self' pollen.

It is apparent from the above reports that both stigmatic and stylar incompatibility barriers have been overcome by a wide variety of methods, the success of each apparently determined by the nature of the incompatibility mechanism involved, and potentially providing further insight into its nature. In many earlier studies such information has led to elaborations of theories attempting to explain the nature of the incompatibility mechanism in the plant involved. One should be cautious of treating such results as universal, even within a family or genus; *Nicotiana*, for instance, has two distinct breeding controls for cross- and self-incompatibility, which apparently act independently (Pandey, 1974). Heslop-Harrison (1975) emphasized this point, asserting that factors contributing to interspecific incompatibility, where there is a biparental mating system, are "in no way comparable with SI [self incompatible] system, and it would be a mistake to refer to them in terms which might suggest homology".

Referring to *Populus*, Knox *et al.* (1972a) point out that the 'recognition' is apparently at the supra species level, and thus it was unlikely that their recognition pollen results would "shed direct light on the effects of the gametophytically determined self-incompatibility systems where the control is imposed at the infra-species level". On the other hand, on the basis that he had located proteins in pollen walls of all the angiosperms that he had tested to that time, it was proposed that "the recognition pollen method can be used to overcome incompatibility barriers in other groups of plants, both where the barrier is interspecific and intraspecific" (Knox *et al.*, 1972a).

Incompatibility is apparently a surface (pollen-stigma) phenomenon in *Populus* judging from the results obtained by Whitecross and Willing (1975) and, to a lesser extent by Knox *et al.*, (1972a,b), and also
evidenced by results presented in Chapter 2. This is characteristic of plants with a sporophytic incompatibility control (Stout 1931), compared with those whose (gametophytic) incompatibility reaction is manifest largely in the style. It is relevant to note here, that where occasional trees of *Populus* have been found which are hemaphroditic, they have, on investigation, been found to be self-compatible (for example Melchior, 1967).

The original intention of this study was not to pioneer methods of overcoming incompatibility, but rather to investigate more fully those already known to be effective with *Populus*. In the course of the work, however, a wide variety of manipulations were attempted, largely in order to determine their effect on pollen-stigma interactions at the microscopic level; in most cases the experimental material was nurtured at least to the stage of production of seeds, and often seedlings. Several of these treatments did result in the circumvention of the incompatibility barrier in *Populus* and their success has been reported in the thesis. Some of the same treatments have been performed independently and with larger samples as since reported by Willing and Pryor (pers. comm. 1975).

**MATERIALS AND METHODS**

**Propagation of *Populus* Material**

The propagation techniques follow those established by Willing (pers. comm. 1973), and outlined in Knox *et al.* (1972a,b); they are discussed in some detail in Appendix II of this thesis. Fresh pollen was used wherever possible although in most cases, dessicated, stored pollen was all that was available at the time of flowering. The method of collection of pollen is described in Appendix II (see also fig. 2.I(a)). All pollinations were effected with (bottle) grafted female material (figs. 2.I(b) and 2.II(b)) for convenience, as trials showed no detectable
Experiments involving pollination of stigmata were carried out between August and October of both 1973 and 1974 at which times female Populus material was in flower.

Controls for each experiment included the pollination of some stigmata from each batch with pollen from *P. alba* var *bolleana* and some with *P. nigra* var *italica* pollen in addition to the more specific controls for each treatment as described in the text.
difference in behaviour between these and those treated, in situ, on the tree itself.

The success of a particular cross was determined by microscopic examination of the pollen behaviour on the stigma (see figs 2.IV(b),(c)) seed set (fig.II(a)), or seed production (fig.2.III(a)). To ensure that true intersectional hybrids had been produced, it was necessary to examine seedlings at the stage of production of the first true leaf (figs. 2.III(b),(c)). Some of the progeny were planted out into an experimental garden as described (Appendix II).

Stigma and Pollen Treatments Used Prior to Pollination

In each case whether pollen or stigma was pretreated, the pollination technique was uniform, being described in Chapter 2. For each test at least 6 inflorescences (with 20-40 flowers on each) were treated, using inflorescences on each of 3 separate grafts. Stigmata used in these experiments were obtained from clones of *P. deltoides* whilst the pollen used was that of either *P. nigra var.italica* ('compatible') or *P. bolleana* ('incompatible').

I. POLLEN TREATMENTS

(a) Irradiation

Either *P. bolleana* or *P. nigra var.italica* pollen was subjected to 100 Krad of gamma radiation from a cobalt-60 source. Each was then either applied directly to the stigma or mixed, (1:1), with non-irradiated pollen of the other species.

(b) Solvent washing

Pollen of either *P. bolleana* or *P. nigra var.italica* was washed in each of the solvents, methanol, acetone, diethyl ether, hexane or water. In each case the procedure consisted of washing the pollen 3 times in an excess of the (previously double distilled) solvent. Each wash was of 10
minutes duration, and was followed by removal of the solvent by syringe or filtration, prior to the next washing or the final drying. In all cases the solvent used in the third wash remained clear. The pollen was finally dried in a stream of cool air to remove any remaining solvent.

(c) Buffer pre-washing

In two experiments pollen was pretreated by immersion in isotonic mannitol-tris-HCl buffer, pH 7.9, (which preserves the integrity of the grain, but allows material to diffuse from it), then used for pollination. In the first experiment, the excess buffer (plus diffusates) was removed from the pollen within 2 minutes of moistening, whilst in the second the pollen was left for 15 minutes before the excess buffer was removed by filtration, using a 'Millipore' syringe attachment. In each case pollination was effected shortly after filtration.

(d) 'False coating'

Pollen prewashed with hexane, then dried as described in (b), was then mixed with hexane washings obtained from an equivalent quantity of pollen from another species, mixed well, then dried with constant gentle agitation. The final effect was to replace the pollen's own hexane-removable coating with that of another as evenly as possible. In addition, the method used by Willing and Pryor (pers. comm. 1975), was tried. This consisted of mixing intact pollen with the dried hexane washings of another Populus pollen species, then effecting pollination by applying this paste to the stigma. In this study 'incompatible' pollen was mixed with 'compatible' washings, and vice versa.

II STIGMA TREATMENTS

All stigma treatments were performed on the grafted material when the stigma was judged to be fully receptive.
(i) Solvent washing

Cleaned sable hair watercolour brushes (No.1) were dipped in a small quantity of the solvent, wiped several times on clean paper until no longer visibly wet, then used to wipe each stigma individually. This procedure was repeated for all of the flowers of each inflorescence. Solvents used in this way included acetone, chloroform, diethyl ether, hexane, methanol and water (as a control). Pollinations were subsequently performed in the normal manner.

(ii) Detergent washing

A 1% solution of the neutral detergent, "teepol", was used in minimal quantities to treat the stigma surface as in (i). The surface was allowed to dry before pollen was applied.

(iii) Enzyme treatments

Each of the enzymes, lipase (from wheat germ, Calbiochem), protease (Type VI, Sigma), and pectin esterase (Sigma) were prepared in concentrations of 0.5 mg per ml of 0.25 M tris-HCl buffer pH, 7.9. The buffer was also used alone as a control. Enzyme solutions and controls were placed in 3 x 1 cm glass vials which were then taped individually to each inflorescence so that it was completely immersed. These vials were left attached for one hour at a (glasshouse) temperature of approximately 25°C, after which the solutions were removed with a syringe and the inflorescences washed twice for 10 minutes each, in buffer alone, prior to removal of the vials. The inflorescences were then left for at least one hour prior to pollination with either compatible or incompatible pollen.

(iv) Cold treatment

Three of the 'bottle grafts' of female *P. deltoides* were placed in a 4°C cold room one week after making the graft (to facilitate its 'taking') and left, with occasional watering, to continue development at a reduced rate. When almost fully developed, albeit markedly chlorosed,
the grafts were 'staged' via the laboratory, for two days at room
temperature, to the glasshouse, where they were left for 2 further days
to become acclimatized prior to their pollination.

(v) Serum treatments

Female flowers were brushed lightly with pure rabbit serum from
each of three sources.

(a) rabbits unchallenged by any plant extract

(b) rabbits challenged with pollen diffusate extract of
P. nigra var. italica.

(c) as for (b), but with extract from pollen of P. bolleana.

After brief drying the stigmata were pollinated with intact pollen,
either P. bolleana or P. nigra var. italica, to produce all combinations
of serum and pollen.

(vi) Concanavalin A treatment

Con A (Boehringer-Mannheim) was diluted with 0.25 M-Tris-HCl, pH
7.9 to a final concentration of 0.125 mg/ml, lightly brushed onto the
stigma surface then allowed to dry for 2 hours before pollination with
either 'compatible' or 'incompatible' pollen.

(vii) Hormone treatments

Solutions of either gibberellic acid (1 x 10^6 p.p.m.) or indole-
acetic-acid (2 x 10^6 p.p.m.) were liberally painted onto the stigmata,
the excess gently shaken off, and the surface allowed to dry partially
before pollinating with either 'compatible' or 'incompatible' pollen.

(viii) Inhibitor treatments

50 µg/ml solutions of the RNA synthesis inhibitor, actinomycin D,
or the protein-synthesis inhibitors chloramphenicol or cycloheximide, were
painted onto the surfaces of the stigmata, then left for approximately
10 minutes, after which the flowers were pollinated with either
'compatible' or 'incompatible' pollen.

(ix) Stigma controls

These included wiping the stigma surface with a sable hair brush moistened with distilled water, 0.25 m tris-HCl buffer, or the same buffer with the tonicity adjusted by the addition of mannitol.

Assessment of the Treatments

The effects of each of the treatments were determined by:

1. transmission electron, scanning electron-, or fluorescence microscopy examination, these methods used in each case being outlined in Chapter 1. These techniques enabled the elucidation of the initial pollen stigma reactions regardless of whether the cross was successful in producing seed set, or not.

2. setting of capsules, although often followed by premature dehiscence, which gave an intermediate indication of the effect of the treatment.

Proof of the success of a cross was obtained where seed could be produced, although where an incompatible cross had been made much of the seed was often wizened or chaffy. Seed was germinated, the seedlings being raised to the stage where the hybrid nature of the cross could be determined (figs. 2.III(b), (c))

Microscopic Examination

In most cases sampling for microscopic examination took place 24 hours after pollination. Three flowers were excised from each inflorescence, after which some were inserted by their pedicels in 6% agar to be examined within a few hours by scanning electron microscope. Most were immediately fixed, then processed for either scanning electron microscopy or fluorescence microscopy as described in Chapter 1 (Materials and Methods).
RESULTS

The results obtained following attempts to overcome the intersectional incompatibility barrier of *Populus*, both in this and earlier studies, are summarized in table 2.1. A description of the events observed at the microscopic level is presented below for each of the treatments.

All fluorescence micrographs show the appearance of the stigma 24 hours after pollination.

**TABLE 2.1**

**A SUMMARY OF EFFECTS OF VARIOUS POLLEN AND STIGMA PRE-TREATMENTS ON THE ABILITY OF 'INCOMPATIBLE' POLLEN TO OVERCOME THE INTERSECTIONAL INCOMPATIBILITY BARRIER OF *POPULUS*.**

(for details see text)

<table>
<thead>
<tr>
<th>I</th>
<th>POLLEN PRETREATMENTS</th>
<th>FLOWER SET</th>
<th>SEED PRODUCED</th>
<th>HYBRIDS PROVEN</th>
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</thead>
<tbody>
<tr>
<td>(a)</td>
<td>irradiated mentor pollen</td>
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<td>+</td>
<td>+ 1.5.6</td>
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<td>(b)</td>
<td>solvent wash</td>
<td>+</td>
<td>+</td>
<td>+ 1.2</td>
</tr>
<tr>
<td>(c)</td>
<td>buffer wash</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(d)</td>
<td>'false-coating'</td>
<td>+</td>
<td>+ 1.</td>
<td>+ 2.</td>
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<tr>
<td>(e)</td>
<td>recognition protein</td>
<td>+</td>
<td>+</td>
<td>+ 4.</td>
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<td>(f)</td>
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<th>FLOWER SET</th>
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<th>HYBRIDS PROVEN</th>
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<td>+</td>
<td>+ 1.2.3</td>
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<td>-</td>
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<td>(vii)</td>
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<td>-</td>
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<tr>
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<td>(ix)</td>
<td>buffer and water controls</td>
<td>- 1.</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**References**

1. This study
2. Willing and Pryor (1975)
3. Whitecross and Willing (1975)
4. Knox *et al.* (1972a)
5. Knox *et al.* (1972b)
6. Stettler (1968)
7. Willing (pers. comm.) 1975

'+' indicates success of the treatment to the stage indicated, whilst '-' is recorded where either the treatment was unable to produce material to this stage, or was not tested beyond this point.
I POLLEN PRETREATMENTS

(a) Radiation

Pollen viability, following 100 Krad. of radiation, was reduced to less than 5% as determined by the proportion which had germinated on the stigma after 24 hours. Radiation of pollen with a dose of 100 Krad. neither prevented the success of normal compatible crosses, nor overcame incompatibility. In agreement with the earlier results of Stettler (1968) and Knox et al. (1972b), intersectional hybrids were produced by the irradiated mentor pollen technique.

(b) Solvent washing

Contrary to the earlier finding of Knox et al. (1972b), that ether killed Populus pollen, this study showed that this pollen was not killed by washing with diethyl ether, hexane or acetone under the conditions described. This result is investigated in more detail in Chapter 4. After washing with any of the above solvents, pollens of both P. nigra var. italica and P. boleana behaved as if compatible (example fig. 2.IV(a)&(b)) and when pollens thus treated were used in 'incompatible' crosses these resulted in the production of demonstrably intersectional hybrids. More detailed reports of success obtained with crosses using this technique, and carried out independently, are presented in a recent paper by Willing and Pryor (pers. comm. 1975).

(c) Buffer washing

No success was obtained following pollinations using pollen previously washed in buffer for periods of either 2, or 15 minutes.

(d) 'False coating'

Willing and Pryor (pers. comm. 1975) found that incompatible pollen could be rendered compatible by mixing it with the dried hexane washings of compatible pollen. Although attempts were made in this study to repeat their experiment these were not successful (the seeds produced
failing to germinate), the fluorescence microscopy results do tend to support their findings. It can be seen that the 'false coated' *P. bolleana* incompatible pollen behaves as if compatible on a *P. deltoideae* stigma 24 hours after pollination (fig. 2.V(a)). The reverse treatment, that of providing compatible pollen with an incompatible coating, gave visibly poor germination, and any pollen tubes which did develop failed to penetrate the stigma surface.

The more involved exchange of hexane-soluble pollen surface materials produced similar results, yet not as pronounced as in the former treatment (fig. 2.V(b)).

II STIGMA PRETREATMENTS

(i) Solvent washing

Of the solvents tried, hexane and diethyl ether proved the most effective in visibly modifying the stigma surface (compare figs. 2.VI(a) and 2.VII(a) with fig. 2.VI(b)) although, as can be seen, diethyl ether caused some damage to the papillae. Chloroform diminished the 'wax structures' to some extent (fig. 2.VII(b)). Tris-HCl buffer was found to have little effect.

Transmission electron micrographs have shown that the hexane treatment of the stigma receptive surface removed (or displaced) the pellicle and caused a reduction in the size of the lipoidal 'wax structures' as well as a disruption of their structure.

After such treatment, both compatible and incompatible pollens behaved as if compatible (fig. 2.IV(c)). Intersectional hybrids were produced following treatment of the stigma surface with hexane, diethyl ether, chloroform, or ethyl acetate thus confirming and extending Whitecross and Willing's (1975) earlier results.
(ii) Detergent washing

1% teepol, used in the same manner as solvents, (see (i)), also reduced the prominence of the 'wax structures', but to a lesser extent than either hexane or diethyl ether (fig. 2.VII(c)). Results were similar to those in (i) above, (fig. 2.VI(c)) and the treatment has been demonstrated to enable the production of intersectional hybrids in Populus, as proposed by Whitecross and Willing (1975) and since confirmed by Willing (pers. comm. 1975).

(iii) Enzyme treatments

Pectin-esterase pre-treatment of the stigma had no apparent effect on either the compatible, or the incompatible, pollen-stigma interactions (fig. 2.VIII(a)).

Protease pre-treatment caused the almost complete inhibition of pollen whether compatible or incompatible (fig. 2.VIII(b)).

Lipase pre-treatment did not inhibit pollen germination as much as did protease, yet caused compatible pollen to behave in the same way as normal incompatible pollen (fig. 2.VIII(c)).

(iv) Cold treatment

After pollination of each of the 3 cold pre-treated grafts, 2 lost all their catkins a short time later due to abscission; the third, pollinated with compatible and incompatible pollen (using different catkins), survived and produced seeds in both cases, whether the catkins were pollinated with pollen of P. bolleana or P. nigra var. italica. Similar, and independent, results were obtained by Willing (pers. comm. 1975) and intersectional hybrids produced.

(v) Serum treatments

Antisera against extracts of either of the two pollens used (P. bolleana and P. nigra var. italica), caused each to behave as if
incompatible, their growth being erratic, and most pollen tubes failing
to penetrate the stigma surface (example fig. 2.IX(b)).

Serum from rabbits unchallenged by plant material caused *P. bolleana*
pollen to behave as if compatible (fig. 2.IX(a)) and had little effect
on the normal compatible behaviour of pollen of *P. nigra* var. *italica*.
Seed was produced from the intersectional cross but failed to germinate.

(vi) Concannavalin A treatment

Con A prevented the germination of either compatible or incompatible
pollen (fig. 2.IX(c)).

(vii) Hormone treatments

Both gibberellic, and indole-acetic acid treatments enabled the
incompatible pollen to penetrate the stigma without adversely affecting
normal compatible pollen behaviour (figs. 2.X(a),(b)). Viable seed was
produced following hormone treatment whether the original pollen was
compatible or incompatible.

(viii) Inhibitor treatments

Cycloheximide prevented further growth of both compatible and
incompatible pollen tubes shortly after their initial contact with the
stigma surface (figs. 2.XI(a),(b)).

Chloramphenicol similarly halted pollen tube development of both
compatible and incompatible pollen but at a later stage, that is, after
extension of the tube. Both compatible and incompatible tubes (when
developed) grew erratically, as is typical of incompatible tubes
(figs. 2.XII(a),(b)).

Actinomycin D similarly caused pollen tubes to grow erratically
and to fail to penetrate the stigma surface (figs. 2.XIII(a),(b)).
Setting of some flowers was apparent after inhibitor treatment, but in each case these abscised, and the effect was most likely parthenocarpic.

(ix) Controls

Treatment of the stigma surface with 0.25 M tris-HCl buffer alone affected neither compatible nor incompatible pollen behaviour or, in the case of compatible pollen, its ability to produce viable seed.

DISCUSSION

It is clear from these results, that a wide variety of techniques may be used to overcome the intersectional incompatibility barrier in *Populus*. In particular, the results have confirmed those obtained with *Populus* using the irradiated mentor pollen technique of Stettler (1968), Stettler and Bawa (1971) and Knox *et al.* (1972b), as well as the solvent stigma treatments of Whitecross and Willing (1975).

The finding that *Populus* could not only remain viable after a radiation dose of 100 Krad, but also produce progeny, does not agree with the report of Knox *et al.* (1972b), that (*Populus*) pollen was "completely" inviable after the same radiation dose.

Despite the fact that radiation alone resulted in production of viable progeny, there was no evidence that radiation treatment of pollen would alone cause incompatible pollen of *Populus* to behave as if compatible. The evidence still supports the proposal of Knox *et al.* (1972b) that incompatible pollen "effectively 'borrows' recognition material" from the irradiated mentor pollen in such treatments.
Willing (pers. comm. 1974) has obtained success in overcoming the incompatibility barrier in *Populus* using pollen mixes alone, thus providing practical confirmation for the earlier suggestion of Stettler (1968) who held that "non-irradiated mentor pollen ... would probably cause equal, if not more, stimulation" in pollen mixes, to that produced by the irradiated mentor pollen in the same situation. Willing found a threshold value for the proportion of (intact) mentor pollen in a mix to be 40% of the total; below this level successful seed set could not be obtained. Willing and Pryor (pers. comm. 1975) interpreted the results using pollen mixes (with or without irradiation of mentor pollen), in terms of the presence, in *Populus*, of a pollen 'P-factor', and a stigma 'S-factor', both involved in the incompatibility mechanism. The combined effect of the two in an incompatible combination was held to result in the incompatibility reaction. Compatible 'P-factor' in mentor pollen, when present in adequate proportions, they held, "presumably nullifies the reaction of the 'S-factor' which would otherwise occur, if incompatible pollen alone were used, by preceding or overriding the incompatible 'P-factor' action" (Willing and Pryor pers. comm. 1975).

No suggestions are made as to the nature of 'P-' or 'S-factors', although by their definition, they are among the materials removed from stigma or pollen grain by certain solvents. They neither promote nor refute the possible equation of 'P-factor' with components of the 'recognition substance' earlier identified by Knox *et al.* (1972b), or with the more purified, 'recognition protein' of Knox *et al.* (1972a) (inferred to be proteinaceous by the method of its extraction and purification and further, by its antigenicity.) The nature of the proteins within the extract was not determined, and it seems probable that these could include enzymes, whose involvement had earlier been suggested by Stettler (1968). It is also reasonable to suppose that many enzymes would retain their activity following the processing described by Knox *et al.* (1972a), and could be
released from either intact, or irradiated pollen. That such enzymes could also be expected to retain their activity following (100 Krad) radiation, is a proposal supported by the observation that following such radiation, *Populus* pollen retains both its compatibility (or incompatibility), as well as its viability; this contrasts with the counterproposal made by Hopper and Peloquin (1968) to explain their results obtained with *Lilium* following the use of radiation treatments to overcome incompatibility.

It remains uncertain whether the 'recognition substance' (or 'protein'), 'P-factor', or solvent pollen washings, are sporophytic or gametophytic in origin; either origin is possible, for although the above entities are extracted from the gametophyte (pollen), in some cases their origin may be sporophytic, as would be the case if they were derived from the anther tapetum at an earlier stage of development (Heslop-Harrison 1968b).

Preliminary attempts to overcome the *Populus* incompatibility barrier by pollinating the cut surface of the style following excision of the stigma lobes, failed to demonstrate germination of the pollen. Such negative results have also been obtained by Willing (pers. comm. 1975). Chemical treatments were more successful, since when *Populus* stigmata were treated with teepol, hexane, ether or chloroform, both compatible and incompatible pollens placed thereon behaved as if compatible, confirming the earlier result of Whitecross and Willing (1975). The present study has demonstrated that these treatments cause the removal of the pellicle, and a substantial modification of the "wax structures" at the stigma surface. This tends to confirm Whitecross and Willing's suggestion that the substance(s) removed forms at least part of the incompatibility barrier in *Populus*. Taken together with the stigma excision results, it is clear that removal of the whole stigma surface does not, by itself, overcome incompatibility. Considering the difficulty found in germinating *Populus* pollen, (and stored pollen in particular), it seems that this requires fairly precise conditions, available neither in many of the normally effective germination media
(Chapter 2), nor in stylar tissue. The stigma surface of *Populus* probably provides the physical conditions, nutrients, or growth stimulants necessary for the efficient growth of the pollen; such a stimulus may well be more readily available to compatible, than incompatible pollen. This suggestion could implicate the 'cutinase' hypothesis of Linskens and Heinen (1962) and Heinen and Linskens (1961). The overcoming of the incompatibility barrier of *Populus* by the partial removal and degradation of the lipidic surface, is not inconsistent with this suggestion. The failure of the enzyme treatments to overcome incompatibility is possibly explained as outlined below.

Pectin-esterase is an inappropriate enzyme for surface degradation, (despite its proposed equation, by Brewbaker (1971), with the 'cutinase' of Linskens and Heinen (1962)), and therefore it is not surprising that it has no effect on the normal behaviour of compatible or incompatible pollen behaviour. Protease would feasibly degrade the pellicle of *Populus* in accord with similar findings by Mattson *et al.* (1974), who demonstrated the dispersal of the pellicle of the receptive stigma surfaces of *Silene, Brassica* and *Raphanus* (all dry stigma plants) using protease. Protease is unlikely to affect the surface lipids, yet any residual protease, not removed by the subsequent washing, could deleteriously affect the enzymes which are released from the pollen in the early stages of germination, and thus inhibit normal development thereafter. This might explain both the present protease results, and those of Heslop-Harrison and Heslop-Harrison (1975), who observed frustration of the pollen tube penetration of the stigma in *Agrostemma* following similar protease treatments - particularly as the latter authors used a 4 minute wash only, after their enzyme treatment.

If lipase does degrade the stigma surface, a possibility not yet proven, then one would expect products of the degradation, as well as material released from the matrix, to be dispersed into the reaction medium and thus unavailable to any pollen subsequently placed on the
stigma whether 'compatible' or 'incompatible'. This could explain the observation that both compatible and incompatible pollens behave as if incompatible after the Lipase treatment of the stigma. The known esterase content of serum (Lewis, Burrage and Walls, 1967) may explain its demonstrated ability to facilitate incompatible pollen tube penetration of the stigma, allowing that serum esterases may assist pollen esterases in this function. Where the serum was obtained from rabbits previously challenged with *Populus* pollen extracts, neither compatible nor incompatible pollen penetrated the surface; this could probably be explained by the presence of 'antienzyme' (Chambers, 1975) antibodies in the serum, hindering the enzyme-mediated penetration of pollen to a degree which could not be compensated for by the normal serum enzymes alone. The enzyme content (including esterases) of *Populus* pollen diffusates is further investigated in Chapter 6, and the ability of esterases to act as antigens, in Chapter 7.

Concanavalin A may enter pollen and bind to mannose-containing carbohydrates, thus diminishing their possible availability as metabolic reserves for the germination process; this could provide a reason for the failure of either compatible or incompatible pollen to germinate on a *Populus* stigma treated with concanavalin A.

The promising results obtained with cold treated female flowers could be attributable to a changed composition of the stigma surface lipids during their slow development. Armstrong (1973) noted that differences in the leaf surface waxes of *Brassica* accompanied growth in different temperature regimes. Both compatible and incompatible pollens produced seed from flowers of *Populus* thus treated, in preliminary trials.

Of particular interest to this study are the independent, yet corroborative results of both this study and that of Willing and Pryor (pers. comm. 1975), that following pre-treatment with several low polarity
solvents, incompatible pollen of *Populus* will overcome the incompatibility barrier and produce demonstrable intersectional hybrids; this study has further found the behaviour of solvent pre-treated compatible or incompatible pollen on the stigma to be similar to that of intact, compatible, pollen. This is, in effect, a complementary result to that obtained following stigma prewashing, and indicates that the *Populus* incompatibility mechanism involves material from both stigma surface (sporophytic) and pollen (gametophytic and/or sporophytic). It will be shown (Chapter 4) that the solvents successfully able to overcome incompatibility (including diethyl ether), penetrate the pollen wall (both exine and intine), but not the cycloplasm itself. On the other hand, methanol or acetone-water mixes (98:2 and 50:50), each enter the pollen cytoplasm and render *Populus* pollen inviable. The ether results do not conform to those of Knox *et al.* (1972b) who reported the preparation of 'recognition' pollen by 'killing' with ether, and also with Roggen's (1974) reported retention of the viability of *Brassica* pollen following treatment with acetone/water (99.8:0.2, 90:1.0, 50:50 and 10:90) mixes. Recently, in agreement with the findings of the present study, Willing and Pryor (pers. comm. 1975) also found that ether did not render *Populus* pollen inviable, but could be used to overcome the intersectional incompatibility barrier of *Populus*.

Willing and Pryor (pers. comm. 1975) further reported that if intact *Populus* pollen were mixed with dried hexane washing of other pollen then the final breeding behaviour of such pollen was determined by the normal behaviour of the pollen from which the coating was derived. It is now apparent that these hexane washings of pollen contain wall-held materials (Chapter 4), possibly exclusively, and this indicates the determinative role of such wall held substances. Knox *et al.* (1972a) believed that pollen wall materials, which they identified as proteins, are concerned not only in pollen germination but also in 'recognition' phenomena. The determination of the nature of pollen wall materials is critical to an
understanding of the incompatibility mechanism in *Populus*, and they are examined in Chapter 4, with attention being focussed on potentially lipidic and proteinaceous components. Lipids are not only present in the hexane washings of pollen and stigmata, but have also been implicated in the incompatibility mechanism by Whitecross and Willing (1975). Protein of pollen has been implicated in a 'recognition' role in *Populus*, and also located in the pollen wall by Knox *et al.* (1972a).

The success obtained in overcoming the stigma 'incompatibility barrier' by the use of gibberellic-, and indole-acetic-, acids in stigma pretreatments, clearly shows that these hormones are acting directly on the pollen of *Populus*, and not indirectly by simply preventing floral abscission, as has been proposed for other genera by de Nettancourt (1972).

As inhibitors of both protein and RNA synthesis also repress the growth of compatible and incompatible pollen, this indicates that the pollen tube growth in both cases may depend on the continued production of new proteins (almost certainly largely enzymic), some of which may be involved in stigma surface penetration. In most cases the inhibition of growth occurred after pollen tube contact with the stigma, suggesting that, either the action of the inhibitor is delayed, or alternatively, that the pollen tube has a more intimate association with the stigma surface than does the grain itself. One should be cautious, however, in making interpretations of the effect of cycloheximide based on its supposed role in inhibiting protein synthesis, for, as McMahon (1975) has recently demonstrated, "cycloheximide appears to have two or three independent inhibitory effects on the cell."

Although not performed in this study, the effectiveness of 'electric-aided pollination', as used by Roggen *et al.* (1972) to overcome the self incompatibility of *Brassica*, is of some interest. Roggen *et al.*
(1972) attributed the success of their technique to any of several possible effects on the stigma surface, including the explanation that "the electric potential difference, which exists in cell walls (including the cuticular layer) may be disturbed as a result of which the permeability is changed." Considering the manner in which the treatment was performed, it seems equally feasible that the electric treatment could be explained in terms of its effect on pollen. In a recent paper by Coster and Zimmerman (1975), it was shown that pulsed currents of 0.85 V caused dielectric breakdown of the membrane of Valonia, which breakdown was shown not to result from membrane damage, and to be followed by a resealing time of approximately 10 seconds. The success of the 'electric-aided pollination treatment' of Roggen et al. (1972) could be explained in terms of a leakage of originally intracellular pollen material through the membrane following such an induced dielectric breakdown. Similarly the success of, for example, hexane washing of Populus pollen may be attributable to increased permeability of the pollen wall following removal of materials from its matrix, a possibility further investigated (and vindicated) in Chapter 6.

This clearly focuses attention on quantitative aspects of pollen diffusates, and might suggest that whilst both compatible and incompatible pollens may be qualitatively similar in the nature of their diffusates, quantitative differences determine which shall be more successful in overcoming the incompatibility barrier in a particular situation. On this basis, solvent washing of pollen may enable release of material from incompatible (and compatible) pollen to a level in excess of that required for 'recognition' in each case. The apparent determinative role of the pollen coating, however, may mean that the compatibility of Populus pollen is partially determined prior to anthesis, then further enhanced, following hydration of the pollen, by some 'switch' or stimulus provided by materials at the pollen, and possibly also the stigma, surface. This proposal is supported by the results obtained in this study, and those of Willing and Pryor (pers.
comm. 1975) using artificially coated pollens. The determinative role of the stigma surface in the incompatibility mechanism of the crucifers, Brassica and Arabis was particularly well demonstrated by Kroh (1966). Kroh showed that the incompatibility barrier could be overcome by first placing pollen on a compatible stigma, then transferring these grains to an incompatible stigma between 4 and 30 minutes later; the result showed that in these (dry stigmata) genera, the compatibility (or determinative) reaction is rapid, taking less than 30 minutes, irreversible, and results in a 'programming' of the pollen grain.

Taking together the results obtained in this study, as well as those of earlier workers, implicate the involvement of stigma surface components, pollen wall materials (see also Chapter 3) and pollen diffusates (see also Chapter 5) in the incompatibility mechanism of Populus. The solvent treatments, in particular, offer a valuable means of isolating some of the components involved in the incompatibility mechanism of Populus, and of gaining a better idea of their composition. This approach would not have been possible prior to the availability of the solvent technique, but should now provide a valuable insight into the incompatibility mechanism, for, as Linskens stated in 1965: "little is known about the biochemical background of cross-incompatibility, both intergenic and intragenic" - a statement which still holds to the present day.
fig (a) Method of collection of pollen. The tubs contain water. The branches are lightly tapped to release the pollen which is then collected immediately from the paper beneath.

fig (b) 'Bottle grafts' of female scions bearing flower buds, onto the already rooted stock.
fig (a) Pollinated catkins of *P. deltoides*. In this example many of the flowers have been successfully fertilized and have set.

fig (b) The graft is made using plastic budding tape, the base of the scion being immersed in water.
fig (a) Capsule dehiscence of female flowers of *P. deltoides* showing release of both seed and 'cotton'.

figs (b) and (c) Hybrid (*P. deltoides* X *P. alba*) seedlings, fig (c) showing one whose first true leaves are chlorosed and distorted.

Hybrid seedlings are distinguishable from those produced in *Aigeiros* intrasectional cross, by the presence of hairs on the abaxial surface (not visible here) and sinuate to lobed margins on the first true leaves.
Pollen on the *P. deltoides* stigma surface.

**fig (a)** S.E.M. of hexane prewashed pollen of *P. bolleana* clearly showing the region of adhesion of the grain to the surface (arrowed) two hours after pollination.

**fig (b)** Fluorescence micrograph of hexane pre-washed pollen of *P. nigra* var. *italica* 24 hours after pollination *in vivo*. (callose stained).

**fig (c)** *P. bolleana* ('incompatible') pollen on the previously hexane-brushed surface of the stigma 24 hours after pollination.
Plate 2.V

Pollen on the *P. deltoides* stigma surface, 24 hours after *in vivo* pollination (callose stained).

**fig (a)**  'False coated' *P. bolleana* (BH1) pollen.

**fig (b)**  'False coated' *P. bolleana* pollen, the original hexane soluble material having been removed prior to its replacement by the corresponding material from pollen of *P. nigra* var. *italic*. 
fig (a) S.E.M. of the hexane brushed papillae of the receptive surface of the stigma of *P. deltoides*. Comparison with fig (b) shows that the 'wax structures' (w.s.) have been largely removed.

fig (c) Fluorescence micrograph of *P. bolleana* pollen on the hexane brushed surface of the *P. deltoides* stigma, 24 hours after pollination (callose stained).
Plate 2.VII

Scanning electron micrographs of the receptive stigma surface of
P. deltoides after surface treatment (callose stained).

fig (a)  After brushing with diethyl ether. Note collapsed papillae.

fig (b)  After chloroform brushing.

fig (c)  After brushing with a 1% solution of 'Teepol' detergent.
Plate 2.VIII

Fluorescence micrographs of the *P. deltoides* stigma 24 hours after pollination (callose stained).

fig (a) Pectin-esterase pre-treatment of the stigma was followed by application of pollen of *P. nigra* var. *italica*.

fig (b) *P. nigra* var. *italica* pollen after prior protease treatment of the stigma.

fig (c) *P. nigra* var. *italica* pollen after prior lipase pre-treatment.
Plate 2.IX

Fluorescence micrographs of the *P. deltoides* stigma 24 hours after pollination (callose stained)

**fig (a)** Stigma treated with serum from rabbits previously unchallenged with plant extracts, then pollinated with *P. bolleana* pollen.

**fig (b)** Stigma treated with serum from rabbits challenged with diffusate extract of *P. bolleana* pollen. The stigma was then pollinated using pollen of *P. nigra var. italica*.

**fig (c)** Stigma surface previously treated with concanavalin A, then pollinated with *P. nigra var. italica* pollen.
Plate 2.X

Fluorescence micrographs of the *P. deltoides* stigma 24 hours after pollination (callose stained).

fig (a) *P. bolleana* ('incompatible') pollen on a stigma previously treated with gibberellic acid.

fig (b) *P. bolleana* pollen on a stigma previously treated with indole-acetic acid (I.A.A.).
Plate 2.XI

Stigmata of *P. deltoides* treated with cycloheximide, pollinated then observed 24 hours later.

fig (a) Fluorescence micrograph of *P. bolleana* pollen (callose stained).

fig (b) S.E.M. of *P. nigra* var. *italica* pollen.
Plate 2.XII

Stigmata of *P. deltoides* treated with chloramphenicol, pollinated with *P. bolleana* pollen, in each case, then observed 24 hours later.

**fig (a)** Fluorescence micrograph (callose stained).

**fig (b)** S.E.M.
Plate 2. XIII

Stigmata of *P. deltoides* treated with actinomycin D, pollinated, then observed 24 hours later.

fig (a)  Fluorescence micrograph (after callose staining)

*P. nigra* var. *italica* pollen.

fig (b)  S.E.M. *P. bolleana* pollen.
CHAPTER 3

THE EFFECT OF THE SOLVENT WASHING OF *POPULUS* POLLEN

INTRODUCTION

Organic solvents are widely used as components of extraction, fractionation and purification media in the biochemical analysis of plant tissues (Pasto and Johnson, 1969; Cherry, 1973; Kates, 1972; Johnson and Davenport, 1971).

The most common solvent combination used for lipid extraction is chloroform-methanol (2:1 v/v) (Folch et al, 1957; Johnson, 1971) which is often modified by the addition of water, as in the method of Bligh and Dyer (1959), and its variants (Kates, 1972; Kates, 1970; Kates and Eberhardt, 1957; van der Horst et al, 1969). Other binary solvents useful for lipid extraction include ethanol-diethyl ether (3:1 v/v) (Johnson and Davenport, 1971), and toluene-ethanol (3:2 v/v) (Schmid et al, 1973). Binary solvent systems are favoured extraction media as they provide optimal conditions for solubility of both relatively non-polar neutral lipids and polar lipids (Schmid et al, 1973), compared with other simple organic solvents such as acetone (Schmid and Hunter, 1971), ether (Mitchell, 1971), and petroleum ether (Troeng, 1955; Benesova et al, 1972). Removal of surface waxes of plants is usually accomplished using diethyl ether (Martin and Bart, 1958; Purdy and Truter, 1969), chloroform (Martin, 1960; Roberts et al, 1961; Whitecross, 1963; Schuck, 1972); or n-hexane (Kolattukudy, 1965; Tulloch, 1974).

For the extraction and dissolving of phenols, the more polar solvents, methanol, ethanol, water and alcohol-water mixes are most commonly used (Harborne, 1964), often with the addition of 0.1% to 1% concentrated hydrochloric acid (Asker and Fröst, 1973; Martin 1970).
Sugars may be dissolved in pyridine (Halligan, 1967) whilst acetone is commonly employed for the precipitation, or extraction, of protein from contaminants. It should be noted that there is a marked tendency for lipid extracts to trap non-lipids, (phospholipids, for example form micelles in organic solvents, Johnson, 1971), and thus further procedures are usually required for separation of lipids from non-lipids.

Recently several workers have demonstrated the practical potential of organic solvent treatment of the seed (Meyer and Mayer, 1971; Triplett and Haber, 1973; Khan et al, 1973; Iwanami and Akizawa, 1974), stigma (Tatebe, 1968; Whitecross and Willing 1975) or pollen (Iwanami, 1972, 1973a,b; Iwanami and Nakamura, 1972a,b; Roggen, 1974); with the apparent retention of viability of the tissue thus treated in each case.

Apparently Millborrow (1963) was one of the earliest workers to suggest the practical potential of organic solvent treatments of seeds. Later research has shown that dichloromethane can be used effectively to introduce substances, including the growth inhibitor coumarin (Meyer and Mayer, 1971; Anderson, 1973), into dry seeds. Both these, and control experiments, revealed that the solvent itself caused little damage to the seed (Meyer and Mayer, 1971; Anderson, 1973; Khan et al, 1973). Khan et al.(1973) applied either germination promotors or inhibitors to the seeds with effect, and further found these effects to be lost if the first application was followed by a second wash with the solvent alone; similar observations were made by Anderson (1973). Khan et al (1973); Iwanami and Akizawa (1974) and Tao and Khan (1974) also showed that solvents other than dichloromethane could be used to obtain parallel results. Iwanami and Akizawa, in particular compared a large number of solvents (polar and non-polar) for their effect on the seeds of four different genera, their results indicating that different genera varied in their sensitivity to solvents, and further, that with some genus-solvent combinations, the solvent treatment alone signific-
antly elevated the germination rate above that of the controls.

Anderson (1973) investigated the depth of penetration of [3-¹⁴C] coumarin into seed when incorporated in dichloromethane, as solvent, and found this tracer to be largely associated with the "pericarp-integument-endosperm complex" surrounding the embryo, and not the embryo itself; this result contrasted with those of controls where tracer was applied in water, after which it became uniformly distributed. Anderson's conclusion was that the endosperm complex acted as a barrier to penetration of the solvent. Brewer and Wilson (1975) similarly showed that dichloromethane was "not capable of penetrating to the inner cells of all types of seeds." In other experiments Tripplett and Haber (1973) showed that ethanol containing appreciable amounts of water (in their example, 8%) rapidly caused loss of viability of the intact seed of *Lactuca*, and further, that where the embryo was exposed directly to any of the organic solvents acetone, ethanol or dichloromethane, it completely lost its viability. This suggests that where acetone does reach the embryo it renders it inviable, as acetone is known to preserve the viability of intact seed (example Iwanami and Akizawa, 1974; Tao and Khan, 1974). Tao and Khan have shown that although acetone penetrates the seed as far as the embryo, its penetration into the embryo itself is superficial only. The general consensus from the solvent-seed studies is thus that penetration of solvents (which preserve seed viability) into the seeds studied, is superficial; this, as Khan et al (1973) point out, may be fundamental to the retention of viability of the seed, and thus the success of these treatments in their ability to introduce biologically active chemicals into the seed.

Mitchell and Whitehead (1941) found that diethyl ether extracts of *Zea* pollen had apparent hormonal activity. Later, hormonal activity was further detected in ether extracts of *Phaseolus* (Mitchell and Livingstone 1968; Mitchell et al 1971), *Brassica* and *Alnus* (Mitchell et al (1970, 1971) which was attributed by Mitchell et al. (1970, 1971)
to the presence, in each of these extracts, of fatty compounds which they
termed "brassins", and which differed qualitatively from similar (ether)
extracts obtained from immature seeds of *Phaseolus* (Mitchell *et al.* 1951,
1971). No reference is made, in their studies, to the viability of either
seed, or pollen, after such extractions nor, it seems, have the authors
allowed for the possibility that their pollen extracts (derived from open
anthers and stigmatic surfaces), may be derived in part from tapetal
material. They did, in one study however, check the possibility of a
contamination of pollen extracts by stigmatic surface materials (Mitchell
*et al.* 1971).

Martin, in a series of papers (1969, 1970a,b,c), has shown that the
extracts made from stigmas, using a number of different solvents, were
mainly lipidic or phenolic - the composition of the extract depending on
the type of solvent used. Martin (1969) found that the amounts of material
extracted from the stigmas varied with the solvents used, and with the
durations of extraction, with usually only small amounts obtained by
aqueous extraction, intermediate amounts with chloroform or acetone, and
the largest amounts with 95% ethanol containing 1% concentrated HCl or
with ether alone. He further noted that substantial quantities of water
were extracted by all of the solvents, but especially by acetone and ethanol-
HCl. His analysis of the extracts obtained with each solvent (Martin 1970a,b)
showed that alcohol-HCl extracts contained anthocyanins and phenolic compounds,
the acetone extracts contained phenolic compounds, and ether extracts
contained esters of fatty acids and lipophilic phenolic compounds. Further-
more Martin and Brewbaker (1971) and Martin and Ruberte (1972) found that
the extracts obtained with different solvents had correspondingly different
degrees of inhibitory effect upon the growth of pollen on agar plates.
Martin and Ruberte did not discount the possibility that some substances
may have been extracted from within the stigma.
It is apparent that, in many studies involving organic solvent extraction of materials from plants, often too little attention has been paid to the degree of penetration of each solvent into the tissue under study. Thus, in some cases, it has apparently been assumed that material has been removed from both intracellular and extracellular sites (Mitchell and Whitehead, 1941; Mitchell et al., 1951, 1970, 1971; Mitchell and Livingstone, 1968; Meyer and Mayer, 1971; Khan et al., 1973; Triplett and Haber, 1973), whilst in others, often using the same solvents, albeit with different plant genera or tissue, largely surface removal has been assumed (Martin, 1960, 1969, 1970a, b, c; Schuck, 1972; Tulloch, 1974). It is generally accepted, for instance, that non-polar solvents such as n-hexane (Kolattukudy, 1965; Tulloch, 1974), or chloroform (Schuck, 1972), when applied to leaf surfaces for short periods of time, will remove only surface materials, mainly waxes. Kolattukudy (1965), for instance, showed that 99% of the radioactively labelled surface wax was released within the first 10 seconds of washing with n-hexane, and recognised the possibility that an unnecessarily prolonged wash could cause leaching of internal lipids. With this in mind, both Tatabe (1968), who treated the surfaces of *Raphanus* stigmata with ether, and Whitecross and Willing (1975), who treated the stigma surface of *Populus* with ether and hexane, in each case used the minimum quantities of organic solvent necessary to overcome incompatibility. The authors of both the latter papers commented on the fact that an excess of solvent caused visible damage to the stigma.

Against this background the results obtained by Iwanami (1972a, b, 1973a, b) and Iwanami and Nakamura (1972) mainly with pollen, warrant closer attention, leading to a re-examination of the effects of organic solvents on plant tissues, particularly their effect on pollen and stigma. Iwanami (1973b) has tabulated the effects of a large number of pure solvents, and of solvents containing different percentages of water, on both pollen tube length and germinability (*in vitro*), of *Camellia japonica* pollen as
a measure of their viability after each treatment. Similar results were
obtained using the pollen of *Petunia hybrida* (Iwanami 1973a), several
*Lilium* species and *Impatiens balsamina* (Iwanami, 1972a; Iwanami and

Iwanami (1972a) noted that of the solvents that he used, the alcohols,
in particular, had an adverse effect on pollen viability; this he ascribed
to the low volatility of the alcohols and thus the probable difficulties
in drying the pollen efficiently prior to its use in pollination.

Iwanami (1973a) reported that there were indications that solvent
pre-treatment of *Petunia hybrida* pollen could diminish the (incomplete)
self-incompatibility barrier in certain crosses - he explained this result
in terms of a possible absorption, by the solvent, of pollen inhibitors.
He concluded: "Hence this technique for pollination by thus previously
stored pollen grains in organic solvents is no doubt useful for removing
of self-incompatibility". The observation of Iwanami and Nakamura (1972)
that, "the nuclei of pollen cells are not injured by the soaking in
certain organic solvents", as evidenced by the continued division of the
generative nucleus after acetone washes, led them to conclude from
these and earlier results that, "the cells of both plants and animals
maintain life in organic solvents if water in the cell can be extracted
without injury to the cells."

Roggen (1974), working with *Brassica oleracea*, reported the maintenance
of viability of pollen after brief treatment with acetone or chloroform,
as measured by germination or vital staining. Roggen also used acetone-water
mixes with acetone content of 10% to 99.8% but, apart from his breeding
results, there is no other clear indication of the viability of the pollen
after each of his treatments. One infers from Roggen's paper that *Brassica
oleracea* pollen is undamaged by any of the acetone-water mixes. Roggen
reports that different solvents, or solvent-water combinations remove different substances from the pollen, and postulates that, whereas pure acetone or chloroform overcome self incompatibility in *Brassica* by "decoding" the pollen, the acetone-water mixtures produce a low seed set in the same crosses by "re-coding" the self-incompatibility. Roggen's reported retention of the viability of pollen after acetone-water mixes seems to be in direct conflict with Iwanami's findings (Iwanami, 1972a; Iwanami and Nakamura, 1972) that water present in acetone in proportions as low as 0.5% reduced the viability of the pollen of *Lilium auratum*, and with those containing greater than 1.5% water, the pollens lost their ability to germinate altogether, even allowing for the fact that the results were obtained with different genera.

The question thus arises - what are the effects of organic solvents on plant tissues? Apart from the obvious fact that in preparation for electron microscopic examination, most tissues pass through an organic solvent dehydration, there is little information available about the effect of solvents alone on the ultrastructure of plant tissues. One exception is a determination, in the study by Armstrong (1973), of the effects of the solvents n-hexane and chloroform on the ultrastructure of the epidermal cells of the leaf of *Brassica* after superficial solvent washing for different periods of time. He found the optimal treatment, both for removal of wax and for preservation of ultrastructure, to involve the dipping of leaves in two consecutive baths of redistilled n-hexane for a total of 8 to 10 seconds - similarly found by Kolattukudy (1965) to be the optimal treatment for removal of wax.

Hensarling *et al* (1970) studied the ultrastructural effects of various lipid-extracting media on the seeds of *Gossypium hirsutum* L. The solvents used included pure hexane and acetone, and the solvent mixes, chloroform-methanol and hexane-acetone, with or without added water. The
authors used hydrated tissue, pieces which were teased out, dehydrated, then treated with the various extracting media, after which they were processed for subsequent electron microscopy. Their fixation was performed by the addition, in each case, of 2% osmium tetroxide to the solvents (or solvent mixes) - the osmium tetroxide was reported as being "both soluble and stable in the solvents of interest." Controls were fixed using osmium tetroxide fumes alone. It was observed that the solvents chloroform-methanol, hexane-acetone, acetone and hexane each presented a similar ultrastructural appearance to that of the controls, with the exception that the oil-containing spherosomes were empty in solvent treated material. The water-containing solvent media however, showed "disruption of intracellular structures without cellular rupture." As the two water-containing solvents, and chloroform-methanol, extracted about 6% more neutral oil than the other solvents, Hensarling and his co-workers postulated a possible correlation between oil extraction and effects on cellular fine structure; they regarded the exception, in the case of chloroform-methanol, as indicating "that cytoplasmic disruption does not necessarily accompany thorough extraction of oil from cotton seed." Their comparison of the abilities of the solvent mixtures to extract water-soluble phosphorus compounds showed that the aqueous solvents extracted over sevenfold more of these compounds than their non-aqueous counterparts. They comment: "whether this occurrence was a cause or an effect of intracellular disruptions has yet to be determined".

Apparently the only examination of solvent effect on pollen at the ultrastructure level, was made by Hess et al (1973), who studied Pinus pollen after treatment with chloroform-methanol (3:1) (as a lipid extraction medium), and revealed a severe disruption of the physical properties, superficial appearance and fine structure of this pollen.
Although Dickinson and Lewis (1973) comment that "in the condensed form, the tryphine is extremely stable..." and that "covalent solvents affect solely the lipidic component, and only prolonged treatment with ionic solvents will remove it in its entirety", they present no evidence to support their statement, which is not consistent with the earlier finding by Johnson (1971) of the marked tendency for lipid extracts to trap non-lipids.

It is clear that although organic solvents have been widely used in treatments of plant tissue including, for example, the extraction of material for chemical analysis, in routine processing for microscopy, and more recently, in attempts to overcome incompatibility, the effect of these solvents on the tissue is still poorly understood. The aim of this chapter is to determine the effects of each of several solvents on the pollen of *Populus*, and in particular, their degree of penetration into, and effect on, the fine structure of the pollen. This, in turn, should provide a better insight into the reason for the success, or otherwise, of solvent treatments of pollen in overcoming *Populus* incompatibility, and may further provide an understanding of the nature of the incompatibility mechanism in this, and other genera.
MATERIALS AND METHODS

Rationale for the experiment and selection of the tracer

In order to determine the degree of penetration of each organic solvent into the individual cells, and to detect any effects that they may have on the fine structure, a 'tracer' with the following properties was sought:

(a) it should be soluble in each of the solvents used, whether they are polar or non-polar.

(b) it should penetrate the cell to the same degree as the solvent which carries it.

(c) it should either be electron-dense itself, or be capable of conversion to an electron-dense form, without disruption of fine structure or perturbation from the position it reaches by solvent transport.

(d) the 'tracer' should neither change the effects of each solvent on the fine structure, nor prevent the proper fixation of the tissue.

A number of types of tracer were considered. Radioactive tracers (Armstrong 1973; Meyer and Mayer 1973) could be suitable but may be unnecessarily complicated, in practice, for this purpose. The dyes used by Triplett and Haber (1973) to determine the degree of solvent penetration into seeds cannot be readily detected at the fine-structural level. Electron-dense protein tracers, such as horseradish peroxidase, may be detected at the fine-structural level (Turner and Harris 1973) yet might be confused with endogenous peroxidases, where present - quite apart from the fact that their size alone may limit the degree to which they may penetrate intact tissues. Colloidal iron, as used by
Rowley (1970) with *Populus* pollen, is apparently not soluble in the low polarity solvents to be used in this experiment.

The 'tracer' finally employed was osmium tetroxide, a heavy metal oxide used as both a fixative and an electron-dense stain in electron microscopy studies. It is soluble to some degree in each of the solvents to be used in the experiment, and thus, overall, has the several properties delineated for the proposed 'tracer'.

**Experimental Procedure**

Pollen was collected as described earlier, (see chapter 2 and appendix II), and dessicated over a 24 hour period. Two pollen species, *P. nigra* var. *italica* and *P. bolleana*, were used. Tracer solutions were made, consisting of 1% (0.1 g in 10 ml) osmium tetroxide in each of the pure solvents, ether, methanol, hexane, acetone (each previously double distilled and then dried over pelleted molecular sieve material, BDH) and distilled water. Similar solvent-tracer solutions were made by dissolving osmium tetroxide in each of the following organic solvent-water mixtures: acetone-water, 50:50 and 98:2; hexane-water, 50:50 and 98:2; ether-water 50:50 and 98:2. All tracer solutions were made with extreme care using protective clothing, a fume hood and a respirator for added safety, considering the fixative properties of the tracer and the volatility of some of the solvents. The tracer solutions were each prepared immediately prior to use as it was found, in preliminary trials, that although the osmium dissolved completely in each solvent, the osmium-hexane and osmium-acetone solutions would darken within an hour of their preparation.

For each trial the treatment consisted of adding 0.25 g of pollen to 10 ml of solvent-tracer solution and allowing it to stand for 30 minutes with intermittent agitation, after which the pollen was washed, for 2
periods of 5 minutes duration each, in the same solvent to remove excess osmium. Subsequent treatment then depended on the nature of the solvent used. Pollen treated in organic solvent-water mixes was staged to the pure organic solvent involved, in the case of 98:2 mixes, directly, while the 50:50 mixes were taken through 75:25, 90:10, 95:5 stages before finally reaching the pure organic solvent involved. Water-osmium treated pollen was dehydrated using a methanol-water series (see chapter 1) until finally immersed in pure (dry) methanol, then both this pollen, and that originally immersed in methanol-osmium, were taken through a methanol-Spurr's resin series (3:1, 1:1 and 1:3) to pure Spurr's resin and embedded as described earlier (chapter 1).

Where pollen had been originally treated with low polarity solvent-osmium mixes, each of the ether and hexane treated pollens were staged (3:1, 1:1 then 1:3) to pure acetone, then both these and the pollen initially treated with acetone alone, were staged through an acetone-Spurr's resin series (3:1, 1:1 then 1:3) to pure resin and polymerized as described earlier (chapter 1). The net result of each of these treatments was that, as far as possible, pollen originally treated with high polarity solvents was processed in stages involving similarly high polarity solvents and *vice versa*. In each case controls were employed where the solvents (or mixes) were used without included tracer.

Sectioning and staining for both light- and electron-microscopic examination was performed as described earlier (chapter 1). For each sample drops of the pollen-resin mix were placed on microscope slides (beneath cover slips) then polymerized *in situ* to form permanent mounts for light microscopic examination.

Sections (0.5 µ and 1 µ thick) of some of the resin embedded pollens were mounted on glass microscope slides and stained for protein, using
1% aniline blue-black in 7% acetic acid (Fisher 1968). Electron microscopy sections were examined, and photographed, using a JOEL. 100C transmission electron microscope.

RESULTS

The light micrographs show that swelling of the pollen occurs only in solvent mixes containing water or in water alone (figs. 3.I(a), 3.V(a), (b), (c), 3.VII(b) and 3.IX(a), (c)). There is no apparent difference, in the degree of swelling, between pollens immersed in water alone, and those immersed in 50:50, low polarity solvent-water mixes (compare figs. 3.I(a) with 3.V(a), (b), (c)). By comparison, 98:2 low polarity solvent-water mixes produce no obvious swelling of Populus pollen (figs. 3.VI(a), (b), (c) and 3.VII(a)). Solvent containing pure organic solvent only, whether polar (methanol), or of lower polarity (acetone, diethyl ether, hexane) does not cause the pollen to swell (figs. 3.I(b), 3.III(a), (b), (c) and 3.IX(d), (e), (f)).

Where the osmium 'tracer' has been added to the solvent used, there is a clear demonstration, in each case, of the degree of penetration effected by that solvent - this is evident in both light, and electron, micrographs. Water and methanol each readily penetrate the Populus pollen grain within 30 minutes (figs. 3.I(a), (b) and 3.II(a), (b), (c)), whereas pure (dry) acetone, diethyl/ether or hexane do not (figs. 3.III(a), (b), (c) and 3.IV(a), (b), (c)). In each of the latter (low polarity solvent treatment) light micrographs, a pollen grain which has taken up the solvent is included in the field to act as a control; these grains, which were not common in the preparations, would almost certainly have been inviable. On the other hand, low polarity solvents including acetone, diethyl/ether and hexane are taken into Populus pollen when they contain
water, whether this comprises 2% or 50% of the total volume (figs. 3.V(a), (b), (c) and 3.VI(a), (b), (c)). Photographs of controls (figs. 3.IX(a), (b)), including hydrated and non-hydrated pollens, but without addition of 'tracer', are presented for comparison with the photographs of 'tracer'-treated material. The difference in appearance between hydrated and non-hydrated *Populus* pollen is clearly seen in the photographs of the controls, taken using interference illumination (figs. 3.IX(c), (d), (e), (f)).

In sectional view electron micrographs show, more precisely, both the degree of penetration of each solvent (or mix), and its effect on the fine structure of *Populus* pollen:

(i) pollen treated with water-'tracer' shows the conventional electron microscopic image of hydrated pollen. The grain is swollen and the cytoplasm vesicular. Osmiophilic material is prominent both within the exine cavities, as well as in association with cytoplasmic vesicles (figs. 3.II(a), (b)), the latter also being located within the generative cell cytoplasm (fig. 3.II(b)). Both by their osmiophilic nature, as evidenced by staining, and by the known composition of solvent-removable pollen wall material of *Populus* (to be determined in chapter 4), both the vesicles and the exine-held substances are judged to consist largely of unsaturated lipids. Fig. 3.II(a) further demonstrates the presence of the wall infolds described in chapter 1.

(ii) methanol-osmium treated pollen, which does not swell, exhibits densely staining cytoplasm, within which one is unable to distinguish organelles other than vacuoles (fig. 3.II(c)). As it would obviously be of interest to determine the fine structural appearance of pollen in its prehydrated state, even after using methanol as the only fixative, 'control' blocks (without inclusion of 'tracer') were sectioned and then
post-stained with either phosphotungstic acid or Reynold's lead citrate alone prior to their examination under the electron microscope; little improvement was found in the image. Osmiophilic material retained within the exine cavities of pollen following water-‘tracer’ washing is removed by the methanol-‘tracer’ washing.

(iii) the low-polarity solvent-‘tracer’ mixes used are not capable of penetrating past the intine of *Populus* pollen within the time allowed, there being no evidence of its entry into the pollen cytoplasm (figs. 3.IV (a), (b)). As with methanol-‘tracer’ treatments, the osmiophilic material has been removed from the exine cavities by these low polarity solvents (acetone, diethyl ether and hexane). In some grains the hexane-‘tracer’ mixture stains regions bordering the inner surface of the intine (fig.3.IV (c)); these osmiophilic materials are not the result of cytoplasmic degradation and may be located between the plasmalemma and the intine of the grain prior to its hydration.

(iv) the addition of only 2% water to each of the low polarity solvents causes dramatic disruption of the pollen cytoplasm (fig. 3.VIII (a)); indisputably this renders the grain inviable.

(v) each of the 50% mixtures of low polarity solvent (acetone, diethyl ether or hexane) and water enters the pollen of *Populus*, and causes the grain to swell. There is clearly some disruption of the cytoplasm, as evidenced by breakdown of vesicular membranes, yet there is not the complete breakdown obvious in 98:2 solvent/water mixes. The intracytoplasmic osmiophilic material, observed in water-‘tracer’ treated pollen, is now dispersed into the adjacent cytoplasm, whilst that of the exine cavities is largely lost to the solvent medium; this is evident in fig. 3.VIII(b) which shows the effect of 50% acetone-water on *Populus* pollen.
Protein staining (aniline blue-black) of 1 µm sections of resin-embedded, unstained, methanol treated *Populus* pollen, has shown that the staining of both intine and exine is slight by comparison with the marked positive staining of the cytoplasm, particularly the regions subjacent to the intine.

**DISCUSSION**

The main conclusion that can be drawn from these results is that solvent treatments which preserve the viability of the pollen grain of *Populus* do not result in the penetration of the solvent into the cytoplasm, as has been proposed (Iwanami and Nakamura 1972), or inferred by previous authors (for example, Roggen 1974). The results of the present study with *Populus* may demonstrate that the ability of the less polar organic solvents to penetrate pollen grains is less than previously thought, and may have wider application to other genera.

The results indicate that the ability of pollen (of *Populus* at least) to withstand immersion in some solvents is probably not simply due to the capacity of the cytoplasmic contents to tolerate these solvents. On the one hand it has been shown that the lower polarity solvents do not penetrate past the plasmalemma, or at least a region close to that membrane; and on the other, that where any one of the organic solvents studied has been shown to enter the grain, disruption of the fine structural organization has been demonstrated. The damage ranges from dissociation of membranes to manifest breakdown and loss of cytoplasm. The explanation by Iwanami and Nakamura (1972), for the retention of viability of pollen following treatment with a number of organic solvents no longer appears valid, namely on the grounds that it has been shown that these solvents will not enter the cytoplasm, and therefore the tolerance
of the intracellular contents to organic solvents is not relevant to their survival—quite apart from the fact that they are susceptible to solvent damage.

The present results thus fundamentally agree with the findings of Triplett and Haber (1973) that the survival of lettuce seeds following organic solvent treatment, is not due to any capacity of the embryo to withstand organic solvents, but rather the fact that solvents are prevented from reaching the tissue, in their case by the pericarp-integument-endosperm complex. This was verified in their study by experiments which showed that direct application of solvents to the embryo rendered it inviable. Similarly Armstrong (1973) showed that the intracellular organization of the leaf epidermis was disrupted by prolonged exposure to organic solvents.

In each study (including the present one), where either ethanol or methanol has been used to treat pollen grains, and the subsequent viability of the pollen tested, these solvents have been shown to render the pollen completely inviable. Similarly, treatments of seeds with methanol were shown by Iwanami and Akizawa (1974) to produce loss of germinability. Ethanol is apparently not as damaging to the seeds as methanol; after its use Iwanami and Akizawa have reported a seed germination rate of between 48% and 66% (depending on the species used) whilst Meyer and Mayer (1971) observed the germination of *Lactuca* seeds to be unimpaired after they were soaked in the same solvent.

Iwanami's (1972a) explanation of his lack of success in retaining pollen viability following its treatment with alcohols, was that thorough removal of the alcohols by drying was difficult due to their low volatility, and thus traces of these solvents might still be present at pollination.
This explanation cannot be supported if one examines the volatilities (Table 3.1) of some of the solvents used in both his, and the present, study; it can be seen that hexane, which can be used to wash pollen with the retention of its viability, has an even lower volatility than either methanol or ethanol.

The currently accepted methods for the efficient removal of leaf surface waxes appear, in the light of the results presented here, to be soundly based, as they commonly use lower polarity solvents (for example, hexane, ether or chloroform) and for seconds only. This treatment would minimize both penetration, and extraction of the epidermal cytoplasmic contents by the solvent.

Similarly, low polarity organic solvents also briefly applied, were found by Tatebe (1959), and Whitecross and Willing (1975), to be optimal in surface treatments of the stigma, and were successfully used for modification of the (in)compatibility barrier in each case. As both the present results and others indicate, this treatment limits the possibility of solvent penetration into, and damage to, the papilla cytoplasm. It would thus appear that such organic solvent treatments of the stigma act by modifying or removing pre-existing extracellular materials, rather than by disturbing the ability of the papilla cytoplasm to produce a positive reaction to the presence of pollen; the latter possibility, however unlikely, cannot be completely discounted.

Both scanning and transmission electron micrographs show pronounced modification of the stigma receptive surface by low polarity solvents with no obvious internal damage. As might be expected from its surface location, the pellicle is also disrupted. One feature of the ether treatment of the receptive stigma surface, as also with prolonged treatments using any of the organic solvents of the study is the collapse of the papilla cells (fig. 2.VII(a))
TABLE 3.1

Physical properties of solvents used in pollen treatments.

Data obtained from Merck (1968) and Handbook of Chemistry and Physics (1960).

<table>
<thead>
<tr>
<th>SOLVENT</th>
<th>Dielectric constant ($\varepsilon_{20-25^\circ C}$)</th>
<th>Molecular weight</th>
<th>Temp. required to produce vapour pressure (18-20$^\circ C$) of 1 atmos.</th>
<th>Surface Tension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>80.4</td>
<td>18.0</td>
<td>100</td>
<td>73.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>32.6</td>
<td>32.0</td>
<td>64.7</td>
<td>22.6</td>
</tr>
<tr>
<td>Ethanol</td>
<td>24.3</td>
<td>46.1</td>
<td>78.6</td>
<td>22.8</td>
</tr>
<tr>
<td>Acetone</td>
<td>20.7</td>
<td>58.1</td>
<td>56.5</td>
<td>23.7</td>
</tr>
<tr>
<td>Chloroform</td>
<td>4.8</td>
<td>119.4</td>
<td>61.3</td>
<td>27.0</td>
</tr>
<tr>
<td>Diethyl ether(ii)</td>
<td>4.3</td>
<td>74.0</td>
<td>34.6</td>
<td>17.0</td>
</tr>
<tr>
<td>Hexane</td>
<td>1.9</td>
<td>86.2</td>
<td>68.7</td>
<td>18.4</td>
</tr>
</tbody>
</table>

(i) this study (with *Populus*), and Iwanami (1972b).

(ii) Knox, Willing and Pryor (1972b) reported that ether killed *Populus* pollen whilst both this study and those of Willing and Pryor (pers. comm. 1975) have since found that this is not the case where pure, dry, solvents are used.
- not evident for example when hexane or ether were applied with a barely moistened brush, as previously described (chapter 2). Such collapse emphasizes the possibility of water loss from, and cytoplasmic damage to, the stigma papillae where solvents are used too liberally.

The present results clearly demonstrate that hexane, ether and acetone penetrate into the pollen cytoplasm, when they are mixed with as little as 2% water. The results of this study show that 50:50 and 98:2 low polarity solvent-water mixes disorganise the cellular structure (with the 98:2 mixes causing complete breakdown of the cytoplasm). These results corroborate, and most likely explain, the complete loss of germinability of pollen resulting from treatments with acetone-water mixes between 98:2 and 70:30, as reported by Iwanami (1972a) and Iwanami and Nakamura (1972). They may also explain the report of Triplett and Haber (1973), that lettuce seed lost its viability after washing in 92 ethanol:8 water mix, yet retained its viability after the same treatment using pure ethanol.

Comparable results were obtained by Hensarling et al. (1970) using cottonseed cotyledonary tissue in one of the few known attempts to determine the effects of organic solvents on the ultrastructure of these tissues following such extraction. Hensarling et al. (1970) showed that, whereas lipid may be extracted from spherosomes by several of his organic solvent mixes, there was not the same degree of cellular disruption caused by mixes containing organic solvents alone as there was when water had been added to them.

Given both the foregoing results and the results of the present study discussed in this chapter, Roggen's (1974) results with the pollen of *Brassica oleracea* are puzzling, in that he records retention of
viability of pollen treated with acetone-water mixes in proportions between 10:90 and 99.8:0.2. Roggen did find, however, that alcohol-water mixes killed the grains. In assessing Roggen's results it is felt that one ought to consider the possibility that the treatments he describes may not have had a uniform effect on each of the grains in the sample; thus effectively, some grains may have been killed, others survived a superficial (surface) treatment with possibly some remaining unaffected. His dropwise application of solvent to pollen on filter paper under vacuum would not, it is proposed, necessarily ensure thorough washing, as is certainly obtained by the methods used in this study - as in those of Iwanami.

The behaviour of pollen in solvent-water mixes should be considered when assessing Iwanami's (1973b) suggestion that plant tissues survive solvent treatment if water can be extracted from cells. This no longer appears to be a reasonable explanation on two counts: firstly, as demonstrated in both this study and others, wherever organic solvents have been shown to penetrate plant cells (including pollen, leaves, or seed embryos), the result in each case, has been some degree of disruption; secondly, as the pollen still contains some water after dessication, and at the time of shedding from the anthers, (see chapter 1), then access of dry solvent to this water by its penetration of the pollen cytoplasm would effectively result in a solvent-water mix, capable, as Iwanami and this study have shown, of destroying pollen viability. On the other hand, if the water is extracted from the grain, as Iwanami suggested (1973b), then the external medium would become a solvent-water mix - again with the potential, in the right proportions, of causing loss of pollen viability.
The results presented here are shown to agree well with Iwanami's findings of the effects of various organic solvents and solvent-water mixes on the viability of intact pollen grains. They do not, however, admit the interpretations made by Iwanami to explain the cause of these effects.

The electron micrographs of solvent-tracer treated pollen show that neither the exine nor the intine of Populus pollen prevents the passage of any of the solvents used in this study. That they have not penetrated past the region of the plasmalemma, in the case of the lower polarity solvents, may be due to the properties of the plasmalemma itself. It is proposed that the membrane may be the ultimate barrier to the penetration of these solvents, including those containing osmium.

The Davson-Danielli membrane model (1935), the basis for most modern models, has a bimolecular lipid leaflet as its core, with protein layers flanking either side. The hydrocarbon region of each of the lipid molecules is towards the centre of the lipid layer, and due to the nature of the bonding there is no net change imbalance, and consequently, this central region of the membrane is hydrophobic. The outer portions of these (largely phospholipid) molecules may become changed in aqueous solutions due to the presence of phosphate and, or, amine groups. Whilst Danielli and Davson (1935) proposed that protein is adsorbed as monolayers on either side of the central lipid bilayer, the Singer-Nicolson model suggests that the proteins are more likely to be imbedded, to various degrees, within the membrane in globular form. This would allow the hydrophilic portions of the lipid molecules to interact directly with the medium either side of the membrane, as would also the hydrophilic portions of the globular proteins imbedded within the membrane matrix. The latter model, in particular, stresses the hydrophilic (and
thus polar), nature of the membrane system. Such a membrane might well have a low permeability to lower polarity solvents, that is, those with a low dielectric constant.

Hypothetically, the membrane would be more permeable to solvents with the following physical properties: a high dielectric constant, a lower molecular weight, a lower surface tension and a higher solubility in water; the last-named property is stressed by Schmid et al. (1973), in their determination of the efficiency of penetration and extraction of plant tissues by various organic solvents. These properties are tabulated for each of the more common solvents (Table 3.1). From the table it can be seen that both water, and each of the alcohols methanol and ethanol, should readily penetrate membranes of the type defined, on theoretical grounds alone, as they may be distinguished from low polarity solvents by their higher dielectric constants, lower molecular weights and lower vapour pressures (hexane being an exception as regards the latter property); it is also clear from the table that the surface tension of each solvent is not a good guide to its pollen (membrane) permeability. The addition of water to the lower polarity solvents (with lower pollen permeability), in even small proportions, possibly enables it to gain access to the lipid core of the membrane, thus causing its dissolution and thereby facilitating further access of the solvent mix into the cytoplasm. As Benson (1974) noted: "many biological membrane systems ... resist simple solvent extraction and require solvation by at least two types of solvent". Gutknecht and Tosteson (1970) studied the effects of pure alcohols (IC to 8C), upon the electrical resistance of an artificial lipid bilayer, and showed that the effect of increasing the concentration of these alcohols was to progressively reduce the electrical resistance of the bilayer.
When the resistance fell below $10^5 \ \Omega \ \text{cm}^2$ (at between 1 and 10 molar concentrations for ethanol), the films ruptured. Clarkson (1974) has proposed that the introduction of even low concentrations of alcohol (in water) to a lipid monolayer, would result in the disruption of hydrophobic bonds between fatty acid chains in the lipid, with a resulting increase in liquidity, and a decrease in the structural order, of the membrane. Although the lower concentrations of alcohol greatly increased the electrical conductance of the membrane, its hydraulic conductivity was not increased in Gutnecht and Tosteson's (1970) experiments. Extrapolating these results to the situation under study, it seems likely that pure alcohols would similarly cause disruption of the plasmalemma, allowing the alcohol to enter the cytoplasm and cause its subsequent disorganization. Methanol is shown to cause loss of viability of the pollen of *Populus*, with the grain later failing to hydrate. The density of the pollen cytoplasm, which results from its low state of hydration, makes it difficult to interpret the degree of ultrastructural disorganization - certainly no membranes are visible and the only structures which are apparent within the grain are vacuoles.

It seems that although there are parallels between the effectiveness of (viability preserving) solvent treatments of seed and pollen, the ultimate barrier to penetration of solvents is different in each case. The barrier in seeds has been demonstrated, in the case of *Lactuca*, to be at the pericarp-integument-endosperm complex, with the lower polarity solvents failing to penetrate to the embryo region. Furthermore many of the seeds can survive ethanol, but not methanol, treatment - a result which may be explained, in part, by the lower molecular weight and higher dielectric constant of methanol when compared with ethanol (Table 3.1).
A re-examination of the work of Mitchell et al. (1970, 1971 for example), now indicates that his ether extracts of seeds would almost certainly have contained largely extra-embryonic components, just as, if 40°C treatment of Phaseolus pollen can be assumed to allow it to remain intact, then one could suggest that ether extracts of pollen may be derived solely from extra-cytoplasmic (that is pollen wall) sources. In this light the results suggest the possibility that Mitchell's "fatty hormones" may be located in the pollen wall. It is held, that had Mitchell et al. tested the viability of their seed after ether 'extraction', they should have found it to be viable; the viability of their pollen following heating, then solvent treatment, is less certain yet quite likely, judging from the solvent-pollen results obtained in this study, and from Iwanami's (1973d) observation that pollen of some genera could survive temperatures up to 90°C without loss of viability.

Although possibly distinct from the 'fatty hormones' of Mitchell et al., the 'pollen growth factors' of Brewbaker and Majumder (1961) appear to have been intracellular in location. Brewbaker and Majumder found that water extracts, but not diethyl ether extracts, showed 'pollen growth factor' activity, which may indicate that the diethyl ether did not penetrate the pollen grain to the degree required to obtain this factor. This proposal would support their suggestion that 'pollen growth factor' normally has an intracellular, rather than an extracellular, location. The localization of 'fatty hormones' and 'pollen growth factors' in pollen certainly warrants further attention. One should be cautious in interpretations of the results of Mitchell et al. (1971) when it is recalled that they obtained their pollen and pollen extracts by washing open anthers and stigmatic surfaces with solvents; the possibility of contamination of pollen with stigma surface materials was reportedly checked, whereas the
inclusion of tapetal material from the anthers as well as extracts from pollen over a wide spectrum of developmental stages, was not. One indication that their seed treatments may have resulted in superficial extraction only, is that when a highly polar solvent mix was used to treat bean seeds, a wider range of materials was extracted.

The variation in the composition of Martin's (1969, 1970,a,b,c) stigmatic "surface" washes may be similarly related to the degree of penetration and disruption likely to be caused by each solvent, as may be inferred from the results of the present study. It now appears probable that many of the solvents, if used in the manner indicated by Martin, could extract material from within the stigma papilla cytoplasm. Thus, whilst the lipidic nature of the surface of the stigma could alone explain the observation that greater quantities of extract could be obtained using chloroform or ether rather than water, the duration of many of the treatments (10 minutes) would almost certainly severely disrupt the papilla cytoplasm (as evidenced by Armstrong 1973), and cause extraction of intracellular materials. That collapse of the stigmatic papillae follows liberal applications of ether to their surface has been demonstrated earlier in this chapter. Hensarling et al. (1970) have also demonstrated that washing with hexane or acetone extracts lipids from intracellular sites of seed cotyledon tissue.

Of all the solvents and solvent mixtures used by Martin, the ethanol-hydrochloric and (70% ethanol:1% HCl) mixture would possibly have had the most drastic effect on the cytoplasm, as this solvent is normally used for the extraction of (intracellular) phenolics from plant tissues (Ribereau-Gayon, 1972). The substantial quantities of water reported by Martin to be extracted by each of the solvents or solvent combinations could come from two sources, namely the cytoplasm, resulting from its disruption by the solvent, and also the interstices of the primary and
secondary cell walls of the papillae.

Roggen (1974) reported differences in the composition of the extracts which he made of *Brassica oleracea* pollen, using a number of solvents including water, chloroform and acetone-water mixes with acetone contents of between 10% and 99.8%. Roggen described these extracts as "removable pollen coat extracts", the pollen coat being equated with "the outer layer of the exine". Roggen reported no loss of pollen viability with any of his solvent treatments (including acetone:water), and apparently assumed that the solvents penetrated no further than the exine in each case. There is a general consensus in the literature that hydration of pollen results in release of material from the pollen grain within a short time of its moistening. It has been proposed that much of this material is derived from the intine. Assumptions that materials would not be released from the cytoplasm of pollen within the first thirty minutes of hydration in isotonic buffer (Knox *et al.* 1972a), will be examined in more detail later (Chapters 5 and 6); the present results, however, do indicate that loss of material occurs from intracellular, as well as from extracellular sites of pollen shortly after such hydration. Chloroform washing of pollen grains was shown in Iwanami's (1972b) study to cause a loss in viability of about 80% of the pollen sample tested. If one proposes that death of these grains resulted from penetration of the cytoplasm by chloroform, then intracellular materials will most likely have been extracted in the "pollen coat" chloroform extract of Roggen (1974). The results presented in this study with *Populus*, and with other genera in studies by Iwanami, show that none of the pollens studied can withstand acetone:water mixes with between 50% and 98% acetone content; it is dubious whether Roggen's extracts, also made with acetone:water mixes, could be free of intracellular extracts. In this light Roggen's (1974) 'table 4', may be reinterpreted. The quantity of his Sudan IV -
stainable material may be simply correlated with the lipid dissolving efficiency of each solvent, water being the least efficient and chloroform or 99.8% acetone, the most efficient. Lowry-positive material is released in equal quantities by water and chloroform; this material, it is believed, could have been partially derived from intracellular sites as well as from the intine where water was used, and from the intracellular sites of the grains rendered inviable by chloroform.

Considering that acetone is not a good protein solvent, being widely used to precipitate protein, the exceptionally high protein content of the 50% acetone extractions (greater than six times that of water washings) may be simply a reflection of the degree of disruption caused by this solvent combination. A confident assessment of Roggen's results is thus complicated by the number of variables apparent, including his method of treatment (discussed earlier).

In conclusion, the results obtained in this study are held to elucidate the effects of organic solvents on pollen grains of *Populus*, and are comparable with those obtained with other genera and other tissues. Pollen is shown to be particularly resistant to the penetration of low polarity organic solvents, when compared with other tissues including seeds, leaves and embryonic tissues as examples. It is held that this resistance contributes for the effectiveness of solvent treatments of pollen in preserving viability and in overcoming incompatibility. Also, for those systems where solvent pollen treatments are effective in overcoming incompatibility, the results of this study should help to determine the location (and possibly the nature) of the incompatibility barrier. That many solvents
can penetrate to, but apparently not through, the plasmalemma, should also assist in the determinations of which pollen components are intracellular, and which, wall held prior to hydration; this, it is held, should still be a point of debate. These aspects will be studied in more detail in subsequent chapters.

The next chapter (4) in particular, will provide further support for the central contention of this chapter - that the lower polarity solvents used in these experiments have not entered the pollen cytoplasm.
At the time of final preparation of this thesis the following paper was kindly sent by Professor Yozo Iwanami: 'Studies on pollen grain and seed in organic solvents' JAP. J. PALYNOL. 15, 3-14 (in Japanese) 1975

As the paper was found, on translation, to be relevant to the present study, and in particular to chapter 3, some of its contents are reported and discussed.

Iwanami's aim was to determine whether the organic solvents diethyl ether and acetone penetrate the pollen of *Lilium* and *Camellia*. The results of his experiments provided evidence both for, and against, entry of these solvents into the pollen cytoplasm, although Iwanami was inclined to believe that they did effect entry. It should be noted that Iwanami's treatments involved immersion of the pollen in solvents for periods ranging from 10 to 300 days, and thus may not be strictly comparable with those of the present study (chapter 3) which involve immersions of no longer than 30 minutes.

Iwanami found that lipid vesicles, apparent in the cytoplasm of *Lilium* pollen, were diminished in size, and possibly number, after immersion of the pollen in acetone or ether for 300 days, the reduction being greater in acetone than in ether. Similar vesicles have been noted in the cytoplasm of intact *Populus* pollen, as has their dispersal following 50:50 acetone-water treatment: on the other hand, 98:2 acetone-water caused complete disruption of the cytoplasm, pure acetone and ether having no effect on the cytoplasm of *Populus* pollen over the 30 minute period of its immersion. Although the difference between the *Populus*, and Iwanami's results could be due to variations in the pollens themselves, the
differences in the periods of immersion may be more relevant; even so, Iwanami's results still do not prove the case for solvent penetration - rather perhaps, they may indicate for example, that there are differences in metabolic behaviour between pollen stored in solvent, and that in air.

In a second experiment, Iwanami found that, over a period of 200 days, pollen of *Camellia* lost both sugar and amino-acids into the acetone, but not the diethyl ether, solvent in which it was immersed.

Iwanami's third pollen experiment involved placing 2 samples of pollen in $^{14}$C acetone, one for 10 minutes, and the other for 10 hours, and found that the rate and amount of radioactivity lost was greater from pollen treated for 10 days, than from that treated for 10 minutes - a result he interpreted as indicating, firstly, that penetration had occurred in each case, and secondly that complete penetration took some time.

In the second part of his paper Iwanami presented evidence showing that these solvents may *not* penetrate the pollen. Thus *Camellia* pollen had normal ultrastructural appearance after immersion in ether for 3 months, there being a change by the end of 1 year, by which time the pollen had lost its viability (germinability).

Secondly, his immunoelectrophoretic comparisons of the proteins from *Solidago* pollen with those of pollen of the same genus after 2 months immersion in diethyl ether showed no difference, either quantitative or qualitative, between the two.

Iwanami also took his observation that *Petunia* pollen could still effect successful fertilization after immersion in the above solvents, as a further indication that solvent had not entered the pollen cytoplasm.
In the latter experiment, he showed that solvent treatment did not overcome self incompatibility in *Petunia*, contrary to his earlier suggestion (Iwanami 1973a).

Although Iwanami (1975) favoured the proposal that diethyl ether and acetone did enter pollen in solvent treatments, he conceded that absolute proof was lacking, and that he was unable to explain how pollen could retain its structure and function after such treatment.

In chapter 3 of this thesis some emphasis has been placed on the importance of membranes in any consideration of the effects of solvent treatments on pollen - Iwanami (1975), too, has recognized their importance. He proposed that 'dry', and hydrated, pollens have membranes differing in structure, such that hydrated membranes are semipermeable; unlike those of dry pollen. On this basis he held that, prior to its hydration, pollen would allow influx of higher molecular weight compounds (including solvents), although membrane phospholipids, being bound to protein in the membrane, are not lost and thus the integrity of the membrane, following solvent treatment, is maintained. He saw only the 'free' compounds of the cytoplasm as being removeable, those which are 'bound' remaining *in situ* after such solvent treatment, and able to resume their normal function upon subsequent hydration.

The solvent-pollen results of Iwanami (1975) do not conflict with those obtained in this study with *Populus*, and neither, considering the difference in the times of treatment used in the two studies, do most of his interpretations. Whilst Iwanami's (1975) proposals for membrane changes following hydration must remain speculative, one may reiterate (see discussion chapters 1 and 3) that the pollen plasmalemma at least, must undergo pronounced changes during the process of initial hydration.
It is maintained that none of the low polarity solvents, acetone, diethyl ether or hexane, can enter the pollen of *Populus* within 30 minutes, although it must be allowed, from Iwanami's results, that the possibility of penetration may exist where longer treatments are employed. The question of whether the entry of such solvents into pollen would allow the retention of its viability, or not, remains debatable.
Plate 3.1

Light micrographs of whole pollen of *P. bolleana* after solvent-osmium treatments. The material is embedded in resin.

fig (a)  Water-osmium treated. The grains have become hydrated and are swollen. The cytoplasm is osmium stained.

fig (b)  Methanol-osmium treated. The grains retain their prehydration appearance and the cytoplasm is densely stained with osmium.

NOTE: The reader is reminded that the following plates are derived from material processed with osmium used primarily as a tracer, and that pH, toxicity and composition of the initial 'fixative' mixture could not be modified without negating the point of the experiment. The resulting micrographs cannot thus be technically optimal.

* In these and subsequent figures *P. bolleana* results are displayed. Identical results were obtained in the same experiments with *P. nigra var. italic* pollenc.
Plate 3.II

Transmission electron micrographs of sections of the same pollen material shown in 3.1. Osmium staining only.

figs (a) and (b) Water-osmium treated pollen. The pollen grain has its normal (hydrated) appearance. Osmiophilic material, apparently lipidic, is apparent both in the exine region and in aggregations associated with vacuoles in the cytoplasm. Similar material is present within the generative cell (G.C.). An infold of the intine (arrowed) is visible in (a).

fig (c) Methanol-osmium treated pollen. The cytoplasm (X) is very densely stained, vacuoles being the only organelles discernable.
Plate 3.III

Light micrographs of whole pollen of *P. bolleana* after solvent-osmium treatments. Resin-embedded material.

fig (a) Acetone-osmium treated.

fig (b) Diethyl ether-osmium treated.

fig (c) Hexane-osmium treated.

In each photo it is apparent that the pollen grains have neither swollen nor taken up osmium stain. In each field a grain which has taken up osmium is used as a guide to the potential effectiveness of the solvent-osmium mix concerned to stain the cytoplasm. Such stained grains were not common, and they are possibly inviable.
Plate 3.IV

Transmission electron micrographs of sections of whole pollen of *P. bolleana* in the region of the wall after the solvent osmium treatments. Sections were taken from the same preparations shown in Plate 3.III.

fig (a) Acetone-osmium treated.

fig (b) Diethyl-ether-osmium treated.

fig (c) Hexane-osmium treated.

In each case the exine and most of the intine is osmium stained. In many of the hexane-osmium washed pollen grains regions immediately inside the intine were stained. Such a region is shown in fig (c). In none of the treatments was there any staining of the cytoplasm.
Light micrographs of whole pollen of *P. bolleana* after solvent-osmium treatments. Resin embedded material.

**fig (a)** Pollen treated with acetone-water (50:50) containing osmium.

**fig (b)** Pollen treated with diethyl ether-water (50:50) containing osmium.

**fig (c)** Pollen treated with hexane-water (50:50) containing osmium.

In each case the grains are swollen to various degrees and the cytoplasm is osmium stained.
Plate 3.VI

Light micrographs of whole pollen of *P. bolleana* after solvent-osmium treatments. Resin embedded material.

**fig (a)** Pollen treated with acetone-water (98:2) containing osmium.

**fig (b)** Pollen treated with diethyl ether-water (98:2) containing osmium.

**fig (c)** Pollen treated with hexane-water (98:2) containing osmium.

In each case the grains have swollen slightly only, and the cytoplasm is stained to varying degrees - depending on the degree of disruption caused by the solvent mix and thus the degree of loss of stained material.
Plate 3.VII

Light micrographs of whole pollen of *P. bolleana* after solvent-osmium treatments. Resin embedded material.

fig (a)  Pollen treated with acetone-water (98:2) containing osmium. The variable staining of some grains is shown to be due to loss of the disrupted cytoplasm resulting from the treatment.

fig (b)  Pollen treated with acetone-water (50:50) containing osmium. The grain has swollen and there is no sign of obvious disruption.
Transmission electron micrographs of sections of *P. bolleana* pollen after solvent-osmium treatment. No further stain was used.

**fig (a)** Acetone-water (98:2) and osmium treated pollen as in 3.VI(a) and 3.VII(a), showing the complete disruption of the pollen cytoplasm (X).

**fig (b)** Acetone-water (50:50) and osmium treated pollen as in 3.V(a) and 3.VII(b) showing some disruption of the pollen cytoplasm including, notably, dispersion of the osmiophilic material (arrowed) formerly (3.II(a) and (b)) associated with vacuoles. The remnants of this material are scattered through the cytoplasm, but also prominently line the tonoplast of the vacuoles with which they were formerly associated.
Plate 3.IX

Light micrographs of whole pollen of *P. bolleana* (resin-embedded) solvent treated but without the inclusion of osmium. These act as controls for the solvent treatments which included osmium as a tracer.

fig (a) Water treatment alone.

fig (b) Methanol treatment alone.

fig (c) As (a) but with interference illumination.

fig (d) As (b) but with interference illumination.

fig (e) Diethyl ether treatment.

fig (f) Hexane treatment.
CHAPTER 4
THE SURFACE COMPOSITION OF POLLEN AND STIGMA

INTRODUCTION

The results presented in Chapter 3 clearly demonstrate that both pollen and stigma surface materials may have a role in the (in)compatibility mechanism of *Populus*.

The composition of stigmata, including surface exudates, has been examined by several authors in studies of other genera, although there have been few studies of the composition of the surfaces of stigmata of the 'dry' type (Heslop-Harrison, 1975).

Similarly I am not aware of any previous study of the materials which have been taken from the pollen wall alone, and are definitely known to be free of cytoplasmic contaminants. Consequently, it is held, an accurate picture of the pre-hydration composition of the pollen wall has to be obtained.

Biochemical assays have been carried out on the stigmatic exudates from several genera, and also on pollen-wall materials, the pollen wall being isolated either after maceration of the pollen (for example Hara *et al.*, 1972) or after a series of chemical degradation processes (for example Brooks and Shaw, 1971). There have been several histochemical studies of 'dry' stigmata (Mattson, *et al.*, 1974; Heslop-Harrison, *et al.*, 1975) and of the pollen wall, usually involving fixation, embedding and sectioning, or else freeze sectioning. These latter studies include several recent works by Knox, Heslop-Harrison and Dickinson (for a recent review see Heslop-Harrison, 1975). Each of the above mentioned approaches has certain weaknesses which may produce potentially misleading results.
This is particularly true of pollen studies where any process which involves moistening of the pollen grains, albeit briefly (as in freeze sectioning), results in rapid swelling, and the consequent possibility of rapid translocation of materials, either from the pollen wall into the cytoplasm, or vice versa. It is apparent that most of the histochemical treatments in the past have been carried out with the grains in an already swollen (that is, hydrated) state. Similarly, studies on the composition of pollen diffusates, obtained from pollen soaked in isotonic buffered solutions (containing calcium) for up to 2 hours (Knox et al. 1972a), have been performed on the assumption that the extracts largely contain wall materials, with little chance of their contamination by cytoplasmic components; particularly, it is claimed, the higher molecular weight proteins (Howlett et al., 1975). The validity of this latter supposition is discussed in more detail in Chapters 5 and 6. On the other hand, treatments apparently designed to make extracts of the whole of the pollen (for example, the ether extracts of Mitchell et al. (1971) may, in many cases, be removing material from the wall only (see Chapter 3 discussion). This cannot be taken for granted as other factors, such as moisture contamination of the solvent, might make some such interpretations unreliable.

The 'framework' of the pollen wall consists of an inner intine, normally cellulosic in nature, and an outer exine, composed largely of the highly resistant biopolymer, sporopollenin. The arrangement of the latter is responsible for the often involved sculpturing of the surfaces of many pollens. Sporopollenin is, in effect, the material which will remain after degradation of most other materials by a variety of treatments, including acetolysis (Brooks and Shaw, 1971). It has been proposed that other pollen wall materials within the intine and exine matrices, result largely from deposition by gametophytic and sporophytic tissues respectively (Heslop-Harrison et al., 1973). The materials inserted into the pollen wall from the outside, have been ascribed to two classes; the first being
the 'tryphine' consisting of the hydrophilic substances, the second being the oily, hydrophobic 'pollenkitt' deposited on the outside of the exine (Echlin, 1971), and frequently filling interbacular cavities (Hoefert, 1969). On morphological evidence alone, Hoefert believed that the breakdown products of the tapetal organelles may be incorporated into the walls of the microspore. Heslop-Harrison, et al., (1973) endorsed the view that the exine-held material is derived from the tapetum, one fraction consisting largely of PAS-reacting material associated with 'non-enzymic protein' injected into the exine cavities, and another (the 'pollenkitt'), which is lipidic in nature and commonly contains carotenoids. These substances, due to their supposed tapetal origin, were regarded as sporophytic in origin, whereas materials that were detected in the intine (including proteins, some of them enzymic), were held to be derived from the haploid, gametophytic tissue of the grain itself. Mepham and Lane (1969) have observed release of gametophytic lipids into the intine of the pollen within the few hours prior to anthesis.

Following earlier suggestions (Knox, 1971; Heslop-Harrison, 1971) that proteins found within the wall had a 'recognition' function, Heslop-Harrison et al.,(1973) proposed that these were held in the exine of plants with sporophytic self-incompatibility, and in the intine where plants had self incompatibility under gametophytic control; they allowed for the possibility that *Populus*, with its interspecific incompatibility, could have recognition proteins held in either site. Heslop-Harrison, (1968a) had earlier allowed that the compatibility materials, including possibly the "cutinase" of Linskens and Heinen (1962), could be tapetally derived. Heslop-Harrison et al. (1973) state, however, that "there is nothing to suggest that the *Pollenkitt* plays any part in recognition reactions". The various functions ascribed to the 'pollenkitt' include its possible roles in insect repulsion, in protection from ultraviolet radiation, or by its
'stickiness', in assisting entomophilous dispersion (Echlin, 1971). Roggen (1972) working with *Brassica*, purported to show the involvement of both stigma surface wax and the pollen surface material in a 'sticking reaction' which he claimed to be characteristic of (and necessary for) compatible, but not incompatible pollen-stigma combinations. The composition of tryphine is even less well known, and Echlin (1971), whilst recognising its tapetal origin and hydrophilic nature, commented on the lack of knowledge about the chemistry of tryphine, noting that "although a detailed survey is lacking it appears that tryphine formation occurs in both insect - and wind pollinated plants" (Echlin, 1971).

The point has been reached where it is important to determine the nature of the pollen and stigma surface components in order to interpret their degree of involvement in the incompatibility mechanism, particularly in view of the success of solvent treatments of the stigma (Tatebe, 1968; Whitecross and Willing, 1975), or the pollen (Iwanami, 1973a; Roggen, 1974) in overcoming incompatibility. The solvent treatments provide a powerful tool with the potential to elucidate the incompatibility mechanism (or barrier) by narrowing the possibilities. It appears, for instance, from the results already presented in Chapters 2 and 3, that in *Populus*, and possibly in *Petunia* and *Brassica* as well, some 'recognition substance' exists in the pollen wall prior to hydration and is not derived from the cytoplasm. This is shown by both the breeding results (Chapter 2) and the observed degree of solvent penetration into *Populus* pollen (Chapter 3). Broadly speaking, this proposition, that some 'recognition substances' may be located in the pollen wall, is in agreement with earlier proposals by both Knox and Heslop-Harrison (reviewed by Heslop-Harrison, 1975). Iwanami (1973) was the first to report the use of solvent treated pollen in an attempt to overcome incompatibility (in *Petunia*), although Roggen (1974) later commented (incorrectly) that "no reference has yet been made in the literature to the effect of changing or removing the outside of the pollen and how this
influences the self- and cross- (in)compatibility". Roggen's proposition, based on earlier reports (for example Christ, 1959; Dickinson and Lewis, 1973b), as well as his own earlier (1972) observations, was that removal of the surface of either the pollen or the stigma could influence the (in)compatibility. He tested the proposal by solvent treatments of Brassica pollen and concluded that the chloroform soluble 'pollen coat' materials were "relevant for controlling the S1 [self incompatibility]" (Roggen, 1974). Roggen also made a rough analysis of the material removed by various solvents, finding that organic solvents removed lipid from the pollen of Brassica, whereas water did not, the reverse being true of reducing sugars. He found the amount of Lowry-positive material in the extracts to be the same, whether chloroform or water was used to wash the pollen. On the other hand, the proportion of acetone and water in mixtures of the two had a pronounced effect on the amount of protein removed. When compared with the amount of protein removed by water alone, both 99.8% and 10% acetone-water mixes removed less protein, whilst 50% acetone removed 6 times as much (Roggen, 1974). Roggen concluded that he had confirmed the presence of recognition substances in the 'pollen coat' of Brassica, that they were proteinaceous (and likely to be lipoproteins), and were similar to the 'recognition substances' defined earlier (Knox, 1971; Heslop-Harrison, 1971).

Whitecross and Willing (1975), following their success in overcoming the incompatibility barrier in Populus by pretreatment of stigmatic surfaces with ether and hexane, suggested that they had probably removed some lipoid substance, and that the incompatibility barrier in Populus "might be in the form of emulsifiable lipids on the surface of the stigma." That this suggestion might have wider currency could be inferred from the success obtained by a number of authors in the past (see Chapter 2 and review by Linskens and Kroh, 1967) in overcoming incompatibility by stigma mutilation, stylar implants or stigma decapitation with several genera.
A. THE STIGMA SURFACE

The stigma exudates, or 'liquid cuticle' (Konar and Linskens, 1966a) of stigmata of the 'wet' type (Heslop-Harrison, 1975) can be obtained with little difficulty, and have thus been examined by several authors. Konar and Linskens (1966b) determined that the stigmatic exudate of Petunia contained no liquid water within it, yet on its removal from the stigma a film of water was apparent beneath it. They showed the exudate to be "composed of pure fat, free from phospholipids, sterols and free fatty acids", and to contain 8 fatty acids, the main ones being linoleic (41.7%), undecanoic (17.2%) and heptadecanoic (14.0%) acids. The exudate contained the sugars - sucrose, glucose and fructose with minute traces of free amino acids, but had no detectable acid phosphatase activity. Konar and Linskens' report of lack of protein in Petunia stigma exudates contrasts with earlier reports of a proteolytic function in the exudate of Epipactis (Lingelsheim 1929), and a later report that Lilium exudate had a 7% (dry weight) protein composition (Labarca et al. 1969). The latter authors also held that muco-polysaccharides contribute to the viscosity of this exudate. Using cytochemical methods, Heslop-Harrison (1975) reported that in most cases some enzyme activity can be detected in the exudates of the stigmata of plants with gametophytic self incompatibility. Martin (1968, 1969), however, had earlier reported that if proteins (including enzymes) were present in stigma exudates, then they were not present in significant amounts.

In his study of the stigmatic exudates of 10 species (including Petunia) Martin (1969) failed to detect any sugars, and although aware of the work of Konar and Linskens (1966), which he cites in another context, states that "probably the old generalization that the stigma secretes a sugary substance is not valid" on the basis of his findings that the principal components of stigma surface extracts consisted of lipidic and phenolic compounds.
Martin and Brewbaker (1971) reported that the lipids of the stigmatic exudate occur as free fatty acids or esters of intermediate chain lengths, being neither volatile liquids nor hard waxes, and proposed that the lipid coating protects the stigma from dessication. Martin (1970a) reported the fatty acids in the exudate of *Strelitzia* stigma to have chain lengths of between 8C and 18C with oleic acid being the most common. The fatty acid composition of the *Zea* stigma surface was similar, though with a lower free fatty acid content (Martin, 1970b). Whilst *Ipomoea* (Martin and Brewbaker, 1971) contains only 2 fatty acids after esterification, including capric (decanoic 10C) and lauric (dodecanoic 12C) acids, the amount of stigma exudate in this case is small, as in *Zea*.

Martin (1969, 1970a,b,c, for example) placed particular emphasis on the presence of a number of phenolics within the exudate extracts of the stigmata. These he extracted by various means, but particularly efficiently by using acidic alcohol, which might allow for the interpretation that his extracts could have contained some substances of intracellular origin (see Chapter 3).

Martin saw the possibility that stigma surface phenolics could have a number of functions, possibly including:

(i) insect and disease resistance (Martin, 1969).

(ii) control of pollen germination, (Martin, 1969), and for growth (Martin, 1969, 1970, 1972), by stimulating or inhibiting metabolic processes.

(iii) limiting the specificity of the stigma to a few types of pollen (Martin, 1969), following an earlier suggestion by Moewus (1950) that flavone glycosides in the stigma may be associated with self incompatibility in *Forsythia*.

Martin (1960) proposed the following chain of events for pollen-stigma interaction:
pollen lands on the stigmatic surface; enzymes diffuse from the pollen to the stigma (Stanley and Linskens 1965; Mäkinen and Brewbaker, 1967); phenolic glycosides are hydrolysed; freed sugars diffuse into pollen; respiration of the pollen grain is stimulated (Dickenson 1967); growth of the pollen tube begins.

Apart from the above-mentioned earlier studies by Martin, which concentrate upon phenolics, the composition of the surface materials of stigmata of the 'dry' type has received relatively little attention. Roggen (1972) noted the presence of structures on the papilla surfaces of *Brassica* which were chloroform-removable and Sudan III-positive, which he described as 'waxy substances'. As already mentioned, Whitecross and Willing (1975) postulated the presence of emulsifiable lipids on the stigma surface of *Populus* on the basis of their results using lipid solvent treatments of the stigma surface.

Heslop-Harrison *et al.* (1975) acknowledged the presence of lipidic material "in patches or droplets" on the papilla surface of Cruciferae, but reported finding "no evidence of the 'waxy coating' said by Roggen (1972) to be present on the stigma papillae of *Brassica.*" This latter interpretation apparently resulted in part from their observation that lipase was unable to disrupt the pellicle of the stigma papillae in *Brassica* or any of the other cruciferous genera that they tested.

This 'pellicle' was described by Mattsson *et al.* (1974) as an extra-cuticular protein layer, and has been observed in a number of families with the 'dry' type of stigma (Heslop-Harrison *et al.* 1974). In this study (Chapter 1) the presence of a pellicle is reported for *Populus* for the first time. In the species investigated by Heslop-Harrison *et al.* (1975), they showed the pellicle to be formed as a continuous layer over the receptive stigma surface, to be antigenic, and to exhibit intense non-specific esterase activity. It was also shown to be disrupted by pronase digestion. The latter authors proposed that a correlation existed between
sporophytic incompatibility, and the 'dry' stigma type, with involvement of the pellicle.

B. THE POLLEN

The chemistry of pollen has been studied in considerable detail and from several standpoints (for a recent and comprehensive review see Stanley and Linskens, 1974).

As mentioned earlier, few of these studies provide a reliable indication of the composition of the pollen wall alone, or prior to hydration, which is of obvious importance for the interpretation of solvent pollen treatments - particularly in the light of the findings of Chapter 3.

(a) Carotenoids

The pollen walls of a number of species were examined following their degradation by various chemical reagents, and by ozonization, by Brooks and Shaw (1971) who determined that *Populus alba* pollen walls had a cellulose content of 4.2% and a sporopollenin content of 5.1%. These authors determined that sporopollenin consists largely of co-polymers of carotenoids and carotenoid esters. Heslop-Harrison (1968) showed that the maximum appearance of coloured carotenoids was well after the period of maximum exine deposition and that, in *Lilium*, Ubisch body formation and release, (involved in sporopollenin formation (Echlin, 1971)), occurs before the pollenkitt materials are released from the tapetum. The pollenkitt of *Lilium* was shown to consist mainly of lipids and carotenoids. There thus appear to be two main stages of incorporation of carotenoids into the pollen wall, the first involving a more stable polymerization, the second a looser deposition. This suggestion is supported by Stanley and Linskens (1974) who reported that "when pollen is extracted successively first with a mild lipid solvent, then by a more intensive solvent refluxing, both solvents remove different quantities and types of carotenoids" and proposed that "this suggests that different carotenoids occur at different
loci in the grain". They further report Troll (1928) as having observed that after the yellow surface oils of pollen of Cyclamen were volatilized the pollen appeared white.

(b) Flavonoids

Stanley and Linskens (1974) report that pollen often contains both carotenes and flavonoid derivatives, and noted that "while extractable carotenes may be absent flavonoid derivatives are always present". Stanley and Linskens (1965) had previously observed the presence of flavonoid compounds in diffusates from pollen of Petunia.

Stanley (1971) found flavonoid pigments in the pollens of 150 species, the main pigments being quercetin, kaempferol and isorhamnetin, and noted that anthocyanins have been reported occasionally in pollen. He cited Tappi and Menziani's (1955) observation that flavonoid-glucosides can be removed after removal of carotenes, and implies their location to be subsurface and possibly within the cytoplasm, commenting that "some pigments, in particular flavones, are water soluble and readily diffuse from the pollen" (Stanley (1971).

Populus pollens have been shown to contain the flavonoids, kaempferol (Wiermann, 1968; Sosa and Pecheron, 1970), isorhamnetin, quercetin and narmgenin (Wiermann, 1968), although from which site in the pollen these originated has not been determined. The possible presence of phenolics in the wall of Populus pollen is consistent with Rowley's (1971) microscopy study of the exine, in which he observed the anionic property of the exine surface to be "beyond question". Southworth (pers. comm. to Rowley 1971) had proposed that this anionic property was not due to a polysaccharide content of the wall but could be due to weakly acidic phenolic compounds, already shown to be present in the breakdown products of sporopollenin (Shaw and Yeadon, 1966; Brooks and Shaw, 1968).
Flavonoids may also be located in the intine of pollen. Wierman and Wienert (1969) studied *Tulipa* pollen development and, as Stanley and Linskens (1974) reported, "they recognized two phases of synthesis in the tapetum with deposition occurring primarily in the intine. Synthesis of flavonols occurs in the whole active tapetum, while anthocyanin synthesis increases during the degeneration of the tapetum, at about the same stage carotenoid biosynthesis reaches its maximum".

Stanley and Linskens (1974) reviewed the evidence for flavonoid involvement in pollen-style compatibility, as originally proposed by Kuhn and Low (1949), and Moewus (1950), and concluded that it was unproven. Linskens (1967) had linked flavonoids with enzymes in a proposed hypothetical compatibility control in *Forsythia*, yet this proposal could not be supported by later experiments (Stanley, 1971).

(c) Elemental composition

Numerous analyses have been made of the elements present in ashed whole pollen (for review see Stanley and Linskens, 1974). The possible roles of the minerals, and particularly of boron and calcium have been reviewed by Rosen (1968), and Stanley and Linskens (1974). The mineral content of whole ashed pollen of *Populus tremula* L. was determined by Knight *et al.* (1972) to be as follows (in m. equiv/100g dry wt.)

<table>
<thead>
<tr>
<th>Element</th>
<th>K</th>
<th>Na</th>
<th>Ca</th>
<th>Mg</th>
<th>P</th>
<th>S</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44</td>
<td>1</td>
<td>36</td>
<td>18</td>
<td>32</td>
<td>18</td>
<td>365</td>
</tr>
</tbody>
</table>

Stanley and Linskens (1974) comment that "methods used to assay mineral content afford little, if any specific information about how the elements are localized in particular parts of the pollen", and further, suggest that "analyses of residues after extraction with ethanol or ether may supply information on carbohydrate or pigment bound elements. Such extractive treatments can provide information about the localization of elements and components in different pollen organelles". They were,
however, wary of the interpretation of these short term extractive procedures as an aid to localization of elements at the organelle level owing to the different effects of each organic solvent on viability as indicated by Iwanami's (1972) results, and also by the apparent differences in response of pollen to rapid elution by buffers and water as noted by Stanley and Search (1971). Stanley and Linskens (1974) concluded that such short term extractive methods are "of limited use in studying localization of bound elements". They further note that whilst radioisotopes have been used for more precise localization of certain elements, the technique had not yet "been coupled to autoradiographic studies of microspores or pollen differentially extracted by solvents or enzymes". Stanley (1971) used electron microprobe analysis in examinations of sections of germinating pollen grains, determining the highest levels of calcium to be in the pollen grain walls.

(d) Growth regulators

Growth regulators, both inhibitory and stimulatory, have been found in pollens - even dead pollen, in some cases, produced a similar effect to exogenously applied-indole acetic acid (Stanley and Linskens, 1974). A number of growth regulators, including the major plant hormones, have been isolated from pollens. The exact function of these remains uncertain. Pollen also contains vitamins, (many recognized as enzyme co-factors) and inhibitors, at least one of which may be phenolic (Tanaka, 1958).

The location of these growth promoters or inhibitors has not been determined. Iwanami (1972, 1973b) reported the accelerated growth of pollen of *Camellia sasquana* following non-polar solvent washing due, he claimed, to the fact that an inhibitor "is diffused out of the pollen grains" (Iwanami, 1972a). The effect of this solvent washing on pollen tube growth varied with the species. Iwanami reached no firm conclusion
about the location of his 'inhibitor', but in earlier papers it is inferred that the solvent is able to penetrate into the cytoplasm (see Chapter 3). If the degree of penetration of this solvent into Camellia is similar to that reported for Populus (Chapter 3), one might reasonably propose a pollen wall location for this 'inhibitor'.

(e) Lipids

The ether-extractable lipoids of pollen have been examined by a number of authors (for review see Stanley and Linskens, 1974). They include "lipids, fatty acids, sterols, branched and straight chain hydrocarbons and their alcohol derivatives. The nonsaponifiable portion of the extract contains the sterols, hydrocarbons and higher molecular weight alcohols" (Stanley and Linskens, 1974); they have been described as a fatty oil (Kerner, 1891), often yellow in colour and comprising between 10% and 20% of the pollen dry weight. Ether extracts of pollen have been shown to include saponifiable and non-saponifiable fractions in the ratio 40:60 (Kwiatkowski, 1964).

Stanley and Linskens (1974) speculated that the "exine-contained lipid material is probably the major source of variation in ether extractable materials" although there was little evidence available at the time to confirm this.

Certainly Roggen (1974) had noted the removal of 'pollen coat' by his solvent treatments, as had Iwanami (1972) before him. Iwanami (1972) reported using diethyl ether, "to remove the fatty substance from the surface of the pollen grains". Neither Iwanami (1972a, b) nor Roggen (1974) indicated that the effective solvents had not entered the pollen grain and neither attempted to determine the degree of pollen penetration effected by each of the various solvents that they used.
Cytoplasmic lipids have been located primarily in spherosomes or elaioplasts by Tsinger and Petrovskaya-Baranova (1967).

The polar lipids of the ether extracts have been shown to include phosphatidyl-choline, lysolecithin, 1yso-3-sn-phosphatidylcholine and phosphatidylinositol (Gunasekeran and Andersen, 1973). The neutral lipids include largely triglycerides and also mono- and di-glycerides, free fatty acids, sterols and hydrocarbons (Crifo et al., 1969; Gunasekeran and Andersen, 1973). In several studies of ether extracts, fatty acids have been recovered from the saponifiable portion, methyl-esterified, then examined by gas-liquid chromatography (for review see Stanley and Linskens, 1974). In their review Stanley and Linskens reported that the fatty acids thus studied were shown to be saturated or unsaturated with most having an even number of carbon atoms in their chain, although odd-numbered fatty acids had been found in extracts of Petunia and Corylus pollens. Linoleic acid was present in all pollens, while most also contained myristic, stearic, palmitic, palmitoleic, oleic and lauric acids. Palmitic acid was reported to be the most abundant fatty acid in pollens of Petunia, Pinus, Corylus and Papaver. Of the fatty acids extracted from Petunia pollen, 77% were reported to be bound primarily as phospholipid. The fatty acid content of the phospholipids of Oenothera was reported by Caron (1972) to include 87% palmitic acid and 13% linolenic acid, whilst the Oenothera glycolipids contained 42% palmitic, and 58% linolenic, acid.

Caron also found that the proportions of phosphatidyl-ethanolamines, -cholines and -inositols could be related to the pollen genotype.

Scott and Strohl (1962) considered the exine and intine to be the main locations of long chain fatty acids, alcohols and waxy esters, whilst lipids of the cytoplasm would be likely to include a triglyceride mixture of linoleic, oleic and palmitic acids.
Mepham and Lane (1970), in an electron microscopy study of the development of pollen, also proposed a distinction between intine-, and exine-held lipids. They noted that whilst the pollenkitt lipids are apparently sporophytically (tapetally) derived there is observed, in the few hours prior to anthesis, a transfer of lipid globules "...apparently derived from the plastids...", which ",...mass in the peripheral cytoplasm and begin to exude through the pollen wall filling the channels through the endexine". Lipid globules thus derived would obviously then have a gametophytic origin. Echlin (1971) commented that "the full significance of the extrusion of lipid material from within the pollen grains is not at present clearly understood" (Echlin, 1971). One must allow for the possibility of such an extrusion into the pollen wall of Populus, in which case low-polarity solvent extracts, being derived from the pollen wall, may contain both gametophytic and sporophytic lipids.

It is allowed that the process of solvent washing of either pollen or stigma surfaces will be capable of removing not only lipophilic, but also lipophobic materials, whether these are within the lipo-soluble matrix and removed with it, or are liberated into the solvent by removal of such a matrix (see Chapter 3 and Johnson, 1971). For this reason, whilst attention has been focussed on the lipidic nature of the extract, one might expect, from existing literature (Roggen, 1974 and, for review, Heslop-Harrison, 1975), to find hydrophilic substances (including proteins) within the nonpolar solvent extracts. For this reason, attempts have been made to determine both the extent to which hydrophilic substances occur in such extracts and their location prior to hydration. Further, a more general comparison is made of the materials extracted by each of a variety of solvents, including solvent-water mixtures, which results should corroborate those obtained in Chapter 3 by other means.
The results obtained in Chapter 2 have placed particular emphasis in this study on the pollen and stigma surface composition, whilst those of Chapter 3 show that the short term effect of the solvents as effective in incompatibility treatments on pollen, is extracytoplasmic. The latter observation indicates that not only can pollen wall materials now be described with confidence using extracts free of cytoplasmic contaminants, but also, fortuitously, there is now available a technique allowing a closer investigation of the part played by (extracytoplasmic) surface materials in the incompatibility mechanism in Populus, and with minimal ambiguity.

The intention of this chapter is, in effect, to exploit this advantage in determining the composition of pollen and stigma surfaces, with the ultimate purpose of clarifying, if possible, the role of material at these locations in the mechanism in Populus.

MATERIALS AND METHODS

X-Ray Analysis of Pollen and Stigma Surfaces

Direct determinations were made of the elemental composition of Populus pollen and stigma surfaces by energy dispersive X-ray analysis. The samples were examined with a Cambridge Stereoscan 180 scanning electron microscope fitted with 'EDAX' energy dispersive X-ray analysis equipment, the analysis being controlled through a remote computer console with a programme provided by EDAX International. Fresh material was fastened to aluminium stubs using colloidal carbon.

All tests were conducted at an accelerating voltage of 10Kv and analyses performed over periods between 100 and 400 seconds at rates of up to 2000 counts per second using 20 eV per channel. Records were obtained from the computer printout and by photographing the 'EDAX' television monitor screen using Polaroid film.
Enzyme Staining of the Intact Stigma Surface

The enzyme activity of the stigma surface was determined by immersing the stigmata of intact whole flowers in each of the enzyme reaction media for periods of between 10 minutes and 1 hour. After staining, the intact stigmata were washed and their surfaces examined directly using either a stereomicroscope or a Zeiss 'Ultraphot' fitted with 'Epiplan' (Zeiss) optics and illumination. Material was also sectioned by hand, mounted in distilled water on glass slides then examined using the conventional light microscope. *P. deltoides* was the only species thus examined.

Reaction media were used for the detection of the following enzymes: acid phosphatase, peroxidase, esterase, lipase, leucine amino-peptidase, aspartate amino-transferase and malate dehydrogenase. The composition of each reaction medium is described in Appendix VI.

Extraction of Surface Materials for Analysis

A. The stigma surface

Whole intact female catkins of *Populus* were individually washed in twice redistilled hexane for 8 seconds each, the washings pooled and filtered through sintered glass Buchner filters under vacuum. The filtrates were pooled, placed in round-bottomed 'Quickfit' flasks, and the hexane removed under vacuum using a rotary evaporator. The residue was washed several times with redistilled chloroform to remove moisture and dried after each wash; finally the air in the flask was replaced with nitrogen gas and the flask sealed, then stored, until required, under refrigeration at -4°C.

Sonication of the flowers with hexane was also tried, with success, as a potentially more efficient way of removing surface materials, but was discontinued and the extracts thus obtained not used, as cytoplasmic disruptions, which would cause contamination of surface materials, could not be discounted.
B. The pollen surface

Extracts were made of pollen surface materials by washing 1 gram quantities of intact dessicated *Populus* pollen in hexane. The washes were of 10 minutes duration and repeated twice. All the washings from each sample were pooled and filtered free of pollen using either a Millipore microsyringe holder fitted to a glass syringe, or a Millipore microanalysis filter holder connected to a suction line. In each case the filters used were Millipore 'Mitex' Teflon filters (5.0 µm pore size), preferred for their inertness to organic solvents (including hexane). The filtrate was collected, dried, concentrated and stored as described for stigma washings (above).

For thin layer chromatographic comparisons of the effects of various solvents on extract composition, other extracts were made by similar means, but using the solvents methanol, acetone and acetone-water mixtures.

Analysis of Surface Extracts

I  C, H and N content

Samples of dried surface extracts of *P. nigra* var. *italica* and *P. bolleana* pollen surfaces and *P. tremuloides* and *P. deltoides* stigma surfaces were submitted for C, H and N analyses on an Hewlett Packard 185B automatic C,H,N analyser. Phosphorous content was determined using the molybdenum blue test.

II  Protein content

The protein content of the pollen washings was tested in several ways:

(a) The Lowry method

The hexane washings of *P. yunnanensis* and *P. bolleana* were collected, dried down, then redissolved in acetone. They were then stored overnight at -4°C to allow the precipitation of any protein present. The precipitate
was removed, redissolved in tris-HCl buffer, pH 7.9, and then analysed by the Lowry method (Lowry et al., 1951) as described in Chapter 5 (and Appendix IV).

(b) Esterase isozyme content

Hexane washings of either pollen or stigmata were dried down then tris-HCl buffer, pH 7.9) added and 'slurried' into the fatty material. More buffer was then added to the slurry and the aqueous material freed of the residue by filtering as described above. These aqueous extracts were then run on electrophoresis gels and then stained to detect any esterase isozymes present, as described in Chapter 5 (and Appendix VI).

(c) Thin layer chromatography

Separation of hexane (and other solvent) washings was followed by spraying with ninhydrin reagent, which stains any free amino groups purple (Kates, 1972).

III Spectroscopy

(a) Ultraviolet and visible spectroscopy

Spectroscopic examination of hexane washings of pollen or stigmata was performed using a Varian techtron, Model 635 dual beam spectrophotometer with hexane in the reference cuvette.

(b) Infrared (IR) spectroscopy

Spectroscopic analysis was performed over the range of wavelengths between 4000 cm\(^{-1}\) to 660 cm\(^{-1}\), including stigma washings of *P. alba* and pollen washings of *P. nigra* var. *italica* and *P. bolleana*, each of the samples having been previously prepared as a solid dispersion consisting of 1% of the dried (solvent) extract in potassium bromide, finely ground and pressed under several tonnes of pressure into a transparent disc (KBr being transparent in the IR region of the spectrum). The disc was examined in a Pye 'Unicam' SP1000 infrared spectrophotometer.
(c) Nuclear magnetic resonance (NMR) spectroscopy

Samples were taken from the same extracts used for IR spectroscopic examination.

The extracts were dried down to remove hexane then redissolved in deuterio-chloroform (CDCl₃). In each case the spectrum was obtained using a sweep width of 250 Hz.

(d) Mass spectroscopy

As the samples tested by mass spectroscopy (MS) were first fractionated by (on line) gas liquid chromatography (GLC), the same samples were used for (GLC-) mass spectroscopy as were prepared for GLC alone. Their preparation is thus described below (see IV.b.(ii)).

The methyl esterified sample, dissolved in hexane, was injected directly into the GLC column of a Varian 'Mat III (GNOM)' GLC-mass spectrometer fitted with a 2mm. internal diameter glass column packed with 2% OV-17 on Gas Chrom Q. Helium was the carrier gas used. For 8 minutes after injection the column was maintained at 185°C, after which the temperature was raised by 8°C per minute until a temperature of 264°C was reached, and maintained to the completion of the run.

As each peak of interest appeared on the GLC chart, its mass spectrum was recorded.

IV Chromatography

(a) Thin layer chromatography (TLC)

Several types of thin layer plate were used for TLC. Silica gel (with, or without fluorescent indicator) was mixed with water in the proportions 50g/100 ml's and applied as a thin (2.5 mm) layer onto glass plates using a 'Shandon' coating apparatus. The more convenient 'prepared plates', with either silica gel or cellulose coating, were also used, including those with aluminium foil (Merck) or poly-ethylene terephalate (Eastman) backing.
Each type of plate had its own disadvantage, the laboratory-made plates proved time consuming to make to the standard required, the Merck plates showing lifting of the stationary phase from the aluminium backing on heating, and the Eastman plates, whilst preferred for this study, incorporated an organic binder preventing one using the concentrated acid test as a general test for the presence of organic compounds (Stahl, 1969; Kates, 1972) on these plates. All plates used were 20 x 20 cm.

Plates were activated at 90°C for one hour then cooled in a dessicator prior to their use.

Of the number of solvent systems tried the most effective, and thus most widely used in this study, were diisobutyl ketone/acetic acid/water (80:50:7) (Kates, 1972); petroleum ether/diethyl ether/acetic acid (50:50:1 or 90:10:1) (Malins and Mangold, 1965); hexane/diethyl ether/acetic acid (70:30:1) (Mangold, 1969) and benzene alone (Kates, 1972). A number of other solvent systems were tried but generally gave poorer results than those listed above.

All plates were run in glass chromatography tanks lined with blotting paper previously soaked in the solvents of the mobile phase and allowed to stand for at least one hour prior to use. The nature of the materials separated by TLC was determined in part by the use of pure standards (including fatty acids, phospholipids, phenolics and steroids purchased from Sigma or Calbiochem), but mainly by the use of spray reagents. The spray reagents used are mentioned in the text, their composition in each case being described in either Stahl (1969) or Kates (1972).

Plates were examined under UV light with, and without, the application of fluorescent indicators prior to their staining with spray reagents and re-examination. Spray reagents were used shortly after they were made or, if bought, after purchase. Staining with vapours of either iodine or osmium
tetroxide took place in large glass dessicators in a fume hood.

(b) **Gas-liquid chromatography**

(i) **Free fatty acid content of the stigma**

Whole stigmata of *P. deltoides* were homogenized using a motorized Potter-Elverheim homogenizer, the extraction technique following that of Johnson and Davenport (1971). This basically involved the extraction of homogenized tissue using chloroform-methanol-water (1:2:0.8) (after Bligh and Dyer, 1959). During all the processes up to the final removal of the lipid-containing chloroform (using a separatory funnel), the tissue was maintained at a temperature of less than 10°C (on ice) as far as possible. The chloroform extract was used either directly, or after redissolving, for TLC and GLC.

(ii) **Total fatty acid content of stigma and pollen surface washes, and of whole pollens with or without hexane prewashing**

Total fatty acid extraction and methyl esterification techniques closely followed those established for yeast by Rogers (1973 pers. comm.) after Gordon (1971). For details see Appendix III.

(iii) **GLC procedure**

The instrument used was a dual column Pye Unicam series 104 chromatograph fitted with a flame ionization detector, the signal being recorded, via an Autolab 6300 digital integrator, on a 'Unicam' AR 25 linear recorder. Nitrogen was used as the carrier gas. The two column types used with most success were 1.7 m x 2 mm 1.D. glass columns packed with 15% Apiezon L (w/w) grease on 100/120 Gas Chrom 'P' support as its stationary phase; also 0.9 m x 2 mm 1.D. glass columns packed with 10% diethylene glycol adipate (DEGA) on 80/100 Gas Chrom 'P' support.

The temperature programmes found to provide optimal separation of sample components were as follows:

*Although this equipment was used to process the signal in each case, the part of the circuit responsible for integration was malfunctioning and hence could not be used.*
1. DEGA columns were run isothermally at 175°C for the duration of each run.

2. APIEZON L. columns were run first at 185°C for 8 minutes, then raised by 8°C per minute until a final (isothermal) temperature of 264°C was reached.

Once established, these programmes were used for the same columns throughout the study, standards being run before, after, and often with, the samples for identification of their components.

Fatty acid methyl ester standards were purchased from Sigma Chemicals Ltd. Unfortunately the integrator was not functional for the duration of these experiments and thus no attempt has been made to make the survey truly quantitative.

RESULTS

X-Ray Analysis of Pollen and Stigma Surfaces

Records are presented (figs. 4.1) of the oscilloscope displays showing the energy spectra for each of the surfaces of both intact, and pre-washed, P. bolleana pollen, (figs. 4.1,(b),(c)) and of the receptive surface of the stigma of P. monilifera (fig. 4.1.(a)).

The order of concentration determined for the elements O, Mg, P, S, K and Ca is summarized in table 4.1, for each of the three samples. This has been further expressed in terms of relative percentages in the case of the stigma surface analysis, where the investigation was more intensive, the display record (fig. 4.1.(a)) showing the results of the analysis after the Brehmsstrahlung background had been removed by computation. The relative percentages of the stigma elements were computed from the background subtracted, and integrated, peaks, correction being made (on a theoretical basis) for atomic number, absorption and fluorescence.
TABLE 4.1

Relative concentrations of the surface elements of pollen and stigma surfaces, as determined \textit{(in situ)} by X-ray analysis.

<table>
<thead>
<tr>
<th>RELATIVE % ORDER OF RELATIVE CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>(STIGMA only)</td>
</tr>
<tr>
<td>Stigma</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>35.94</td>
</tr>
<tr>
<td>16.58</td>
</tr>
<tr>
<td>7.47</td>
</tr>
<tr>
<td>3.61</td>
</tr>
<tr>
<td>0.10</td>
</tr>
<tr>
<td>0.00</td>
</tr>
<tr>
<td>7.47</td>
</tr>
<tr>
<td>3.61</td>
</tr>
</tbody>
</table>

The X-ray energy (Ko) for each element is given in brackets in the stigma column.
It is emphasized that even with the more detailed stigma surface results the confidence limits are ±15% due to the uncertainty of the take-off angle from the (rough) surface examined.

Table 4.1 includes the silicon ($K\alpha = 1.48$ KeV) and aluminium ($K\alpha = 1.74$ KeV) results for the stigma sample. Silicon was found in appreciable quantities on the stigma surface only, whilst aluminium may be regarded as a contaminant, almost certainly due to the use of an aluminium stub for mounting the specimen. The oxygen result is probably not reliable, being close to the limits of detectability.

The table shows that intact pollen and stigma surfaces of each of the species tested have similar relative concentrations of the elements listed, with the exception of Mg which is present at a relatively higher level at the stigma surface. The high K and Ca levels, in both cases, are particularly notable. Hexane washing of the pollen is shown to have an effect on the elemental composition, which presumably reflects that of the newly revealed surface.

The percentage (w/w) of material removable by hexane washings from non-hydrated pollen is shown for each of several pollen species below (Table 4.2).

### Table 4.2

Proportion of total mass removed from pollen by hexane washing.

<table>
<thead>
<tr>
<th>POLLEN SPECIES</th>
<th>PERCENTAGE WEIGHT HEXANE REMOVABLE/TOTAL %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leuce</td>
<td></td>
</tr>
<tr>
<td>P. bolleana</td>
<td>2.44</td>
</tr>
<tr>
<td>P. tremuloides</td>
<td>1.51</td>
</tr>
<tr>
<td>Aigeiros</td>
<td></td>
</tr>
<tr>
<td>P. nigra var. italica</td>
<td>1.34</td>
</tr>
<tr>
<td>P. deltoides var. angulata</td>
<td>1.84</td>
</tr>
<tr>
<td>Tacamahaca</td>
<td></td>
</tr>
<tr>
<td>P. yunnanensis</td>
<td>2.79</td>
</tr>
<tr>
<td>P. sp.</td>
<td>2.62</td>
</tr>
</tbody>
</table>
The hexane washings of both pollen and stigma each, in their dried form, have a consistency between that of butter (pollens, particularly the extracts) and a soft wax (stigma, particularly the extracts).

The colour of pollen and stigma hexane washings ranges in both types of sample, from that of pale straw, in white poplar extracts, to a yellow to orange colour in those of *Taoromahaca* and black poplars.

**Enzymes of the Intact Stigma Surface**

The results, presented in table 4.3, show that several enzymes besides esterases, may be detected at the *Populus* stigma surface. That the stigma staining was superficial was confirmed by examination of hand sections by light microscopy which showed the staining where present, to be at the surface only. A closer examination of the stigma surface, following esterase staining and using Zeiss 'Epiplan' optics, revealed that the activity of this enzyme is prominent in the region of the 'wax structures; described in Chapter 1. This, preliminary, study is consistent with the conclusion that enzymes detected are located in the pellicle - though this is not proven.

**TABLE 4.3**

*Stigma Surface Enzymes as Observed after Superficial Staining.*

<table>
<thead>
<tr>
<th>POSITIVE</th>
<th>POSSIBLE</th>
<th>NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>α Esterases</td>
<td>Acid Phosphatase</td>
<td>Malate Dehydrogenase</td>
</tr>
<tr>
<td>β Esterases</td>
<td></td>
<td>Glutamate Dehydrogenase</td>
</tr>
<tr>
<td>Lipase</td>
<td></td>
<td>Alcohol Dehydrogenase</td>
</tr>
<tr>
<td>Leucine Aminopeptidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transaminase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
I

C, H and N Analysis

The analysis was performed on the crude dried hexane washings of a number of species including: the stigmata of P. tremuloides, P. sp. (a Tenmu-baana), and P. alba, and pollens of P. bolleana, P. nigra var. italica, P. yunnanensis, P. deltoides var. angulata and P. tremuloides. The results were of the same order in each sample; some are presented in table 4.4, below.

TABLE 4.4

C, H and N composition of the hexane washings of Populus pollen and stigmata.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>Balance</th>
<th>C:H ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLLEN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. nigra var. italica</td>
<td>77.79</td>
<td>11.21</td>
<td>Nil</td>
<td>11.00</td>
<td>6.94</td>
</tr>
<tr>
<td>P. bolleana</td>
<td>81.34</td>
<td>11.41</td>
<td>Nil</td>
<td>7.25</td>
<td>7.13</td>
</tr>
<tr>
<td>STIGMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. deltoides</td>
<td>84.40</td>
<td>13.31</td>
<td>Nil</td>
<td>2.29</td>
<td>6.34</td>
</tr>
<tr>
<td>P. tremuloides</td>
<td>80.77</td>
<td>12.00</td>
<td>Nil</td>
<td>7.23</td>
<td>6.73</td>
</tr>
</tbody>
</table>

Significantly, it is apparent that the hexane washings of both stigma surface and pollen contain no detectable nitrogen. This suggests that either protein is not present, or that if present, it comprises only a small proportion of the total mass of each extract.

Comparison of the C and H percentages of these extracts with those of a number of natural products (diagram 4.1, after Brooks 1970) shows their composition to be comparable with those of waxes.
RELATIONSHIP OF COMPOSITION OF KEROGEN, SPOROPOLLENIN AND NATURAL PRODUCTS.

II Tests for the presence of protein in hexane washings of pollen

(i) Lowry test.

Lowry-positive (protein) material was not detected in any of the extracts derived from the hexane washings of Populus pollens.

(ii) Isozyme test.

The activity of isozymes could not be detected in the extracts of material originally removed from pollen by hexane washing. This is further discussed in Chapter 5.

(iii) Ninhydrin test.

As shown by TLC (fig. 4.VI(d)), the hexane extracts of Populus pollens do not contain ninhydrin-positive materials, by contrast with pollen buffer washes for example, which do.

III (i) Ultraviolet and visible spectroscopy

UV and visible absorption spectra (not presented) showed major peaks to occur at the wavelengths ~212 and ~325 (UV light) and ~450 (visible light) in crude acetone washings of each of the pollens tested. As already shown in Chapter 3 these washings contain materials which are normally wall-held in the Populus pollen grain prior to its hydration.

The results allow for the interpretation that these washings contain aromatic compounds which possibly include carotenoid pigments (DMS UV. Atlas of Organic Compounds).

(ii) Infrared Spectroscopy

Differences between the spectra of hexane washings of P. bolleana and P. nigra var. italica pollens are slight (figs. 4.II(b) vs. 4.II(c)). By comparison, the spectrum obtained from the hexane washings of the stigma of P. alba is simpler than those of either of the pollens (fig. 4.II(a)).
All three samples had prominent peaks in the region 2750 - 3000 cm\(^{-1}\) (indicating the presence of CH\(_2\) and CH\(_3\) groups), and 720 cm\(^{-1}\)((CH\(_2\))\(_n\) where \(n\) is greater than 4); peaks in these regions are characteristic of lipids (Johnson and Davenport, 1971). The presence of ester linkages, again in all three samples, is shown by the presence, in each, of peaks at 1740 cm\(^{-1}\), albeit not as pronounced in the pollen, as in the stigma, washings.

Free fatty acids (1710 cm\(^{-1}\)) may also be present in each, in small quantities. The unsaturated nature, particularly of pollen extracts, is indicated by the peak at 3020 cm\(^{-1}\).

The peak at 1600 cm\(^{-1}\) in washings of P. nigra var. italica pollen allows for the possibility that aromatic compounds are present in this sample. (Fleming and Williams 1966).

None of the three samples shows signs of the presence of cholesterol (3350, or 1045, cm\(^{-1}\)) phospholipids (3100, 3600, 1200-1250, or 1000-1100, cm\(^{-1}\)) or of compounds containing amide linkages (1640 cm\(^{-1}\)) (Davenport 1971).

(iii) Nuclear Magnetic Resonance Spectroscopy

NMR spectra (figs. 4.11(a), (b) (c)) have been interpreted (Armarego pers. comm. 1975) as demonstrating the presence of unsaturated long chain fatty acid esters in the hexane washings of pollen of both P. bolleana and P. nigra var. italica, the stigma hexane washings also containing long chain fatty acids, but in this case, with a lesser degree of unsaturation and less indication of ester linkages.

(iv) Mass Spectroscopy

GLC-mass spectroscopy analysis of stigma hexane washings revealed that among the methyl esterified materials prepared for GLC, are both fatty acid and terpenoid esters. The stigma surface fatty acids include those with chain lengths of 27C and 29C.
IV (i) Thin layer chromatography (TLC)

Preliminary Studies

In preliminary trials it was found that of the organic solvents used to wash *Populus* pollen (including acetone, diethyl ether, hexane and methanol), methanol extracted the widest spectrum of compounds, including both polar and low-polarity lipids.

TLC comparisons of extracts of different species of pollen, using the same solvent for each comparison, showed that for each solvent used the same major lipid classes were represented. There were slight quantitative differences in composition between species but these did not correspond to differences in the compatibility groupings.

The identity of each of the phospholipids obtained by methanol extraction of pollen was determined by running each extract on TLC plates with the known standards. The most prominent phospholipids present were phosphatidylcholine (lecithin), phosphatidylinositol and phosphatidic acid.

Most of the low-polarity solvent extracts of *Populus* pollen contained at least one, and often two, regions which were identified as phospholipids both by their Rf values, as well as their phosphate staining (Vaskovsky and Kostetsky, 1968).

The phenolic content of pollen washings of *P. nigra* var. *italica* was tested using, in turn, each of the solvents listed above as well as the solvent which Martin and Telek (1971) found most effective in the removal of phenolics from the stigma (surface?), viz. methanol - 1% HCl. These washings were run on silica gel coated plates together with the standards including the flavonols, kaempferol and quercetin; the cinnamic acid derivatives, p-coumaric, caffeic, sinapic and chlorogenic acids; also stigmasterol and the hydrolysable tannin component, ellagic acid. The following positive results were obtained:
(a) there was a small quercetin content in ether and acetone extracts but none apparent in those obtained using hexane, methanol or methanol-HCl.

(b) caffeic acid is present in non-polar solvent washings but not in those containing methanol.

(c) compounds found in methanol and methanol-HCl washings only included the hydrolysable tannin component, ellagic acid, and both sinapic and p-coumaric acids.

**Composition of hexane washings of Populus pollen**

Hexane extracts of pollen of *P. bolleana* and *P. nigra* var. *italica* were run on silica gel coated thin layer plates with each of the four solvent systems described, their composition then being determined largely by the use of spray reagents, as described below.

No glycolipids were detectable in the hexane washings of either *P. bolleana* or *P. nigra* var. *italica*, using the α-napthol stain for glycolipids (Siakotos and Rouser, 1965).

Steroids could not be detected following TLC runs of the hexane washings of several pollen species. This was determined either by the comparison of the washings with standards, including cholesterol and stigmasterol, or by their reaction to the spray reagents, ferric chloride (Lowry, 1968) or antimony (III) chloride (the 'Carr-Price reagent', Stahl, 1969).

Non-specific iodine vapour staining (Kates, 1972) on plates on which the hexane washings of *P. bolleana* and *P. nigra* var. *italica* were run, revealed the presence of one major staining region (Rf. 0.92) and three others on plates run using benzene as the mobile phase. The major region also remained as an opaque band after the plate was sprayed with water, and
was the only band to stain strongly in the presence of osmium tetroxide vapour, indicating a degree of unsaturation in this compound (or group of compounds) (Kates, 1972). It is proposed that the major (neutral lipid) spot detectable on thin layer plates using benzene as a solvent, is by its \( R_f \) value (0.92) an ester (Purdy and Truter, 1968) which is unsaturated, a proposal confirmed by its positive ester staining (see below), and by its IR and NMR spectra (in this chapter).

Of the many solvents tried, a mixture of petroleum ether-diethyl ether-water (50:50:1) (a solvent mixture to which no reference was found in the existing literature), provided the best separation of the pigments of the hexane washings of pollen. Using this system 6 pigment bands, naturally visible by their yellow colouration, were separable. No attempt was made to identify these, but it is suspected that they possibly include carotenes and/or xanthophylls, these pigments having been previously found in the lipid fractions of a number of pollens (Stanley and Linskens, 1974).

Comparison of several solvents by their extracting ability

It is apparent from the preliminary studies that the nature, and apparently the quantity, of material extracted from pollen varies, depending on the nature of the extracting solvents; this effect was previously noted, for example, by Martin (1971), with stigma extracts, and Roggen (1974), with extracts of pollen, - each study using a variety of solvents.

It should be recalled that in Chapter 3, it was demonstrated that various solvents (and mixtures of solvents) penetrate to different degrees into the pollen grains of *Populus*. The low polarity solvents acetone, ether and hexane each apparently penetrate to, but certainly not beyond, the plasmalemma, whilst solvents which are more polar by themselves (methanol or water), or made so by addition of water (organic solvent-water
mixes), penetrate the cytoplasm. Of all the solvents used those shown (Chapter 3) to be most disruptive are the organic solvent-water mixes with a low water content (2%). The results obtained in Chapter 3 should be borne in mind when considering those presented below.

The composition of the materials removed by the solvents hexane, methanol, acetone-water (50:50), acetone-water (98:2) and 'extracting buffer' (isotonic calcium-mannitol tris-HCL, pH 7.9) were compared on (silica gel) thin layer chromatograms with those obtained in the acetone homogenate of the whole pollen. The results on any single plate are both quantitatively and qualitatively comparable, with the exception of acetone-water (50:50) extracts. Solvents used in this experiment included benzene; petroleum ether (B.P.60-80°C)-diethyl ether-acetic acid (50:50:1) and di-isobutyl ketone (DBK)-acetic acid-water (50:50:1) - these providing optimal separation for hydrocarbons, neutral lipids and amphiphilic lipids respectively (Kates, 1972).

Immediately after each run, the plates were examined under UV light to detect the autofluorescence of the separated components. Plates were then sprayed with Rhodamine 6G, examined again under UV light and the fluorescent regions of the plate marked. Photographs of plates thus marked after running in each of the three mobile phases described above, are presented (figs. 4.IV(a),(b),(c)).

The thin layer chromatograms which were run with benzene as the mobile phase exhibited the following features (fig. 4.IV(a)):

(a) diffusate buffer extracts contained fewer bands than are evident in any of the other samples; further, unlike each of the other samples, this extract fails to show the presence of material of intermediate Rf. Polar (low Rf) and low polarity (high Rf) materials are both present.
(b) hexane washings of pollen contains no polar material (low Rf) yet do contain several of those with intermediate mobilities, which are most likely neutral lipids, including esters (as also found by IR- and NMR-spectroscopy).

(c) as expected, extracts of macerated whole pollen contains all the bands detectable in those obtained by each of the other treatments, including 'diffusate buffer' and hexane washings.

(d) extracts of macerates of pollen which had been hexane pre-washed contain each of the bands present in buffer diffusates yet lack some found in hexane washings (as expected) and others found in macerates of previously intact pollen. Material absent in hexane pre-washed pollen but present in intact pollen may be found in the hexane washings.

(e) extracts of macerates of whole intact pollen, and methanol and acetone-water (98:2) washings each had similar composition.

(f) acetone-water (50:50) extracts are similar to those of macerated whole pollen, except for the absence of higher mobility (lower polarity) compounds.

Similar results were obtained with 'ether' and 'DBK' mobile phases, (figs. 4.IV(b) and (c)). It is apparent, particularly on ether-run plates, - that the acetone-water (50:50) extracts do contain the more mobile compounds not apparent in extracts of the same type run under similar conditions with benzene as the mobile phase. Possibly the reasons for the poorer separation of the acetone-water (50:50) extracts by either benzene or 'DBK' mobile phases, could be due to either sample overload, or a failure to dry the (water containing) extract properly, prior to running the plate.
The demonstrated differences in the composition of the various extracts were further examined using spray reagents.

(i) **Phospholipids** were detected using the phosphate stain of Vaskovsky and Kostetsky (1968); some were further identified both by their Rf values and their staining reactions to the ninhydrin reagent (Marinetti 1964) and the Dragendorff stain for choline (Beiss, 1964). At least three phospholipids are common to extracts of each of the pollen homogenates and methanol washings. The same phospholipids are present, though in smaller quantities, in acetone-water (98:2) extracts. The buffer diffusate was the only extract with no demonstrable phospholipid content (figs. 4.V(a), (b)).

One phospholipid, absent in hexane extracts, but present in those of macerated pollen and in methanol washings, was identified as phosphatidylcholine by its Rf (0.4 with DBK solvent mix); its yellow colour under UV light after spraying with Rhodamine 6G; its positive staining with Dragendorff's reagent (fig. 4.V(c)) and its failure to stain with ninhydrin reagent (Kates, 1972) (fig. 4.V(f)).

A band of Rf 0.93 (with DBK as the mobile phase) stains positively with both phosphate, and Dragendorff, spray reagents in all but the buffer diffusate and acetone-water (50:50) extracts, however the latter does stain positively with phosphate reagent alone. This region is believed to include several compounds including phosphatidic acid (accounting for its phosphate content) and the esters located earlier (see also 4.VI(b)), the latter producing a positive reaction to Dragendorff's reagent (fig. 4.V(c)) possibly, in part, due to its unsaturated fatty acid content (Kates, 1972). The unsaturated nature of this region was also evidenced by its osmiophilic staining.

(ii) **Esters** were detected using the spray reagents of Skidmore and Entenman (1962), on plates run using benzene as the mobile phase (fig.4.VI(b)).
They are resolved in all extracts except those of buffer diffusates, and the acetone-water (50:50) mix. Hexane pre-washed pollen homogenate shows a weaker reaction to the stain than does that of the intact pollen. When the plates were placed in an osmium vapour chamber, the same (ester-positive) spots darkened.

(iii) reducing lipids and steroids may be detected using the phosphomolybdic acid spray reagent (Seher, 1959) which is a nonspecific stain (Johnson and Davenport, 1971) for reducing and unsaturated lipids (Stahl 1959). This reagent produces a strong reaction with components of the extracts of pollen homogenates, as well as with hexane, methanol and acetone-water (98:2) washings. There was little, or no, reaction from buffer diffusates or acetone-water (50:50) washings, there being only a slight reaction evident in low mobility fractions of the latter washings. Apparently most of the molybdenum blue staining material is present in the pollen wall, as evidenced by its markedly strong staining in fractions of hexane washings and whole pollen homogenates, compared to the low staining of hexane pre-washed pollen homogenate. The methanol washings have an identical phosphomolybdic acid pattern to that of intact pollen homogenate extracts. Spraying of the plates with stannic chloride reagent for sterols and steroids (Scheidegger and Cherbuliez, 1955), produced negative results.

(iv) vitamin D a fat soluble vitamin may be present, particularly in buffer diffusate and acetone-water (50:50) extracts, but not in hexane washings, as indicated by its muddy green colour after staining with concentrated sulphuric acid and its low Rf value of 0.06 when DBK was used as the mobile phase.

(v) protein was detected using ninhydrin spray reagent (Kates, 1972), which detects amido groups, and by its zero Rf (with the 'ether mix' as mobile phase). It was found in all of the samples but the hexane washings.
Gas Liquid Chromatography

Photographs of the GLC chart records are presented (figs. 4.VII, VII, IX, X and XI). The peaks have been identified as far as was possible using the methods described earlier. Although samples were run on DEGA and APIEZON L columns, the results were comparable and thus only the results obtained with APIEZON L columns are presented. Each sample was run for at least 2 hours to ensure that the compounds with higher retention times would not be overlooked.

(1) Free fatty acid content of the stigma homogenate

The predominant fatty acids found in total stigma homogenate (T.S.H.) extracts of *P. deltoides* were palmitic (16C), and unsaturated 18C, fatty acids, including oleic (18:1), linoleic (18:2) and linolenic (18:3), acids. Other fatty acids present included stearic acid and unsaturated 12, 13, 14 and 15C fatty acids (figs. 4.VI(a),(b)).

(2) Total fatty acid content of pollen and stigma hexane washings and of whole pollen macerate extracts with, and without, hexane pre-washing

The hexane pre-washed pollen homogenates are found to contain predominantly 16C and 18C unsaturated fatty acids, as in the stigma homogenates. The chromatograms of the extracts from each of the pollens are virtually identical (figs. 4.VIII(a),(b),(c),(d)).

Hexane washings of pollen have as their major peaks, 16C and unsaturated 18C fatty acids, the relative proportions of each varying with the species from which it was derived (figs. 4.IX(a),(b),(c)).

By contrast, the hexane washings of *Populus* leaves from representatives of the three breeding groups under study (*Aigeiros*, *Tacamahaca* and *Leuce*), each have the same, or at least very similar, fatty acid content, with 16C and unsaturated 18C fatty acids predominant (figs. 4X(a),(b),(c),(d)).

The fatty acid composition of extracts of hexane washings of the stigma is notable in that in each case 16C, and unsaturated 18C, fatty
acids no longer predominate, there being at least 7 'major' peaks in each sample including both these mentioned above and several with chain lengths greater than 22C. For each species the relative areas of the last two 'major' peaks, which shall be referred to as 'A' and 'B', correlate with the compatibility group to which each species belongs; thus the ratio of the area of peak 'A' to that of peak 'B' is greater than one, in the case of poplars in the section Leuce (P. alba and P. tremuloides), and less than one in those of the sections Tacamahaca (P. sp.) and Aigeiros (P. deltoides and P. monilifera) as is evident in figs. 4.XI(a) to (e) inclusive, and summarized diagrammatically in fig.4.XII. Preliminary studies using mass spectroscopy, as mentioned earlier, have shown that the hexane washings of the stigma surface of Populus contain fatty acids of chain length up to 29C.

DISCUSSION

The results obtained in this chapter, using solvent extracts of pollen, integrate well with those of Chapter 3 in their demonstration of the removal of extracellular material from pollen by several low polarity solvents; those of higher polarity, (including organic solvent-water mixes) are shown to be capable of the extraction of material from the cytoplasm as well.

Of the solvents, and solvent systems, tested and shown to penetrate the cytoplasm, acetone-water (98:2) is clearly the most disruptive in its effect on pollen. The evidence for disruption was not as definite following washing of pollen by the acetone water (50:50) mix, although the TLC results show that a wide spectrum of materials has been extracted, apparently from within the cytoplasm, and the ultrastructure is shown (Chapter 3) to be disturbed by this solvent. Methanol clearly disrupts the grain, the extracts being shown to contain the bulk of materials apparent in whole pollen homogenates which suggesting cytoplasmic disruption. The latter effect could be only inferred from the earlier microscopy studies (Chapter 3) owing
to the density of the (non-hydrated) tissue. At this juncture one should
also consider the reported (Martin and Telek, 1971) efficiency of methanol
(and methanol-HCl) in removing phenolics from the stigma surface. The
results presented in this chapter show that a number of substances
(including, for example ellagic, synapic and p-coumaric acids) have been
extracted from intact pollen only where more polar solvents have been used.
This suggests, in retrospect, that these were likely to have been intra­
cellular, as were probably those extracted by Martin and Telek under similar
conditions.

Hexane extracts of pollen could not be shown, on TLC, plates to
contain polar materials, bearing in mind that phospholipids are correctly
described as amphiphilic, rather than polar, (Johnson and Davenport, 1971).
These extracts do, however, contain neutral lipids including pigments
and traces of phenolic substances all of which are extracellular, rather
than cytoplasmic, as demonstrated by their absence in macerates obtained
from hexane prewashed pollen. Hexane treatment is shown in Chapter 3 to
retain the viability of the *Populus* pollen.

Diffusate buffer extracts of the pollen, by contrast, have fewer
neutral lipid components (largely pigments and traces of esters) and contain,
principally, more polar materials including proteins, the protein content of
such extracts being noted previously by Knox *et al* (1972a).

That disruption is caused by methanol and acetone-water mixes (98:2
and 50:50) is shown by the presence of phosphatidylcholine in extracts
obtained using these solvents, but not in either hexane or 'diffusate'
extracts. This phospholipid is known to be a prominent component of membranes
(Thompson, 1965). The results of this chapter and those of Chapter 3 are
consistent with Iwanami's (1972a,b), results which showed that both
methanol, and any of the acetone-water mixes containing more than 1.5%
water, rendered the pollen of *Lilium auratum* completely inviable. The
present results highlight the importance of the dryness of the low-polarity solvents for effective breeding treatments using the solvent technique (Chapter 2) and, in addition, emphasize the significance of the addition of water to solvent extraction media (for example, that of Bligh and Dyer, (1959)). The results further show that comparisons of the ether extracts of pollen cannot, and should not, be made unless one is certain of the purity of the ether used, and particularly of its dryness in each case. If the ether (or certain other low polarity solvents) used to treat pollen is pure, the extract should contain extracellular materials only, whereas if impure it may be effectively extracting material from the whole grain; this has been shown to be true of Populus, at least.

Even allowing for possible variation in the pollen of different genera in their permeability to solvents, the results obtained with Brassica pollen by Roggen (1974) remain an enigma, as already suggested in Chapter 3. Roggen (1974) washed Brassica pollen with a variety of solvents including chloroform, acetone and acetone-water mixes (10:90, 50:50, 90:10 and 99.8:0.2) and reported finding "no significant differences either in germination or in vital staining ... between untreated and washed pollen". These results are in direct contrast to those obtained by Iwanami (1972a) with Lilium, as well as with the present Populus results. A possible explanation of Roggen's viability results, based on the nature of his solvent treatment, is presented in Chapter 3 - failing this one must also allow for either a possible basic difference in reaction to solvents, between Lilium and Populus pollen on the one hand, and Brassica, on the other, due perhaps in part, to differences in exine structure.

Roggen held that the composition of the removable 'pollen coat' material varied with the solvent used, which proposal is in accord with the TLC results reported in this chapter. Thus his acetone-water (90:10 and 50:50) mixes extracted more sugar and protein than did either
water or chloroform alone, and chloroform extracted lipids (Sudan IV-positive) which water did not — yet even with chloroform, Roggen records the extraction of Lowry-positive material from *Brassica* pollen. By contrast, the present study is unable to demonstrate the removal of proteins from *Populus* pollen using low polarity solvents.

Obviously the material extracted from *Populus* pollen by low polarity solvents is more characteristic of 'pollenkitt' (*sensu* Echlin, 1971) in its composition, than of tryphine; that is, it consists largely of hydrophobic lipids and carotenoids, rather than the hydrophilic proteins or carbohydrates, observed for instance, by Roggen in *Brassica*. This brings into question whether material similar to that classed as tryphine can truly be said to exist in the wall of *Populus* pollen prior to its hydration, and there may even be wider implications, particularly where 'pollen-wall' proteins are concerned. This will be discussed further in Chapters 5 and 6.

The lipoidal materials extracted from the pollen wall by low polarity solvents could include those of both sporophytic (tapetally derived), and gametophytic (from the haploid grain itself), origin — a possibility already evidenced by Mepham and Lane (1969). The proportion of the total pollen dry weight extracted by hexane (approx 2%) is in agreement with that obtained by other authors using ether extraction that is between (1 and 20%), albeit towards the lower end of the scale. Apart from intrinsic differences between pollen species, the higher percentages of ether extract obtained by some other authors could also be due to their use of contaminated solvent, or even of damaged pollen. The appearance of hexane, and other low polarity solvent, extracts of *Populus* pollen agrees with that described for other pollens using ether; that is, they are yellow in colour and 'fatty' in nature (Stanley and Linskens, 1974). The *Populus* extracts have a simpler composition than those of the pollen of other genera reported, and reviewed by Stanley and Linskens. For example *Populus* pollen washings
contain no detectable steroids and are primarily composed of unsaturated long chain fatty acid esters. The fatty acid composition, with its predominance of palmitic, oleic, linoleic and linolenic, acids is typical of the ether extracts of most pollens studied so far (Stanley and Linskens 1974). The relative proportions of the various fatty acids within the pollen, as detected by GLC in this study, are remarkably constant in all Populus species studied. This is in contrast with those of the pollen wall, which are shown to vary from species to species. This supports Stanley and Linskens (1974) proposal, that "exine contained lipid material is probably the major source of variation in ether extractable materials."

The distribution (wall or cytoplasm) of lipids in Populus pollen appears to be close to that proposed by Scott and Strohl (1962) for pollens in general. The intine and exine lipids are shown to consist largely of long chain fatty acid esters (mainly unsaturated), with some evidence of alcohols but no sign of glycerides. The cytoplasmic lipids have a prominent phospholipid content, and possibly contain glycerides with palmitic, oleic and linoleic acids being the predominant fatty acids present.

Phospholipids are not prominent in the Populus pollen wall although phosphatidic acid is present. Populus pollen cytoplasm contains phosphatidylcholine, phosphatidylinositol and, to a lesser extent, lysolecithin, as do several other pollen species (see, for example Caron, 1972, Stanley and Linskens, 1974). It is worth noting that there are indications from the present TLC studies, of differences in cytoplasmic phospholipid content between pollen species, at least quantitatively, and possibly qualitatively, such a correlation between pollen genotype and phospholipid content having been noted in Oenothera pollen by Caron (1972).

The presence of carotenoids in the pollen wall of Populus, which is an anemophilous genus, contributes to the proposal by Stanley and Linskens (1974) that an earlier-suggested carotene-entomophilous correlation is an oversimplification.
Carotenoids are present, not only in the 'pollenkitt' of Populus, (probably deposited, in this and other genera, during later stages of development (Heslop-Harrison, 1968b)), but also possibly in a more stable form within the wall, as evidenced by its residual pale yellow colour after the organic-solvent-extractable material has been removed. These remaining carotenoids are probably incorporated into the sporopollenin. Evidence for this may be obtained from Southworth (1974) who showed that the sporopollenin of Ambrosia pollen is insoluble in a series of organic solvents, both polar and non polar, and further by Brooks and Shaw (1971) who showed that sporopollenin contained carotenoids in a wide range of species.

The presence of the (fat-soluble) vitamin D within the cytoplasm of Populus pollen (but apparently not in the wall) is of interest considering its possible potential as an indirect regulatory substance. At this point one should recall that Mitchell et al. (1970, 1971) obtained the fatty hormone, 'brassin' in diethyl ether extracts of pollen. In an earlier study by Mitchell and Whitehead (1941), similar growth stimulating substances were removed from Zea pollen using ether, but the pollen had been preheated at 80°C. Later studies (Mitchell et al., 1970, 1971) make no mention of such preheating. They did not determine if their pollens (Zea, Brassica, Alnus and Phaseolus) could withstand a temperature of 80°C and remain viable, although Iwanami (1973d) later reported that Camellia, Erythrina and Cyclamen pollens could survive oven temperatures of 80°C for 20 minutes without loss of either viability or vigour. Whether fatty hormones are wall-held or cytoplasmically located must remain an open question, yet this may now be readily solved by means of solvent treatments and bioassay.

Quercetin is apparently located within the pollen wall of Populus, its presence in the pollen confirming the earlier finding of this flavonol in Populus pollen by Wierman (1968). Caffeic acid is also wall-held. The cinnamnic acid derivatives p-coumaric, and sinapic acids, and the hydroly-
sable tannin component, ellagic acid, are each extracted by methanol or methanol-HCL washings which may indicate that they are located within the cytoplasm of Populus pollen.

Of the elements assayed by X-ray analysis of the pollen and stigma surfaces, potassium and calcium are present in the greatest proportions, and with the exception of the higher level of magnesium at the stigma surface, the order of relative concentration is the same in each case, corresponding to that of ashed Populus pollen as determined by Knight et al. (1972). That the pollen X-ray analyses do not simply reflect the total elemental composition of the pollen is indicated by the relative drop in potassium and calcium levels which follows hexane washing of the pollen. The high calcium level in the Populus pollen wall parallels Stanley's (1971) observation that calcium is present in greatest concentration in the wall of Pyrus pollen. It is proposed that a high calcium level at either, or both, surfaces may limit leakage from what appears, in the case of pollens at least, to be highly secretory membranes. Such a role for calcium was put forward by Dickinson (1968).

The demonstrated presence of protein in pollen (buffer) 'diffusates' and its absence in hexane washings, is an important result as it confirms the presence of proteins in the short term buffer diffusates (further investigated in Chapters 5 and 6), yet indicates that proteins are not wall-held prior to the moistening of the pollen of Populus. Whilst the result should be treated with caution, by itself, and cannot be assumed to apply to other pollen genera, it may well encourage reinterpretations of a number of earlier studies which have demonstrated the presence of protein in the wall as well as the cytoplasm of hydrated pollen grains and assumed it to be present in the wall prior to hydration. Such proteins have been considered to be derived from tapetal deposition in the case of materials apparent in the exine (after processing), whilst those *since it was concluded in Chapter 3 that hexane does not penetrate to the cytoplasm.
of the intine are regarded as having been deposited prior to pollen shedding (Heslop-Harrison et al., 1973).

No attempt was made, in this study, to determine the non-lipid composition of the stigma surface materials of *Populus* although, judging from the chromatographic and spectroscopic evidence, neutral lipids predominate. Mass spectroscopy results also determined that terpenoid esters are present. The neutral lipids are mainly composed of long chain fatty acid material, largely saturated, and including esters.

As has been mentioned, the exudates of 'wet' stigmata (Heslop-Harrison, 1975) have been shown to contain largely 8C to 18C fatty acids. This is in marked contrast to the stigma surface lipids of *Populus*, which consist largely of fatty acids of chain length greater than 18C and up to 29C, although 16C and 18C fatty acids are still present in significant proportions. Thus, in accord with the definition of Kates (1972), the stigma surface lipids of *Populus* are classed as waxes. By their appearance on the receptive surface of *Populus*, these surface lipids appear similar to those on the surface of *Brassica* papillae, which were described as 'wax' structures by Roggen (1972), and (to a lesser extent), to the stigmatic secretions on *Forsythia* papillae (Dumas 1974b). The 'bumps' on *Populus* appear to be more uniform in size, shape and distribution than those of the above genera (Chapter 1). Although Heslop-Harrison et al. (1975) claimed that they could find "no evidence of the 'waxy coating'" reported by Roggen (1972) on the *Brassica* receptive surface, both their presence (Chapter 1) and their 'waxy' composition, are clearly demonstrated in *Populus*. Apparently one of the reasons for the doubts Heslop-Harrison et al., 1975 expressed about the presence of the 'wax' coating was their observation that "lipase digestion does not disrupt the pellicle of the stigma papillae of *Brassica* or any other of the cruciferous genera tested". This observation need not discount the claimed waxy nature of these structures.
Evidence presented earlier in this thesis (Chapter 2) has implicated hexane-soluble pollen and stigma surface materials in the incompatibility reaction of *Populus*. It should be noted that both extracts contain components characteristic of 'pollenkitt' (carotenoids and lipids). This suggests that *Populus* 'pollenkitt' may be involved in the incompatibility mechanism although there has been no evidence from previous studies for the involvement of 'pollenkitt' in incompatibility responses (Heslop-Harrison *et al.*, 1973). Although the 'sticking reaction' (Roggen, 1972) occurs to some degree following compatible and incompatible pollination of *Populus*, it is held that the pollen and stigma surfaces may still participate in (in)compatibility reactions as suggested by Roggen (1972) and earlier authors (for instance Christ, 1959; Linskens and Kroh, 1967). Whitecross and Willing (1975) have already proposed that lipoids on the stigma surface may act as a barrier to incompatible pollen. In this light the fact that the present results show differences in the composition of lipids of both stigma and pollen surfaces may be related to the incompatibility mechanism. This is particularly evident in the case of the stigma surface lipids of *Populus*, where the proportions of longer chain fatty acids categorize the breeding groups, rather than the section or species alone, in the species of *Populus* examined. Any proposal for a role for such lipids in the incompatibility mechanism must remain speculative at this stage - this is discussed further in Chapter 7.

Although phenolic substances have been implicated in the incompatibility reaction by several authors (Kuhn and Low, 1949; Moewus, 1950; Linskens, 1967 and Martin, 1969) the proposal has not been supported in later experiments (Stanley and Linskens, 1974). When one considers the nature of the solvents shown to be most effective in removing 'stigma surface' phenolics notably the alcohols, and alcohol-mineral acid mixes (Martin, 1969), it seems likely that many of the phenolics reported to be present at the stigma surface could, in fact, be derived from the cytoplasm.
In this chapter the material located in the wall of *Populus* pollen prior to its hydration has been examined. Much of this material would have been sporophytically derived and would thus possibly be implicated in the sporophytic (in)compatibility control of breeding in the genus *Populus*.

The widely-employed technique of examining wall materials in post-hydration pollen has been shown to be potentially misleading, causing unnecessary complications if one is to determine the nature of sporophytically derived materials free, as far as is possible, of intracellular gametophytic compounds. Such pollen buffer diffusates clearly fail to reflect the prehydration composition of the pollen wall, both by their apparent inclusion of material from the cytoplasm, and by their omission of the less polar constituents of the wall - the major pollen wall components in *Populus*. Results presented here have shown that there is no evidence of pollen wall proteins in *Populus* prior to hydration and thus possibly no sporophytic proteins at this location. Sporophytic proteins, even if present in the wall, need play no part in the incompatibility mechanism at all; also although the incompatibility control of *Populus* is probably sporophytic, it need not be sporophytic proteins that are involved.

Certainly some of the diffusate components, including proteins, are involved in the incompatibility mechanism as shown by Knox *et al.* (1972a,b) and these shall be discussed in more detail in Chapters 5 and 6.

There need be no contradiction between the observed effect of diffusate protein on *Populus* incompatibility, the lack of either sporophytic or gametophytic protein in the prehydration-pollen wall, or of the involvement of non-proteinaceous pollen wall substances in the incompatibility mechanism. This is discussed further in Chapter 7.
Plate 4.I

Records of the oscilloscope displays showing the energy spectra resulting from energy dispersive X-ray analysis of pollen and stigma surfaces.

fig (a)  *P. monilifera* stigma surface in region of a 'wax structure'.

fig (b)  *P. bolleana* pollen surface.

fig (c)  *P. bolleana* pollen surface after hexane pre-washing.
Plate 4.II

Infrared (I.R.) spectra of hexane-removable pollen and stigma surface materials.

fig (a)  *P. alba* stigma surface extract.

fig (b)  *P. bolleana* pollen surface extract.

fig (c)  *P. nigra* var. *italica* pollen surface extract.
Plate 4.III

Nuclear magnetic resonance (N.M.R.) spectra of hexane-removable pollen and stigma surface materials.

fig (a)  *P. alba* stigma surface extract.

fig (b)  *P. bolleana* pollen surface extract.

fig (c)  *P. nigra* var. *italica* pollen surface extract.
Plate 4. IV

Thin layer chromatograms (T.L.C.) of *P. nigra* var. *italica* pollen extracts run on silica gel plates with several solvents, sprayed with fluorescent indicator (Rhodamine 6G) then examined under UV light. The UV visible regions are outlined on the plates.

The extracts were obtained in each case (from left to right) with:

1. isotonic buffer
2. buffer after maceration of whole pollen.
3. buffer after maceration of hexane pre-washed whole pollen.
4. hexane alone.
5. methanol alone.
6. acetone-water (50:50)
7. acetone-water (98:2)

**fig (a)** Mobile phase of petroleum ether-diethyl ether-acetic acid (50:50:1).

**fig (b)** Mobile phase of di-isobutyl ketone-acetic acid-water (50:50:1)

**fig (c)** Mobile phase benzene alone.
Plate 4.V

T.L.C. silica gel plates run with extracts (see plate 4.IV) of pollen of *P. nigra* var. *italica*.

**fig (a)** Mobile phase petroleum ether-diethyl ether-acetic acid (50:50:1). Stained for phospholipids.

**fig (b)** Mobile phase benzene. Stained for phospholipids.

**fig (c)** Mobile phase petroleum ether-diethyl ether-acetic acid (50:50:1). Stained with Dragendorf's (choline) spray reagent.
Plate 4.VI

T.L.C. silica gel plates run with extracts (see plate 4.IV) of pollen of *P. nigra* var. *italica*.

**fig (a)** Mobile phase benzene. Plate stained with iodine vapour.

**fig (b)** Mobile phase benzene. Plate stained for esters (darker stained region towards the front).

**fig (c)** Mobile phase benzene. Plate stained with phosphomolybdic acid spray reagent.

**fig (d)** Mobile phase petroleum ether-diethyl ether-acetic acid (50:50:1). Plate stained with ninhydrin spray reagent. Note the complete absence of a positive reaction by the fourth sample. (hexane removable pollen materials).
Plate 4.VII

Gas liquid chromatograms of extracts of homogenized whole
P. deltoides stigmata. These were run on Apiezon L. columns
as described in the text.

fig (a) Stigma homogenate extract alone.

fig (b) Stigma homogenate extract run with fatty acid standards
8C to 22C (10C and 22C are marked as reference points)
to produce additive peaks.

The gas-liquid chromatograms which follow (4.VIII to 4.XI) were
produced using extracts alone. The peak identifications were made,
however, on the basis of identical runs but with known standards included
(see text).
Plate 4.VIII

Gas-liquid chromatograms of extracts of whole pollen (hexane prewashed in each case).
Run under identical conditions on Apiezon L. columns.

fig (a)  P. bolleana (BH).

fig (b)  P. tremuloides (TH).

fig (c)  P. yunnanensis (YH).

fig (d)  P. nigra var. italica (NH)

The major peaks (16C and unsaturated 18C fatty acids) are marked in (a), being the same for each sample.
Plate 4.IX

Gas-liquid chromatograms of methyl esterified hexane washings (wall material) of pollen. Run on Apiezon L. columns.

The 16C (palmitic acid ester) peak is marked as a reference point in each case.

(a)  *P. bolleana* (section *Leuce*)

(b)  *P. yunnanensis* (section *Tacamahaca*)

(c)  *P. nigra* var. *italica* (section *Aigeiros*)
Plate 4.X

Gas-liquid chromatograms of methyl esterified hexane washings of leaf surfaces. Run on Apiezon L columns. The 16C (palmitic acid ester) peak is marked as a reference point in each case.

(a) \( P. \) bolleana (section Leuce).

(b) \( P. \) yunnanensis (section Tacamahaca).

(c) \( P. \) simonii (section Tacamahaca).

(d) \( P. \) nigra var. italic\( a \) (section Aigeiros).
Gas-liquid chromatograms of methyl esterified hexane washings of the stigma surface. The 16C (palmitic acid ester) peak is marked as a reference point. Peaks 'a' and 'b', referred to in the text, are marked in fig (a), the corresponding peaks also being present in each of the figures (b) to (e).

(a) \textit{P. alba} (section \textit{Leuce}, subsection \textit{Albidae}).

(b) \textit{P. tremuloides} (section \textit{Leuce}, subsection \textit{Trepidae}).

(c) \textit{P. sp} (of the section \textit{Tocamahaca}).

(d) \textit{P. monilifera} (section \textit{Aigeiros}).

(e) \textit{P. deltoides} (section \textit{Aigeiros}).
Plate 4.XII

Diagrammatic representation of the relative areas of peaks 'A' and 'B' for each of the chromatograms presented in plate 4.XI. The height of each column represents the proportion of the area of the peak it represents, to that of 'A' and 'B' combined, expressed as a percentage.
SECTION LEUCE

P. alba

\[
\begin{aligned}
\% & 75 \\
\% & 50 \\
\% & 25 \\
0 & 0 \\
A & \phantom{} \\
B & \phantom{}
\end{aligned}
\]

P. tremuloides

\[
\begin{aligned}
\% & 75 \\
\% & 50 \\
\% & 25 \\
0 & 0 \\
A & \phantom{} \\
B & \phantom{}
\end{aligned}
\]

SECTION TACAMAHACA

P. sp.

\[
\begin{aligned}
\% & 75 \\
\% & 50 \\
\% & 25 \\
0 & 0 \\
A & \phantom{} \\
B & \phantom{}
\end{aligned}
\]

Key

- Peak A
- Peak B

SECTION AIGEIROS

P. monilifera

\[
\begin{aligned}
\% & 75 \\
\% & 50 \\
\% & 25 \\
0 & 0 \\
A & \phantom{} \\
B & \phantom{}
\end{aligned}
\]

P. deltoides

\[
\begin{aligned}
\% & 75 \\
\% & 50 \\
\% & 25 \\
0 & 0 \\
A & \phantom{} \\
B & \phantom{}
\end{aligned}
\]
CHAPTER 5

POLLEN DIFFUSATES

INTRODUCTION

That the early events following pollen stigma contact are critical for the successful pollination of the flower is well established (for review see Linskens and Kroh, 1970), provided that: the stigma is of the same species as the pollen or closely related to it (Martin, 1969, Linskens, 1967); the stigma is in a receptive phase (Stanley, 1964); and the pollen is in a mature and viable condition. In many instances pollination must occur within a short time of shedding to be successful, as pollen longevity varies with the species, and is governed by a complex of factors including flowering time, temperature, relative humidity and light (Rangaswamy, 1963, and for review see Johri and Vasil, 1961).

The early events following pollen-stigma contact, or the 'dialogue' between pollen grain and stigma papilla (Heslop-Harrison et al., 1975), has been followed in Gramineae (Watanabe, 1955) and Cruciferae (Heslop-Harrison et al., 1975). The sequence is basically that of pollen hydration, followed by emissions from the pollen grain, apparently enabling the pollen grain to stick to the surface (Watanabe, 1955), after which the pollen germinates. It is relevant to note here that although Roggen (1972) reported an absence of the 'sticking' reaction in incompatible pollen-stigma combinations in Brassica and regarded the 'sticking' as having been bought about by 'recognition' processes, Heslop-Harrison et al. (1975) have made no reference to any such distinction in their discourse of events based on observations of members of the Cruciferae. That pollen grains release material into the environment following moistening has been widely recorded, for example: Green's observation of the liquification of starch paste by such exudates (1894); Brown and Bennotti's (1941) observations of the release
of particulate matter associated with fine mucilagenous threads from the grooves (colpi) of pollen; Watanabe's (1955) comments on the secretory role of pollen and Heslop-Harrison's (1971) report of the immediate dispersal of the 'pollenkitt' (Knoll, 1930) when the pollen is brought into contact with water. Heslop-Harrison proposed that the latter effect was due to the lipid of the 'pollenkitt' being associated with surface proteins with detergent properties.

One of the earliest attempts to determine the nature of the pollen diffusate (or exudate) was that of Green (1894) who demonstrated that diastase (amylase), present in the exudate, caused the liquefaction of starch paste.

Apart from observations of the proteinaceous nature of pollen exudates, which are noted below, and despite the many determinations of the composition of macerated whole pollen (for review see Lunden, 1954, Johri and Vasil, 1960), the non-proteinaceous components of pollen exudates have received little attention. Some exceptions include the observations that thiamin, biotin, pantothenic, and nicotinic acids are present in secretions from pollen (Kakhidze and Medvedeva, 1956), as well as flavonoid compounds (Stanley and Linskens, 1965), glycoproteins (Knox et al., 1975) and carbohydrates (Kirby and Smith, 1974). To these may be added the components of the 'pollenkitt', which lipophlic outer coating of the exine (Echlin, 1971) consists mainly of lipids and carotenoids (Heslop-Harrison, 1968a; Heslop-Harrison and Dickinson, 1969). As mentioned above, pollenkitt materials were shown to be dispersed upon moistening of the pollen (Heslop-Harrison, 1971). Knox (1973) notes that "it has long been known that sugars, pigments and lipidic materials are lost" from moistened pollen grains.

The 'population effect', consisting of an enhancement of pollen germination in artificial culture by a high density of pollen, is widely reported (Brink, 1924; Beck and Joly, 1941; Borriss and Krolop, 1955;
Visser, 1955; Iwanami, 1970). Brewbaker and Kwack (1964) postulated the existence of a pollen growth factor (PGF) in pollen exudates of some species, concluding that this was calcium. Cook and Walden (1965), however, could not support this proposal from their results obtained with Zea pollen. As shown in the previous chapter (4), the results of this study demonstrate the existence of calcium at the pollen surface of Populus. The observation remains that in some studies (Cappelletti and Tappi, 1948; Schwarzenbach, 1955) pollen exudates have been reported to have a stimulatory effect on the stigma, and secondarily, on pollen grains. Indirect evidence for this may be provided by the success obtained in pollen mix experiments using live (Michaj'lova, 1950; Polyakov, 1950), or killed (Stettler, 1968), pollen. The component of the exudate which causes the stimulatory effect is still uncertain, although clues to its identity could be found in observations that the pollen exudate increases the permeability of stigmatic papilla cells (L'Vova, 1950; Heslop-Harrison, 1971), the human nasal mucosa (King et al., 1967) and the vasculature of non-sensitized mice (Kind et al., 1964).

Antigens have been reported to occur in the pollen diffusates of a number of genera (Lewis, 1952; Ciffò and Iannetti, 1969; Knox, 1971; Knox and Heslop-Harrison, 1971b; Belin and Rowley, 1971), including Populus (Knox et al., 1972 a & b). The antigenic nature of components of the pollen diffusates will be discussed in more detail in the following chapter. (Chapter 6).

That pollen diffusates have allergenic activity in many cases, has been widely demonstrated (for example, reviews by Newell, 1941; Wodehouse and Coca, 1946; Bernton, 1949; King and Norman, 1962; King et al., 1964, 1967; Underwood and Goodfriend, 1969; Griffiths and Brunet, 1971; Belin, 1972), with particular emphasis placed on antigens E and K which were originally identified in Ambrosia pollen (King and Norman, 1962; King,
et al., 1964). Recently an antigen E-like protein has also been detected in *Cosmos* (Howlett et al., 1973).

The activity of hormones, including auxins (Laibach, 1932; Lund, 1956; Johri and Vasil, 1961), gibberellins (Barendse et al., 1970) and the fatty hormones 'brassins' (Mitchell et al., 1970, 1971), has been detected in pollen. The exact function of these hormones *in vivo* has not been determined although it is known, for instance, that pollination has the same effect as indole-acetic acid (IAA) in preventing abscission of the style in some plants (La Rue, 1936; Stanley, 1964) and it has been proposed by Stanley (1964) that "this extension of receptivity may explain the results of experiments now in vogue involving multiple and mixed pollination" (Stanley, 1964). Of particular interest to this study is La Rue's (1936) observation that the pollen diffusate of *Populus* has the same effect as auxin (-lanolin) pastes, in inhibiting abscission of debladed *Coleus* petioles.

Barendse et al. (1970) reported that the results of their studies of the gibberellin content of pollen showed that this hormone was utilized by the pollen during germination, rather than in tube elongation. The stimulatory effect of gibberellin on pollen germination had also been noted by Kaurov and Vakula (1961), whilst the failure of either auxins or gibberellin to promote pollen tube growth was reported by Vasil (1960).

That timing of release, and quantitative aspects of pollen diffusates might be critical to successful pollination has been proposed. Stanley and Search (1971), comment that: "pollens release protein and other chemical moieties to the solubilizing solutions of the environment at different rates and in different quantities, depending primarily upon the species, and, to a lesser extent, upon the extracting solution", further noting that "... the compatibility response of plants will presumably be
influenced by such differences." Kirby and Smith (1974), in a study of elutable substances from the pollen of 42 species, including the genus *Populus*, have made quantitative assays of the proportions of carbohydrate and protein present. Together these two groups of compounds comprised little more than 4% of the total eluate dry weight, with carbohydrate exceeding protein content. Kirby and Smith (1974) reported that binucleate and trinucleate groups of angiosperm pollen "differ quantitatively in the surface compounds that have been both gametophytically and sporophytically contributed".

Of the diffusate components studied, enzymes have received the most attention; particularly since the study by Stanley and Linskens (1965). One of the earliest reports of enzyme content in pollen diffusates (as opposed to that of macerated whole pollen), was that of Green (1894) who reported the activity of diastase outside the grain after its hydration. He further reported that the content of both diastase and invertase in extracts of macerated pollen increased with time after germination. Although, according to Dixon and Webb (1958), diastase came to be used as a general term for enzymes up to 1898, Green makes it clear in his paper that the diastase which he reports should be equated with amylase. Other evidence for the liberation of enzymes from moistened pollen grains has been presented in studies by: Lopriore (1928) and Euler *et al.* (1948), for the release of catalases; Elser and Ganzmöller (1931), of several enzymes; Ostapenko (1961) of oxidative enzymes and Poddubnaya-Arnoldi *et al.* (1961) of acid phosphatases, for example.

Brewbaker, in a review of the enzymes which have been found in pollen, notes that "comparatively few enzymes of plant tissues have been sought without success in pollen or pollen tubes" (Brewbaker, 1971; see also Paton, 1921, and Mäkinen and Macdonald, 1968). Tsinger and Petrovskaya-Baranova (1961) found dehydrogenases, cytochrome oxidase and acid
phosphatase in the walls of pollen grains using histochemical stains. Later Petrovskaya-Baranova and Tsinger (1962) reported that acid phosphatase was released into the external environment of germinating pollen. On the other hand, Gorska-Brylass (1965) reported that "I have never observed any penetration of acid phosphatase into the environment of the pollen tube", claiming that this confirmed Haeckel's (1951) opinion that acid phosphatase 'represents a 'closed', intracellular enzyme" (Gorska-Brylass, 1965). Stanley and Linskens (1964, 1965) showed that the release of sucrose metabolising enzymes occurred within minutes of moistening of Petunia pollen and this preceded germination.

Following the finding (Heinen and Linskens, 1961) that the stigmatic cuticle of several Cruciferae (of known surface incompatibility) could be degraded by an enzyme preparation from Penicillium spinulosum, Linskens and Heinen (1962) showed that diffusates from intact pollen of several species are capable of degrading isolated cutin, and thus have a 'cutinase' activity (Linskens and Heinen, 1962). The earlier study (Heinen and Linskens, 1961) had involved titrimetric determination of 'cutinase'-liberated fatty acids, whilst the second (Linskens and Heinen, 1962), in view of the possible contamination of the end product with fatty acids from burst pollen grains, was performed by measuring (titrimetrically) the degree of liberation of carbonyl groups. Brewbaker (1971) later inferred this 'cutinase' to be polygalacturonidase ('pectinase'), noting that addition of pectinase to growth media of Pyrus pollen stimulated pollen tube growth.

The presence of the enzyme, β-fructo-furanosidase external to the pollen cell membrane, was found by Dickinson (1967), who also reported the differential permeability of this membrane during germination. Mäkinen and Brewbaker (1967) followed the release of the isozymes of esterases, leucine amino-peptidases, acid phosphatases, catalases and amylases of Oenothera organensis pollen at different times after immersion in a
calcium-supplemented culture medium. Their results led them to agree with Ostapenko's (1961) suggestion that the enzymes pass from pollen to stigma by "exosmosis" possibly, they suggested, influenced by the metabolic products of the stigmatic tissues. They also observed the diffusion of enzymes to occur immediately upon moistening, their production not being dependent on germination of the grain, (in agreement with Green's (1894), original observation). Mäkinen and Brewbaker (1967) further proposed that the inactivation of the enzymes by stigma or stylar components could be an incompatibility reaction providing an effective barrier to pollen tube growth, following earlier proposals for a similar such mechanism (for example, Christ, 1959; Lewis, 1960).

Lewis et al. (1967) showed that 40% of the total protein in pollen of *Oenothera* (including both amylase and invertase), was lost within an hour of moistening; thus, as an earlier immunological study by Lewis (1952) had shown the incompatibility reaction in this genus to occur within 30 minutes, Lewis et al. (1967) proposed a "highly significant correlation between the immunological reactions, the enzyme activity, the speed of diffusion, and the incompatibility reactions".

Pandey (1967) found a correspondence between S-gene type and the peroxidase isozyme pattern in the pollen of *Nicotiana* species and argued that the production of certain peroxidase isozymes may be one of the last acts of the S-gene complex, these peroxidases being activated tetramers formed only in an incompatible combination - in accord with Lewis's (1965) 'dimer-hypothesis.'

Roggen (1967) made a close examination of enzyme activities in the style during the progamic phase in *Petunia* and demonstrated the presence of an 'activation wave' of enzyme activity in front of the growing pollen tubes in the style, with increased carbohydrate metabolism in the region of growth, possibly forming a chemotropic gradient.
Roggen (1967) held that glutamate dehydrogenase (GDH) was activated in the style, "under the influence of a high molecular (sic) substance presumably a protein or RNA-like substance of the pollen tubes". He further proposed that "proteins and RNA can alter the configuration of the enzyme molecule, thus affecting its catalytic activity (Nichol, 1964) as was demonstrated for GDH by Roberts (1966) and Holzer and Hierholzer (1963)."

Whether the increase in enzyme activity with germination is due to \textit{de novo} synthesis, or to an activation of pre-existing enzymes, has not been established. Brewbaker (1971) considered that, as many inhibitors of protein synthesis do not inhibit pollen germination, it would appear that germination can proceed without synthesis of new enzymes.

The enzymic content of pollen diffusates has been confirmed, as has their probable involvement in "pollen germination, stigma penetration and pollen tube growth" (Knox and Heslop-Harrison, 1971). Recently there appears to be a revival of interest in the possible involvement of enzymes in the compatibility mechanism, a proposal which has been put forward a number of times over the last half century (for example: East, 1929; Straub, 1947; Lewis, 1949; Lewis and Crowe, 1958; Christ, 1959; Linskens, 1960; Mäkinen and Lewis, 1962; Linskens and Heinen, 1962; Pandey, 1967). Kroes (1973) has proposed an 'enzyme theory' for self-incompatibility, which offers the possibility that each S allele determines the lack of a corresponding enzyme, thus pollens with different S alleles might be considered to differ not in the substance they produce, but rather the substance (enzyme) they lack. This theory has been neither promoted nor discounted since its presentation, and does indicate that we have by no means exhausted the number of possible explanations for existing results.

Bredemeijer (1974) and Bredemeijer and Blaas (1975) made a detailed investigation of the possible involvement of enzymes in incompatibility
reactions. The peroxidases of *Nicotiana* were studied (Bredemeijer, 1974) because of the reported correlation between S-genotype and peroxidase isozymes in *Nicotiana* (Pandey, 1967), and also because of the known involvement of peroxidases in the inhibition reaction of certain resistant plants to invading mycelial growth (Macko *et al.*, 1968; Seevers and Daly, 1970 and Hislop and Stahmann, 1971). This was, in part, because Hogenboom (1973) had drawn a parallel between the latter interactions and those of the pollen and stigma. Bredemeijer's (1974) results caused him to discount this parallel between fungi-host and pollen-stigma, as far as peroxidases were concerned, but he did notice an increase in enzyme activity after pollination, as had Roggen (1967), and further noted that the increase in enzyme activity following cross-pollination was slightly greater than that following self-pollination (the incompatible situation). Bredemeijer also found that an extra peroxidase isozyme was produced after crossing, one that was not produced after selfing. In further studies with the same system Bredemeijer and Blaas (1975) found increasing activity of certain peroxidase isozymes being transmitted, in a wave, from the stigma to the basal end of the style. Linskens (1964) had divided the incompatibility reaction into a 'recognition reaction' and a 'rejection reaction' and Bredemeijer and Blaas suggested that while the peroxidases that Pandey (1967) studied might be involved in the 'recognition reaction', the peroxidases that they detected in the style were more likely to be involved in a 'rejection reaction' in an incompatible situation.

The particular relevance of pollen diffusates to this study is established by the findings of Knox *et al.* (1972b) who showed that the diffusate of *Populus* pollen may act as a 'recognition substance' which when mixed with otherwise incompatible, but intact pollen, apparently enables it to behave as if compatible and to complete fertilization. Howlett *et al.* (1975) used similar diffusates to overcome the sporophytic self-incompatibility of *Cosmos*. 
That proteins may be detected within the pollen wall by histochemical means was first demonstrated by Tsinger and Petrovskaya-Baranova (1961). This has since been demonstrated by other workers using whole pollen (Sood, 1974) or isolated pollen walls (Tsinger and Petrovskaya-Baranova, 1961, and Hara et al., 1972); also by the study of pollen grains either immuno- or histochemically stained after freeze sectioning (Knox and Heslop-Harrison, 1969, 1970, 1971; Knox et al., 1970, 1972a) or after embedding in araldite/epon (Heslop-Harrison et al., 1973) Heslop-Harrison et al. (1973) proposed that recognition substances involved in sporophytic control would be found within the exine, whilst those involved in gametophytic control would be located within the intine, as might logically be supposed from the presumptive origin of material in each of these sites.

Howlett (1973) found protein in the exine of pollen of a species of *Cosmos* which exhibited sporophytic self-incompatibility control; she took this protein to be tapetally derived and commented: "the exine proteins may also control interspecific incompatibility since Pandey (1970) has suggested that this type of incompatibility is determined sporophytically in *Nicotiana* species." Howlett et al. (1975) believed that the exine-held proteins mediated in the early events of the (self-)incompatibility reaction of *Cosmos*. Heslop-Harrison et al. (1974) similarly believed the protein detectable in the exine to be involved in the incompatibility response of the sporophytically controlled self-incompatible crucifer, *Iberis*. Knox et al. (1972a & b) believed that their recognition substances are intine-held in *Populus* pollen and that they constitute a large part of the protein located within the intine (Knox et al., 1972a). Thus in recent studies emphasis has been placed on the role of proteins which are apparent in the pollen wall (either within the exine or the intine) as 'recognition substances'.

Although isozymes of acid phosphate and esterase have been found in
"pollen-wall proteins" (Knox and Heslop-Harrison, 1970, and Howlett et al., 1975), emphasis is placed on the non-enzymic proteins as mediators in the recognition response (Knox and Heslop-Harrison, 1971). The latter authors comment that "there is reason to believe that most of the intine held proteins are non-enzymic, but are concerned rather as recognition substances in inter- and intraspecific incompatibility reactions."

It should be recalled that the *Populus* pollen washings were shown, in Chapter 2 (and by Willing and Pryor pers. comm., 1975), to be involved, either directly or indirectly, in the *Populus* incompatibility mechanism. In Chapter 3 it was shown that these hexane washings could only have come from the pollen wall; they were later found (Chapter 4) to be free of protein. By contrast Knox et al. (1972a) extracted protein (described as wall-held) from *Populus* pollen which they implicated in the incompatibility mechanism. The experiments described in both this chapter and the next will attempt to determine, firstly, whether the composition of the protein of intact *Populus* pollen may be related to the incompatibility group to which the pollen belongs, and secondly, what effect hexane-prewashing of pollen has on subsequent protein release. By these means one may gain a better insight into both the nature of the proteins involved in the incompatibility mechanism, and the reason (or reasons) for the effectiveness of the hexane pre-treatments of pollen and thus, in turn, a better understanding of the *Populus* incompatibility mechanism.

This chapter concentrates, in particular, upon the enzymic activity of the proteinaceous diffusates - Chapter 6 will make a similar study of their antigenicity.
MATERIALS AND METHODS

The difficulty experienced in attempts to germinate *Populus* pollen *in vitro*, at any time beyond the first week or so of its anthesis, means that results of *in vitro* studies of stored pollen cannot accurately reflect the *in vivo* behaviour of pollen. For this reason *in vitro* diffusates were collected within the first 30 minutes of pollen hydration only, and not beyond, in order to minimize the inevitable misrepresentation of normal (*in vivo*) events.

I Quantitative assay of proteins from pollen diffusates

Quantities (1.5g) of dry, viable, stored pollen of *P. nigra* var. *italica* and *P. alba* var. *bolleana* (*P. bolleana*) were either used directly or pretreated as follows:

(a) by washing 3 x 10 minutes in redistilled hexane, then filtering using either a 'Millipore' micro-syringe filter holder and glass syringe, or a 'Millipore' microanalysis filter holder attached to a suction line. In each case Millipore 'Mitex' (5.0 μm pore size) teflon filters were used for their inertness to organic solvents. The pollen was then allowed to dry at room temperature before use.

(b) alternately pollen treated as in (a) was then 'false-coated' as described in Chapter 2.

Treatments (a) and (b) are the same as those used in Chapter 2, the hexane-rewashing being described in that chapter. Pollen of each of the 2 species of *Populus* used, whether intact or after treatments (a) or (b), was placed in 8 mls of mannitol-buffer solution composed of 0.025M tris-HCL, 0.025M CaCl₂ and 0.5M mannitol, adjusted to pH 7.9 and described as 'diffusate buffer' (Howlett, 1973). After 30 minutes in this buffer the pollen was separated from the slurry by filtration using the 'Millipore' filter equipment (see above) and cellulose ester filters (0.22μm pore size).
The pollen was then washed twice with 2 ml quantities of the same buffer with 0.25% polyvinylpyrrolidine (PVP) added, and the combined filtrate collected. The residues obtained from each sample were separately ground with a pestle and mortar with 3g of fine sand, washed with mannitol-PVP-buffer as before, and the filtrates collected. The residues were discarded. The filtrates obtained from either diffusates, or macerated pollen, were then placed in test tubes to which were added equal volumes of saturated (60% w/v) ammonium sulphate, then allowed to stand overnight at 4°C.

The following morning samples were centrifuged at 5000 Xg for 10 minutes, the supernatant was discarded, and the precipitate resuspended in 4 mls of 60% ammonium sulphate. A 0.3 ml aliquot of the suspension was placed in a microfuge tube then centrifuged at 6000 Xg for 10 minutes. The supernatant was removed with a syringe, and the pellet finally dissolved in 0.3 ml of a medium consisting of 50 mM HEPES-KOH buffer, 10 mM β mercaptoethanol, 0.25% polyvinyl pyrrolidine and 0.1 mM magnesium chloride at pH 7.5.

A 20 µl sample of this suspension was used for each assay.

(i) Total protein estimations were made by the Folin-Ciocalteau phenol reagent method of Lowry (1951).

(ii) The enzyme activities tested, and the reaction media used for each, are outlined in Appendix IV. The enzyme nomenclature used in this thesis is derived from the recommendations of the Commission on Biochemical Nomenclature on the Nomenclature and Classification of Enzymes (revised 1972).

II Gel electrophoresis

(i) Gels used

Both starch (13%) and acrylamide (7% and 10%) gels were used in this study. Their preparation is described in Appendix V.
(ii) Sample preparation and application

Fresh leaves, flowers and pollen of a number of poplars were macerated (as described below) and applied to gels. Stigmata were removed from several flowers in each inflorescence, sampled, combined and macerated. To enable intact and hexane washed flowers to be compared, some stigmata were brushed with hexane prior to their placement on gels.

Comparisons were also made between untreated pollen, solvent washed pollen and 'false coated' pollen, pretreated as described earlier.

Where the composition of materials diffused from pollen over different time intervals was studied, the (isotonic) buffer diffusate was collected as a filtrate at each of the specified times. Methods for the collection of buffer diffusates of pollen were described earlier. After each collection the pollen was briefly washed with buffer prior to its replacement with further fresh buffer, and sampling in the next stage of the process. This intermediate washing ensured that each sample was not contaminated by diffusate released from the pollen at an earlier stage.

Wherever possible, and particularly where samples were run together on a gel, it was contrived to make the sample concentrations of each comparable.

Fresh, solid plant material (leaves, stigmata, flowers or pollen) were placed in a pre-cooled mortar or crucible on ice with a small quantity of acid-washed sand and a few drops of dithiothreitol (D.T.T.) added. The sample was then ground with a pestle.

Dithiothreitol was also added to the diffusates prior to application to the gels. Wicks of 'Whatman 3MM' chromatography paper in 5mm squares were soaked in the prepared sample, then lightly blotted to remove excess moisture. The wicks were then inserted into an origin line cut across the starch gels at a distance of 5 cm from the cathodal end, or individually
into slots punched along the origin 4 cm from the cathodal end of acrylamide gels. About 30 samples could be run on each gel.

(iii) **Electrophoresis**

Electrophoresis was performed under similar conditions to those described by Brewbaker *et al.* (1968), after modification by Moran (1975). The tank buffer used for acrylamide gels was borate buffer pH 8.8, whilst buffer '126 A', pH 12.2, was used for starch gels. The electrophoresis tanks were maintained at 4°C for the duration of the run. Starch gels were run for approximately 5 hours, at 300 V and 60 mA, by which time the front was 9 cm from the origin. Acrylamide gels were run for about 4 hours at 250 V, the front having moved about 8 cm over this period.

On completion of the run, gels were removed from the electrophoresis tanks and each cut horizontally into two slices (the bottom slice producing the marginally better result) and stained as soon as possible - in most cases within half to 1 hour after their removal.

(iv) **Enzyme staining of gels after electrophoresis**

Generally acrylamide gels were used for esterases and peroxidases, starch gels being more effective for amino peptidases, dehydrogenases, phosphatases and transaminases. Staining of aminopeptidases, peroxidases and transaminases was effective within 1 hour at room temperature, whilst dehydrogenases and esterases stained within 1 hour at an incubation temperature of 37°C. The optimum staining condition for acid phosphatase was overnight incubation at 37°C. Scoring of gels (and photographic recording) was done promptly, as any delay allowed the stain to diffuse rendering the bands indistinct.

The preparation of the reaction media used in this study is described in Appendix VI.

Ingredients were stored at the refrigerator temperatures recommended
by the manufacturers, and each stain was mixed freshly before use. As
before, the nomenclature is in accord with the recommendations of the
Commission on Biochemical Nomenclature (revised 1972).

Stains were prepared and used for the detection of the following
enzymes.

1.1.1.1 Alcohol dehydrogenase (ADH)
1.1.1.37 Malate dehydrogenase (MDH)
1.4.1.2 Glutamate dehydrogenase (GDH)
1.11.1.7 Peroxidase
2.6.1.1 Aspartate aminotransferase
(syn. glutaric oxaloacetic transaminase (GOT))
3.1.1.6 Esterase
3.1.3.2 Acid phosphatase
3.4.11.1 Aminopeptidase-cytosol (syn. leucine aminopeptidase (LAP))

RESULTS

I Quantitative assay of diffusates

(i) Total Protein

The measures of the total protein content, determined in 30
minute diffusates, are presented (fig. 5.1) for both P. bolleana (B) and
P. nigra var. italic (N)*. For each species, total amounts of protein
solvent released, following either pre-washing (BH and NH)* or 'false
coating' (BHN and NHB)* pre-treatments, are compared with those of each of
the intact pollens (B and N). Total protein is expressed as mg of protein
(Lowry-positive) per gram of pollen (fresh weight).

* For convenience these abbreviations will be used in this, and the
following chapter.
P. nigra var. italica (N) is shown to release a slightly greater quantity of total protein per gram than P. bolleana (B). Solvent pretreatments of both species (BH and NH) resulted in the release of more total protein than was produced from normal untreated pollen.

Pollen with false coatings (BHN and NHB), on the other hand, show decreased release of total protein over the same period, the quantity being less than that from either the respective solvent-washed or the intact pollen samples.

The quantity of total protein released from the intact pollens is in reasonable agreement with the levels determined under the same conditions by other means (see Chapter 6).

One may also compute the theoretical maximum protein content in hexane washings of pollen from the results of the elemental analysis (Chapter 4), which reported no detectable nitrogen with an accuracy of ± 0.3%. As pollen washings comprise approximately 2% of the fresh weight of pollen (Chapter 4), then adopting a factor of 6.25 (Geigy Scientific Tables, 1962) to convert nitrogen to protein, the maximum possible protein content of the hexane washings would be less than 0.4 mg protein per gram of fresh pollen.

(ii) Enzyme activity

The results of the quantitative enzyme assays are presented graphically (Figs. 5.I, II, III) for the pollen species P. nigra var. italica and P. bolleana. Pollen type and pretreatment will be referred to using the same notation as in the Total Protein section (above).

The activity of each enzyme is reported in micromoles of substrate converted per hour per milligram of diffusate protein.

Of the enzymes assayed, acid phosphatase is released in greatest
quantity. Leucine aminopeptidase was present with the lowest activity of those detectable, whilst in none of the samples could any breakdown of α-napthol palmitate be detected. Sulphatase activity was relatively low except in solvent washed pollen of *P. nigra* var. *italica*.

It is apparent that in only some cases (aminotransferase (GOT), leucine aminopeptidase lipase and myristate-esterase) do the pattern of enzyme activities in different pollen treatments reflect the pattern of total protein released (Plates 5.I, II). Among the esterases, the effects of solvent washing and 'false coating' on enzyme activity vary both with the substrate used and the pollen species. Thus, for example, solvent washing of *N* or *B* pollen depresses the stearate-esterase activity of resulting diffusates yet enhances their ability to degrade most other esterase substrates. 'False coating' of these pollens in both cases (*BHN* and *NHB*) not only restores, but also apparently enhances, the stearate-esterase activity in subsequent diffusates.

That solvent treatment may have a different effect on different pollen species is well demonstrated by the studies of laurate-esterase activity; this is enhanced by the 'false coating' of *P. bolleana* pollen (*BHN*) while the reverse effect is produced with *P. nigra* var. *italica* pollen (*NHB*).

Similarly each of the solvent treatments (*BH, NH, BHN, NHB*) has an opposite effect on the sulphatase activity of diffusates from each of the two pollen species.

II Acrylamide and starch gel electrophoresis

Results are presented in diagrammatic form, as 'zymograms', each isozyme being referred to in terms of its retardation factor (Rf), being the distance it has moved relative to the front.
It is found that macerated pollen of *Populus* may contain isozymes of glutamate, malate and alcohol dehydrogenases, peroxidase, aspartate aminotransferase, esterase, and phosphatase and leucine aminopeptidase (Plate 5.IV, V, VI, VII). The same isozymes, with the exception of the dehydrogenases, were detected in buffer diffusates from intact pollen. Aminotransferase, however, was only represented by the presence of a (scarcely visible) single isozyme in diffusates of intact pollen, aminotransferase isozymes being more prominent in diffusates from hexane pre-washed pollen.

Isozymes detected in extracts of macerated stigmata of *Populus* include those of peroxidase, esterase, aminotransferase, acid phosphatase and leucine aminopeptidase. Glutamate dehydrogenase is present in stigmata of the section *Leuce*, but could not be detected in corresponding extracts from either of the sections *Aigeiros* or *Tacamahaca*; malate dehydrogenase was found in *P. tremuloides* stigma extracts only (Plate 5.IV).

In no case could enzymes be detected in extracts derived from hexane washings of any of the *Populus* pollens examined. This contrasts with the appearance of at least one esterase isozyme in similar extracts of hexane washings of the stigma surface of *P. deltoides*. The fact that the extraction procedure used for hexane washings of both stigma and pollen was the same in each case, and that esterase was detected in the stigma extracts suggests that esterase activity would have been evident in pollen hexane washings, had this enzyme been present.

Although the isozyme pattern of pollen is often species specific, some isozymes characterize the sections to which each of the assayed pollens belongs. It is further apparent that some isozymes may characterize the breeding group; this is evident, for instance in the case of pollen esterases and acid phosphatases (Plates 5.VI and 5.V respectively).

Stigmatic isozymes may similarly characterize the breeding groups
in some cases. A glutamate dehydrogenase isozyme is (Rf .27) is, as mentioned, prominent in extracts of stigmata of *P. alba*, *P. tremuloides* and *P. hickeliana*, all of the section *Leuce*, yet is not found in those of either of the sections *Aigeiros* or *Tacamahaca* (comprising another breeding group).

Breeding-group specific differences are also apparent in the stigmatic isozyme patterns of the enzymes: acid phosphatase (Rf .43, .65; Plate 5.V) leucine aminopeptidase (Rf .57, .67; Plate 5.VII) and peroxidase (Rf .54, .74; Plate 5.IV).

On the other hand, other isozymes are common to tissue from all three sections; for example the stigma enzymes: peroxidase (Rf .60 and .66; Plate 5.IV) esterases (Rf .70; Plate 5.VI) and acid phosphatase (Rf .55; Plate 5.V), and the pollen enzymes malate and glutamate dehydrogenase (all isozymes; Plate 5.IV), peroxidase (Rf .13; Plate 5.IV), aminotransferase (Rf .30; Plate 5.V) and leucine aminopeptidase (Rf .49, .51, .62; Plate 5.VII).

Where the isozyme pattern of stigma extracts were compared with those of extracts of the whole female flower, the latter were found to have a broader spectrum of isozymes.

Hexane prewashing of pollen had no effect on the isozyme content of macerated whole pollen either qualitatively or, apparently, quantitatively. The isozyme content of the pollen diffusates was affected, however, by hexane prewashing. This was evidenced, firstly, by the greater concentration of many isozymes in hexane-prewashed-pollen diffusates compared with those in diffusates of untreated pollens and secondly, by the greater number of isozymes in diffusates of pollen previously hexane washed (Plate 5.VI). The first effect is also evident from the earlier quantitative enzyme results and may itself explain why, in most cases, extra isozymes are visible in diffusates of pollen previously hexane washed, if one considers that their concentration is raised to the level where they are rendered visible by staining.
The time course of release of diffusate isozymes of \( \alpha \) and \( \beta \) esterases, acid phosphatase and aminotransferase was followed over the first 30 minutes following pollen hydration to determine whether there were any changes, either qualitative or quantitative, over this period. In each case there was no evidence of any qualitative change, as the isozyme pattern established within 4 minutes of hydration was maintained to the end of the 30 minutes (figs. 5.VIII(a), (b)); this was true of pollen of both \( P. \) bolleana and \( P. \) nigra var. italica whether intact, hexane pre-washed, or 'false-coated' (BHN or NHB). Quantitative differences were apparent over the same period of time, however. While intact pollen released its isozymes at a fairly constant rate over the half hour period, the release from hexane pre-washed pollen was relatively rapid over the first 10 minutes, apparently at a faster rate than from intact pollen; thereafter the rate of release is lower than that from intact pollen. 'False coated' pollen showed a slightly higher isozyme release slightly higher than that of intact pollen initially, after which the rate of release differed little from that of intact pollen over the corresponding period. Both \( \alpha \) and \( \beta \) esterase-stained gels showed these effects, as did those stained for acid phosphatase or aminotransferase, although the level of staining of diffusates was poor in the latter case. The esterase results are representative of each of the other enzyme results and are presented (figs. 5.VIII (a),(b)).

Of particular interest was the effect that hexane prewashing had on the esterase isozyme pattern of diffusates released by \( P. \) nigra var. italica pollen. This, to me, indicates de novo production of an isozyme of low mobility within 4 minutes of hydration of both hexane prewashed and 'false coated' (NHB) pollens as this isozyme is apparent neither in any of the pollens macerated immediately upon moistening, nor in diffusates of intact \( P. \) nigra var. italica pollen (fig. 5.VIII(a)). This new isozyme may correspond to a prominent isozyme apparent in \( P. \) bolleana extracts (fig. 5.VIII(b)).

* A second possibility, that this isozyme is normally membrane-held, is less likely as this isozyme is not present in extracts (which include membranes) of macerated whole pollen.
A similar result was obtained in a gel stained for leucine aminopeptidase (AP) in which the LAP content of 30-minute buffer diffusates of *P. bollea*na (B) and *P. nigra* var. *italica* (N) were compared, both with each other, and with the corresponding diffusates from 'false coated' pollens of the same species (BHN and NHB).

An isozyme is present in BHN diffusate which is not detectable in B alone. This isozyme may correspond with one of similar (though slightly greater) mobility in both N and NHB 30 minute diffusates run on the same gel.

**DISCUSSION**

The results show that pollen of *Populus* behaves in the same manner as pollen of other genera, releasing enzymes into its environment shortly after hydration (Stanley and Linskens, 1964). The enzymes classes detectable in pollens of *Populus* have each been found in pollen of other genera using electrophoretic, histochemical and biochemical methods. In particular, lipase activity is demonstrated in 2 species of *Populus* pollen. This enzyme was reported to be present in pollen of several species by Kammann (1912) and later, by Paton (1921), though lipase has not been found in pollen of any species since then (Görska-Brylass, 1965; Macdonald, 1969).

*Populus* pollen and stigma enzymes have not been described previously, to my knowledge.

Alcohol dehydrogenase (ADH) has been previously found in pollen (Okunuki, 1940; Poddubnaya-Arnoldi *et al.*., 1961). Mäkinen and Macdonald (1968) doubted its presence in pollen, as reported by Poddubnaya-Arnoldi, *et al.*, partly on the basis that Dickinson (1968) had reported that "lily pollen lacks ADH capable of oxidizing pentaerythritol since the compound remains unchanged within the cells." The finding of ADH in *Populus* pollen confirms the earlier proposals for its presence in some pollens, at least.
Malate dehydrogenase has been found in other genera besides *Populus* (Thunberg, 1925; Linskens, 1966; Veidenberg and Safonov, 1968).

Both *Populus*, and *Petunia* (Roggen, 1967), pollens each have a single isozyme of glutamate dehydrogenase (GDH), whereas *Lilium* has two (Veidenberg and Safonov, 1968). The GDH isozyme of *Populus* is common to all the pollen species as well as the stigmata of the section *Leuce*, unlike the situation in *Petunia* where stigma and pollen GDH isozymes differ (Roggen, 1967). A further difference between the two genera is the pre-existence of GDH in *Populus* pollen before its germination, this enzyme being produced *de novo* in *Petunia* pollen during tube growth according to Roggen (1967). Roggen did, however, comment that: "extra increases of all enzyme activation after pollination can be ascribed to *de novo* synthesis of pollen enzymes." He held that the *Petunia* pollen tube GDH is activated in the style by a protein-protein interaction.

As GDH and aminotransferase enzymes are jointly involved in the transamination of glutamate to aspartate (Roggen, 1967), it is perhaps not surprising that these enzymes are present together in the stigmata of *Leuce*, yet neither is present in stigmata of either of the sections *Aigeiros* or *Tacamahaca*. Although Roggen linked GDH composition to S-genotype in *Petunia* there is no basis, at this stage, for such a correlation in *Populus*.

The low level of peroxidase staining of *Populus* pollen, and in particular, of the pollen relative to that of the stigma and leaves, may accord with Brewbaker's (1971) general comment that the activity of this enzyme is "low relative to other plant tissues and isozymic polymorphism is similarly low compared to that of vegetative tissues". Thus although King (1960) has shown that the viability of some pollens may be determined by peroxidase assay, viability determinations involving esterase activities (Heslop-Harrison and Heslop-Harrison, 1970) might be better suited to *Populus*. 
As observations in the present study allow for a correlation between peroxidase isozymes of the *Populus* stigma (but not the pollen), and the breeding group to which each belongs, this allows for the involvement of this enzyme in the incompatibility mechanism of *Populus*. Although this proposal must remain speculative at this stage, Bredemeijer and Blaas (1975) have already found evidence for the involvement of peroxidase isozymes of *Petunia* in the rejection of incompatible pollen tubes.

Although GDH and aminotransferase isozymes may be used to distinguish stigmata of the section *Leuce* from those of *Aigeiros* and *Tacamahaca*, *Populus* pollen aminotransferases differ from one species to the next, with no indication of such a correlation. Brewbaker (1971) noted that aminotransferases had been studied in comparatively few species. He reported a personal communication by Macdonald and Brewbaker, whose results with *Zea* pollen led them to propose that aminotransferases had a dimeric structure. It is interesting to record that Thimann (1972) reported that transamination of tryptophan was required for the formation of indole pyruvic acid which is, in turn, transformed by oxidative decarboxylation to indoleacetic acid, a growth hormone.

The presence of acid phosphatase in *Populus* pollen was to be expected, as this enzyme has been found in practically all the pollens in which it has been sought and, as in this study, in relatively high levels (Stanley and Linskens, 1974). The acid phosphatase activity of *Populus* pollen diffusates was markedly higher than those of any of the other enzymes presently studied (by a factor of about 50).

Acid phosphatase has not been involved in any model for the incompatibility process in the past, and apart from the determined correlation between pollen and stigma acid phosphatase isozymes, and the breeding groups from which they are derived, there is no evidence for their involvement in the incompatibility mechanism in *Populus*. The feasibility of their involve-
ment can, however, be argued. It has been demonstrated that buffer
diffusates of two genera of pollen, namely the 'wall protein' of *Cosmos*
(Howlett *et al.*, 1975), and the 'recognition substances' of *Populus* (Knox
*et al.*, 1972b), are involved in a 'recognition' process leading to pollen
acceptance or rejection. In each study the method of collection of the
active substance was basically the same. Howlett *et al.* (1975) observed
that their *Cosmos* extracts were able to retain their ability to stimulate
selfing after heating at 60°C for 10 minutes, implying that these
'recognition substances' should be grouped among the non-enzymic proteins
of the diffusate - an inference which should be treated with caution. Both
acid phosphatase and esterase enzymes might be expected to survive such
heat treatment, as already demonstrated by Lewis *et al.* (1967) who showed
that there was no inhibition of acid phosphatase activity after this enzyme
was maintained at 80°C for 24 hours, and that it took 4 hours at 60°C for
esterase activity to be completely destroyed. As Dixon and Webb (1958)
comment, there is a "tendency for smaller molecules to be comparatively
heat-stable." It is clearly not improbable that the active components of
these buffer-diffusate 'recognition substances' (Knox *et al.*, 1972b;
Howlett *et al.*, 1975) may well be (or include) enzymes.

The finding that leucine aminopeptidase (LAP) isozymes are present
in *Populus* pollen (and stigmata) is consistent with Mäkinen and Macdonald's
(1968) report that LAP isozymes were present in pollens of most of the
species that they had studied. The Rf values of the LAP isozymes recorded
by Mäkinen and Brewbaker are remarkably similar to those found in *Populus*
pollen using similar techniques (those of Brewbaker *et al.*, 1968). The
present study has shown that LAP isozyme patterns may characterize extracts
of different pollen or stigmata of *Populus*. Similarly Mäkinen and
Macdonald (1968) found that the isozymes of the same enzyme could be used
to discriminate the pollen of various *Zea* hybrids, yet the same authors
found that LAP isozymes could not be used to distinguish between
different incompatibility clones.

Aryl sulphatase, found by quantitative assay in *Populus* pollen diffusates, has also been located, by histochemical means, in the pollen of both *Campanula* and *Hyacinthus* by Górska-Brylass (1965). There is no indication of this enzyme having been sought and not located in other pollens. Its presence, albeit in small quantities, in *Populus* pollen may be related in some way to the (low) polyphenol content of pollen (Chapter 4).

Of the enzymes examined, the esterases have received the greatest attention, being found in *Populus* pollen macerates and diffusates as well as macerates of the anthers, stigmata, ovaries and leaves. Esterase activity has been found to be high in the pollen of most of the species studied (Brewbaker, 1971), although exceptions are known, including the reported absence of esterase in pollen of some species of *Pandanus*, *Petunia* and *Solandra* (Mäkinen and Macdonald, 1968). As generally observed in pollen of other genera, *Populus* pollen esterases exhibit a high degree of polymorphism, the isozyme pattern being distinctive for each of the *Populus* pollen species studied. Some *Populus* isozymes have been shown to characterize the section to which the pollen belongs. That the pollen esterases of *Populus* have different substrate specificities is evident from the quantitative esterase results, as also from electrophoretic studies using different substrates, including α and β napthyl esters of several fatty acids. Similarly Macdonald (1969), in a study of the esterases of *Zea* pollen found that these could be subdivided into acetyl-acetic esterase, carboxylesterase (B esterase) and arylesterase (A esterase) groups; Stanley and Linskens (1974) commented that most studies would have reported these activities as one hydrolase.

Although acrylamide and starch gel electrophoresis has been widely used to demonstrate the presence of isozymic differences between the different tissues of plants, between species and their hybrids, and tissues
at different ages or stages of development, isozyme studies of enzyme involvement in the incompatibility mechanism of plants have been relatively few. Those noted include the reported correlation of S-genes and certain peroxidase isozymes of *Nicotiana* (Pandey, 1967), and glutamate dehydrogenase isozymes of *Petunia* (Roggen, 1967). Bredemeijer (1974) and Bredemeijer and Blaas (1975) presented preliminary evidence which, they suggested, implicated one of the peroxidase isozymes in the stigmatic rejection of incompatible pollen tubes of *Nicotiana*. By contrast, Desborough and Peloquin (1968) could find no association between the self-incompatibility reaction and isozymes of esterase, peroxidase or dehydrogenase of the germinating pollen tubes of *Lilium*.

Similarities in isozyme pattern of pollens belonging to the same *Populus* breeding group, evident in the case of several of the enzymes, may suggest their involvement in the mechanism ensuring the breeding isolation of these groups. The earlier report of the ability of pollen to degrade, or simply soften, isolated stigma surface wax (Chapter 1) indicates some action on this material by one or more of the enzymes released in the normal diffusates of pollen. Such a softening process may be involved in the 'sticking reaction' already described by Roggen (1967) and regarded by him as an event exclusively associated with compatible pollen-stigma contact in *Brassica*. Considering the lipidic nature of the *Populus* stigma surface materials (Chapter 4), and possibly those of *Brassica*, it is reasonable to propose that the diffusate esterases may be involved in the softening process which was demonstrated in *Populus*. One should recall that this study has shown (Chapter 4) that stigma surface lipid composition may be related to the breeding group to which the stigma belongs. Taken together the stigma surface composition results, and the isozyme results (particularly esterases), may suggest that the enzymes released by the pollen belonging to a particular *Populus* breeding group are appropriate for the breakdown of the stigma surface material of the same group only, and are
incapable, under normal conditions, of degrading the surface lipids from
the stigmata of the groups with which the pollen is incompatible. This
speculation will be discussed further in Chapter 7.

There is no evidence, from the results of this chapter, of de novo
synthesis of isozymes by intact *Populus* pollen within the first 30 minutes
of its moistening. As mentioned earlier, the pattern of enzyme release
from *Populus* pollen was not followed beyond the first 30 minutes, in order
to avoid gathering potentially misleading results. In this respect one
should note that although Makinen and Brewbaker (1967) followed the time
course of isozyme release over a 19 hour period their pollen had apparently
not germinated over this period. Their study did, however, show that an
isozyme of esterase (their band G) was produced de novo by *Oenothera* pollen
5 minutes after its initial hydration - de novo in the sense that this band
was not detectable in the pollen initially. Although the present study could
not demonstrate de novo synthesis of esterase, acid phosphatase
or aminotransferase isozymes in intact *Populus* pollen diffusates over the
first 30 minutes, such synthesis was evident in solvent treated pollen.

Within 4 minutes of hydration of hexane prewashed pollen
of *P. nigra* var. *italica*, buffer diffusates could be shown to contain a new
isozyme not present in either, the corresponding diffusates of the intact
pollen, or in extracts of pollen of the same species macerated just after
hydration. The same isozyme appeared, also de novo, within 4 minutes of
hydration of the false coated (NHB) pollen. This 'de novo' α esterase
isozyme produced by *P. nigra* var. *italica* following each of the solvent
treatments, may correspond to the strongly staining, low Rf band of
*P. bolleana* pollen macerates and diffusates. The reciprocal effect, that
of the enhancement of typically *P. nigra* var. *italica* isozymes appearing
in *P. bolleana* diffusates after each of the solvent treatments, is not
apparent in gels stained for α esterase isozymes; there were, however,
indications of the reciprocal effect in gels stained for $\beta$ esterase, but again, not as marked as in the example presented above. It is recognized firstly that gel electrophoresis is not really appropriate for critical quantitative comparisons and secondly, that variations in quantity of the total sample, rather than relative differences with the sample, may confuse the picture.

Despite these apparent limitations, the approach should be continued in detail at some future stage, for there are indications that the hexane-removable pollen wall materials (which are known to be free of protein - Chapter 4) are capable of influencing both the quantity and the composition of the diffusates released from pollen, particularly the enzymes within these diffusates. These results, taken together with those of Chapter 4, show that not only is there a rapid, and possibly less discriminate release of proteins from the pollen during its early 'leaky membrane' stage (Stanley and Linskens, 1965) immediately post hydration, but also that within minutes of achieving osmotic stability the pollen may selectively regulate its subsequent enzyme release. The mechanism for this, latter, regulation is not clear, although a feedback control could be feasible.

The foregoing proposal, that pollen wall materials may affect the behaviour of the grain itself, is not new. Following studies of *Populus* itself, for example, Knox *et al.* (1972a, b) proposed that pollen wall proteins acted as 'recognition substances' indirectly determining the behaviour of the pollen after their interaction with materials at the stigma surface. The proposition arising from the present results, however, is that *Populus* pollen wall materials may directly affect the production of materials (including enzymes) by the same pollen upon its hydration. Allowing for a sporophytic origin of at least some of the pollen-wall components, the conclusion could be that these materials may be exerting the apparent sporophytic control of incompatibility in *Populus* (Knox *et al.*, 1972a; Hamilton and Langridge, 1975).
The results of the quantitative protein assays confirm that one of the effects of solvent prewashing of *Populus* pollen is to increase (rather than decrease) the total amount of protein which diffuses from the pollen within 30 minutes of its hydration. The corollary to this observation is that these wall-held, hexane-soluble materials of pollen normally retard the release of diffusate protein from within the pollen of *Populus*. It has been inferred (Knox *et al.*, 1972a), or suggested (Howlett *et al.*, 1973), that isotonic buffer diffusates obtained from pollen within 2 hours of their immersion contain only extracytoplasmic ('pollen wall') proteins. If true, then solvent prewashing of this pollen would logically be expected to diminish the rate of protein release. That the reverse occurs may suggest that the *Populus* pollen, at least, contains little or no wall protein, as evidenced earlier in Chapter 4, where hexane washings were shown to be free of protein or even nitrogen. As mentioned earlier, histochemical evidence for a wall location of proteins in pollen may be misleading, considering the evident hydrated nature of the pollen at the time of examination.

Heslop-Harrison *et al.* (1973) reported a potential complication arising in the freeze sectioning process where the antifreeze DMSO was incorporated in the gelatine embedding medium, as in several studies of 'pollen-wall' proteins up to that time. They comment that: "this compound has been found to increase the permeability of the pollen membranes and to permit the rapid loss of some cytoplasmic proteins". This, in addition to the apparently normal 'leakiness' of the pollen (plasmalemma) at the time of its initial hydration, could help to explain the appearance of protein in the pollen wall as observed in microscopy studies, including those using the freeze-sectioning technique. In particular, one could expect the protein (including enzymes), to move freely into the intine but to be restricted in their passage through the exine (except where the pollen has been previously solvent washed). The net result would, feasibly, be an accumulation of protein within the intine matrix. Prior to hydration of
*Populus* pollen the free, or mobile, proteins would be accumulated under the plasmalemma (as evident in fig. 2 of the paper by Knox *et al.* (1972a)) and steadily lost from this site to the wall, and then to the immediate environment. This proposal is supported by the combined results of Chapters 3 and 4 in which it is shown that hexane-soluble materials from the pollen wall (Chapter 3) do not contain protein (Chapter 4). One must still consider the possibility, however, that freely diffusable proteins are present in the pollen wall but that hexane does not remove them. This counter proposal was discussed earlier (Chapter 4) and held to be unlikely, considering the pronounced extra permeability of the pollen wall following such washing (as shown in this chapter), and also that the proteins, if present, would most likely be removed along with the hexane-removable matrix anyway.

The quantitative enzyme results, which showed the release of most enzymes to be enhanced following hexane prewashing of the pollen, were to be expected. It is significant, however, that this was not universally the case, the same treatment causing the depression of release of some enzymes furthering the earlier proposal, based on the electrophoresis results, that the pollen wall may have some feedback effect on enzyme production. This effect obviously varies with the enzyme studied and the pollen species concerned. These experiments must be regarded as preliminary, considering both the limited number of pollen species, and enzymes studied - clearly though, they raise some very interesting possibilities.

*Although Knox has shown that some pollens, freeze sectioned during anther development, reveal the presence of antigens and enzymes in wall sites, yet this cannot be assumed to indicate that these will be retained at these sites when the pollen matures.*
Quantitative assays of the protein and enzyme content of the pollen diffusate during the 30 minutes of their immersion in isotonic buffer.

Diffusates were obtained from *P. bolleana* (B) and *P. nigra* var. *italica* (N) pollens, including those hexane pre-washed (BH and NH) and 'false-coated' (BHN and NHB).

Total protein is expressed as mg. of protein (Lowry-positive) per gram (fresh weight) of pollen.

Enzyme activity is reported in terms of micromoles of substrate converted per hour per milligram of diffusate protein.
Quantitative assays of the enzyme content of pollen diffusates of *P. bolleana* and *P. nigra* var. *italica*.

Activity is expressed (along the ordinate axes) in terms of µ mole of substrate converted per hour per mg. of protein.

Esterase activity is measured using different substrates.
Quantitative assays of the enzyme content of pollen diffusates of *P. bolleana* and *P. nigra* var. *italica*.

Activity is expressed (along the ordinate axes) in terms of µmole of substrate converted per hour per mg. of protein.
Esterase
Acetate substrate

Sulphatase

Esterase
Oleate substrate

Lipase
Plate 5.IV

Zymograms of electrophoresis gels stained for:

(a) alcohol dehydrogenase (top left)

(b) glutamate (G.D.H.) and malate (M.D.H.) dehydrogenases (top right).

The G.D.H. isozyme has an Rf of 0.27 in each case.

(c) peroxidase (bottom).

The Rf scales are marked along the ordinate axes.

Top left: A.D.H. isozymes of whole pollen.
Top right: G.D.H. and M.D.H. isozymes of whole pollen (B,T,D,N) and whole stigmata (A,T,H).
Bottom: Peroxidase isozymes of whole pollen (B,T,D,N) and whole stigmata (A,T,H,D,P.sp(T)).

The abbreviations used for Populus species in this plate, and plates 5.V, VI, VII are as follows:

A  P. alba (Leuce)
B  P. bolleana (Leuce)
D  P. deltoides (Aigeiros)
H  P. hickeliana (Leuce)
N  P. nigra var. italica (Aigeiros)
P.sp(T) a species of Tacamahaca
T  P. tremuloides (Leuce)
Y  P. yunnanensis (Tacamahaca)

Where the abbreviation includes the suffix, H, this indicates that the pollen sample has been hexane prewashed.
ADH
GDH and MDH

Peroxidases

B
N
B
T
D
N
A
T
H

Pollen
Pollen
Stigmata

B
T
D
N
A
T
H
D
P.sp(T)
Plate 5.V

Zymograms of electrophoresis gels stained for:

(a) aminotransferase (top)

and

(b) acid phosphatase (bottom)

Rf values are marked along the ordinate axes.

Top: Whole pollen isozymes (B,T,D,N).
    Whole stigma isozymes (A,T,H,D,P.sp.(T))
    Whole leaf isozymes (N,A)

Bottom: Whole pollen isozymes (B,T,D,N)
    Whole stigma (st) and whole female flower (w.f.)
    isozymes (A,T,H,D,P.sp(T)).
Plate 5.VI

Zymograms of electrophoresis gels stained for esterases. Rf values are marked along the ordinate axes.

Top: Using α and β napthyl acetate substrate and samples of pollen diffusates collected after 30 minutes. Diffusates from intact, and hexane prewashed (-H) pollens are compared side by side.

Bottom: Whole pollen isozymes (A,T,H,D,P.sp) and whole leaf isozymes (A,N) in each case stained using α napthyl acetate substrate.
ESTERASE (diffusate)

$\alpha + \beta$

<table>
<thead>
<tr>
<th>Pollen</th>
<th>B</th>
<th>BH</th>
<th>T</th>
<th>TH</th>
<th>D</th>
<th>N</th>
<th>NH</th>
<th>Ta</th>
<th>TaH</th>
<th>Y</th>
<th>YH</th>
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<tr>
<td>Rf</td>
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<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

ESTERASE (homogenate)

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<thead>
<tr>
<th>Pollen</th>
<th>A</th>
<th>T</th>
<th>H</th>
<th>D</th>
<th>Psp</th>
<th>A</th>
<th>N</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rf</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Plate 5.VII

Top: Photograph of 10% acrylamide gel on which 30
minute diffusates of intact (B and N), and
'false coated' (BHN and NHB), pollens were run.
Gel stained to reveal leucine amino peptidase (L.A.P.)
isozymes.

Bottom: Zymograms of electrophoretic gel stained for
leucine amino peptidase. Rf values are marked along
the ordinate axis.

Left: L.A.P. isozymes of whole pollen (B,T,D,N).

Right: L.A.P. isozymes of whole stigmata (A,T,H,D,P.sp(T)).
Photographs of 8% acrylamide gels stained for esterases using α-napthyl acetate as substrate. The samples in each case were pollen diffusates collected at 4, 10, 20 and 30 minutes after their initial immersion in isotonic buffer. These are compared with whole pollen macerate (MAC) extracts taken from pollen immediately on its hydration, and 30 minutes later (30 MAC).

Top: (a) *P. nigra* var. *italica*

Bottom: *P. bolleana.*

Samples from intact (B,N), hexane prewashed (BH, NH) and 'false-coated'pollens. Following each treatment (from left to right) samples treated as follows:

1. pollen macerated on moistening, and diffusates from pollen after
2. 4
3. 10
4. 20
5. 30 minutes in isotonic buffer
and
6. pollen macerated after 30 minutes in isotonic buffer.
CHAPTER 6

IMMUNOGENIC PROPERTIES OF POLLEN AND POLLEN DIFFUSATES

INTRODUCTION

The serological examination of plant tissues

Serology is widely accepted as being of benefit to plant taxonomic studies (for example Jensen, 1973; Hawkes and Tucker, 1968). Cowan (1968) notes that "serology is really a physical demonstration of chemical similarity or difference". A serological approach will thus complement the more specific determinations of protein by electrophoresis and chromatography, yet is limited by its dependence on the ability of the protein (or otherwise) to act as an antigen. Further, as Cowan (1968) notes, serological reactions "are not specific, an antiserum will react not only with the homologous antigen(s) but also with partial antigens (haptens) that have some of the same, or very similar, determinant groups".

Erdtman (1967) noted that some very complex compounds may be synthesized by different pathways and should not be regarded as homologous in different organisms unless these pathways are the same. Even this, as Cain (1967) stressed, would not pinpoint their degree of homology, as in the case of several known compounds (for example, haemoglobin), the derivation of a compound "by the same enzyme pathways may therefore have occurred independently many times" (Cain, 1967).

The first serological-type approach to the study of plants was that of Picado (1921), who showed that fruits of Opuntia produced an agglutinin in response to injections of either water or sucrose suspensions of the pollen of Zea.
The injected pollen was interpreted as having stimulated antibody production in the juices of the developing fruit. Picado's work has apparently not been repeated, and the production of plant protein antibodies has not been demonstrated since to my knowledge.

The immunological emphasis in incompatibility studies of plants was effectively initiated by Jost (1907) who referred to the similarity between the self sterility response in plants and an immunological reaction. This observation by Jost, commented Bhatta charjya and Linskens (1955): "combined with a great number of experiments carried out by him and his school, promoted East (1929, 1934) to propose his classical 'immunity theory'". Basically this theory (East, 1929) assumes that the pollen tube secretions contain materials antigenic in an incompatible situation, and thus, in an incompatible style, stimulate the production of antibodies against them which, in turn, inhibit the growth of the pollen tube. Straub's (1947) consumption theory ('Verbrauchstheorie') supposes that an immunity reaction takes place in the self-incompatible situation and prevents the digestion of stylar conducting tissue by a substance from the pollen tube. He proposed that the immunity reaction inactivated the specific pollen tube substance required for this digestion.

Keszytus (1950, 1957) used serological techniques for the comparison of pollen extracts of various cultivars of members of the Rosaceae. He showed that pollen antigens may specify the taxonomic groupings, there being an immune reaction developed between pollen antigens and the antisera of related species and not with pollen of other genera in the same family. Cultivars within species showed no differences in their reacting properties (Keszytus, 1950).
Serology and the incompatibility mechanism

Most immunological studies of the compatibility phenomenon have focussed on self-incompatible plants, so that the following brief review of existing immunological studies is made with the understanding that these results need not relate directly to the present study on the incompatibility mechanism in *Populus*.

Lewis (1952), using serological techniques with pollen of *Oenothera*, investigated the possibility of "a substance in the pollen which reacts with a complementary stylar substance having the same specificity", this reaction blocking pollen tube growth. His antigen was prepared from macerated pollen (previously extracted with ether to remove lipoids), and the extracts used to challenge rabbits. Reactivity of the antiserum to various antigens was assessed by the 'ring test' in microtubes with different titres of antiserum. Lewis reported that "antisera which had been fully absorbed with pollen extracts heterologous in respect of S alleles gave strong reactions only with S alleles in common with the antisera" (Lewis, 1952), and proposed that specific antigenic substances are produced in *Oenothera* pollen by the incompatibility alleles. He cautions that "it is always possible however, that the antigenic substances are not the end products but are enzymes controlling intermediate stages in the synthesis of incompatibility substances", maintaining that the great specificity of the incompatibility reactions does "almost demand a molecule of the complexity of a protein or polysaccharide" (Lewis 1952). He was unable to locate complementary plant 'antibodies' in the style. Linskens (1960), using similar techniques, produced antiserum to extracts of stylar conducting tissue and pollen of *Petunia* and showed that in precipitin tests of 'sexual' combinations, all homologous extracts showed strong reactions, heterologous combinations producing little, or no, reaction. Linskens interpreted these results as being in agreement with
the hypothesis that S alleles produce specific antigens in pollen and style. Linskens proposed that these antigens might be instrumental in the inhibition of pollen tubes in incompatible pollen-style combinations, through the intermediary of 'Abwehr-Korper' (defence or ward bodies). These were held to be specific proteins detectable, after an incompatible cross of Petunia, as band 'Z', whose mobility was determined by paper electrophoresis and found to differ from the corresponding proteins (bands X and Y) produced following a compatible cross (Linskens 1958, 1959).

Linskens (1960) also suggested that the 'Abwehr-Korper' were enzyme-anti-enzyme compounds involved in the incompatibility reaction. Using the more sensitive double-diffusion technique of Ouchterlony (1949) in studies of Oenothera, Mäkinen and Lewis (1962) found that the precipitin lines formed between the protein extracts from pollen of various S genotypes and the corresponding antisera were characteristic for each of the S alleles. That these 'S-proteins' readily diffused from the pollen grain was indicated by the results showing that the precipitin lines were of equal intensity, whether the extracts were obtained from pollen macerates or diffusates. Mäkinen and Lewis found that the pollen of the 'S₆' genotype of Oenothera and that of its self compatible mutant form, 'S₆''', had identical S-proteins as evidenced by their serological identity. They saw some support, in these results, for Lewis' (1960) proposal that the S-gene complex consisted of two cistrons, one determining the specificity of its incompatibility and serological reaction and the other determining its protein activity. Mäkinen and Lewis also held that their results supported Linskens (1960) enzyme-anti-enzyme hypothesis by interpreting the 'S₆''' mutant as having the appropriate enzyme (S protein) but not its activator. Staining and heat denaturation tests demonstrated that the S substance was proteinaceous but did not preclude the possibility that it might include a small polysaccharide component. Without strong
evidence they claimed that "S-protein is a substantial part of the total protein" obtainable from macerated pollen (Mäkinen and Lewis, 1962).

Hagman (1963) studied the self-incompatible Betula, finding serological differences between pollen of the two species that he examined, which "seemed to agree fairly well with the incompatibility and compatibility of different crosses made" (Hagman, 1964). He also found that some of these serologically detectable substances were not proteins, but he suggested they could have been polysaccharides (Hagman, 1964).

Linskens (1965) considered the evidence, firstly, that tube growth was inhibited at higher temperatures than normal (Lewis, 1952; Straub, 1958 and Hagman, 1962) and secondly, that respiration of pollen was increased in an incompatible situation (Linskens, 1955). He believed that these results agreed with the general finding that antigen-antibody reactions have high energy consumptions (Haurowitz, 1950), and that taken together, the combined results provided support for an antigen-antibody hypothesis for the mechanism of self-incompatibility.

Lewis et al. (1967) determined the antigenic activity of the diffusates of individual pollen grains of Oenothera by placing them on a thin layer of agar which contained antiserum developed against extracts of pollens of several S-genotypes, including the self compatible mutant, S6'S6'. Their results were comparable with the earlier findings of Mäkinen and Lewis (1962). They also studied the enzyme release from the same pollens and their combined results led them to conclude: "it is therefore probable that there is a highly significant correlation between the immunological reactions, the enzyme activity, the speed of diffusion, and the incompatibility reactions. Whether the S protein is one of the enzymes detected or whether it is an enzyme is not known from these results" (Lewis et al., 1967).
Nasrallah and Wallace (1967b) tested the antigenicity of crushed anthers and stigmata from inbred homozygous genotypes of *Brassica oleracea*, a plant with a sporophytic self-incompatibility system. They reported the strong antigenicity of all the anther homogenates, no genotype having less than 7 precipitin bands, yet conceded their failure to demonstrate consistent and unequivocal differences between genotypes. By contrast, the reactions of pistil homogenates to anti-pistil sera yielded fewer bands than the corresponding anther tests, there being discernable differences between those from different incompatibility genotypes. As antigenic material was also shown to be capable of diffusing from the intact stigma of *Brassica*, they proposed that these antigens may play a role in the (self)incompatibility response, particularly since these authors could show a correlation between the appearance of particular antigens with the appearance of incompatibility in a developmental sequence.

Nasrallah and Wallace (1967a) attributed the differences between the stigma diffusate antigens, of several genotypes of the self incompatible *Brassica*, to their S-genotype. These results parallel those of Lewis (1952) and Linskens (1960), who found S-gene-determined antigens in pollen of *Oenothera* and *Petunia*, both plants having a gametophytically determined self incompatibility. Heslop-Harrison (1975) commented that the observed rapid loss of S-antigens from the stigma in *Brassica oleracea*, "strongly suggests that the S-gene related fractions are on or very near the surfaces of the stigma papillae; there is an obvious possibility that these are species of proteins present in the pellicle", as was also apparent from his own studies of the same plant (Heslop-Harrison *et al.*, 1975). While Nasrallah and Wallace (1967b) could not determine the nature of the antigens of stigma homogenates, they observed that their incubation at 60°C for 20 minutes destroyed antigenicity, and that the antigens could be precipitated by ammonium sulphate, and would migrate in an electric field. They later, (1969), showed that these S-gene-determined antigens could probably be
equated with a unique protein band observable in polyacrylamide gels. Heslop-Harrison (1975) regarded the presence of S-genotype proteins in the stigma, but not the pollen, extracts as an enigmatic result considering the widely recorded involvement of pollen proteins in the self-incompatibility system. He suggested that the immunological method might not be capable of detecting differences among proteins of *Brassica* pollen, possibly due to masking. Nasrallah and Wallace (1967a) themselves commented that "most hypotheses on incompatibility assume identity of pollen and stigma antigens. This cannot be reconciled with our data unless it is assumed that the pollen antigens of cabbage, unlike *Oenothera* and *Petunia*, are associated with membranes and cannot be extracted by aqueous methods".

Following earlier demonstrations of the presence of enzymes in the walls of pollen grains after hydration (for example Tsinger and Petrovskaya-Baranova 1961; Knox and Heslop-Harrison 1969, 1970; Knox et al. (1970) showed that antigens, too, could be demonstrated in the walls of *Gladiolus* and *Ambrosia* pollens after freeze sectioning, followed by localization of antigens using either direct or indirect methods. They comment that: "in emphasizing the wall sites as the source of the rapidly leached antigens, we do not suggest that saline extraction does not release proteins and other materials from within the vegetative cell of the pollen grain, but merely that these may not contribute very greatly to the total antigenicity of extracts" (Knox et al., 1970).

The antigenicity of pollen diffusates has also been demonstrated for pollen of *Ambrosia* (Knox and Heslop-Harrison, 1971a), *Phalaris* (Knox and Heslop-Harrison, 1971a), *Iris* (Knox, 1971), *Populus* (Knox et al., 1972a), *Cosmos* (Howlett et al., 1973) and *Phleum* (Nielsen et al., 1974).
The nature of pollen antigens

Knox and Heslop-Harrison (1971b) used immunofluorescence techniques to demonstrate that antigen-E, an active human allergen, was one of the antigens apparent in the pollen wall of *Ambroëia*.

Nielsen *et al.* (1974), in a detailed study of the antigens of *Phleum* pollen extract demonstrated the presence of 28 different antigens, some of whose antigenic precipitin lines were rather blurred and diffuse, "an observation which is usually characteristic of glycoproteins". All but five of their antigens had molecular weights between $10^4$ and $10^6$, and some appeared to be allergens. These antigens survived lyophilization and their allergenic activity increased as the temperature was increased above $4^\circ C$, with the appearance of some new precipitin lines and loss of others. Two antigens were shown to retain their activity after being subjected to temperatures beyond $82^\circ C$. Nielsen *et al.* found that some antigenic and allergenic activity appeared within a few minutes in simple pollen diffusates and most of the antigens were evident in diffusates by the end of the first 30 minutes. Fifty percent of the allergenic activity of the homogenates of pollen was obtained within 8 hours of the initial moistening of the pollen.

Whilst it is widely recognized that pollen antigens may include enzymes and/or allergens, the proportions of each in the pollen diffusate has not been determined. Mäkinen and Lewis (1962) asserted that their tests showed that S-protein is a substantial part of the total protein released from macerated whole pollen, yet did not produce any really quantitative evidence for this.

Lewis *et al.* (1967) recognized the potential correlation between S-gene antigens and enzymes, commenting that: "in view of the extremely strong esterase activity in the diffusate of the pollen it was thought
that a large amount of the protein diffusing from the pollen would be esterase and therefore could be the most important antigen". They attempted to determine if antiserum would inactivate the enzyme effect on starch, but found the results to be inconclusive, owing to the strong esterase activity in the antiserum itself.

Knox (1971) referred to his earlier unpublished results (Knox and Jacobsen), which apparently showed that the activity of several enzymes (not named but apparently hydrolytic) was low, relative to the total protein content. Knox then proposed that although hydrolytic enzymes may be among the total antigens of diffusates, "it is possible that they do not contribute greatly to the total antigenic activity of leachates".

As further evidence for the proposed low enzyme content of pollen diffusates, Knox cited an earlier study (Knox et al., 1970) which showed that although Gladiolus and Ambrosia pollens had several enzymes in common, the antisera to each exhibited only a trivial level of cross reaction. Knox et al. (1970) had allowed that this could have been attributable to "immunological differences in proteins possessing the same enzymic properties in the 2 species", but held that it was "more readily explained on the assumption that the bulk of the antigenic material has other functions".

Knox (1971) and Heslop-Harrison (1971) suggested that much of the rapidly released 'diffusate' was concerned with compatibility reactions. They later (Knox and Heslop-Harrison, 1971a) referred to the diffusate components with this function as 'recognition substances' involved in inter-, and/or intra-, specific incompatibility systems, and held that although "enzymes from the wall sites may be among the antigens ... most may be recognition substances". Knox and Heslop-Harrison (1971a), discussing the results of Lewis et al. (1967) commented: "The fact that
these workers are able to detect S gene segregation in diffusion tests using antisera against total pollen extracts, can only mean that the S protein forms a major part of the total, otherwise the segregation would be lost against the background of reactions involving non-specific proteins - including, of course, the enzymes. Clearly Knox and Heslop-Harrison (1971a) did not equate enzymes with 'recognition substances', the possibility allowed earlier by Lewis et al. (1967). Following their demonstration of a recognition role for lyophilized 1% saline extracts of *Populus* pollen diffusates, Knox et al. (1972a) asserted that they had "used conditions of extraction which precluded the loss of high-molecular weight compounds from the pollen protoplasts, suggesting that the active substances were leached from the wall". In the same study, the authors demonstrated that these diffusates included antigenic material, and reported the results of immunodiffusion tests which had found partial immunological identity between diffusates of pollen of *P. deltoides* and *P. alba*, that is, representatives from separate breeding groups. They were able to resolve at least 5 antigens in *Populus* pollen diffusates. Using the immunofluorescence technique in microscopy studies, Knox et al. (1972a) showed that these antigens were present in the intine region of freeze-sectioned (hydrated) pollen, yet there was also evidence of their presence within the pollen cytoplasm (their fig.2). They reported that the same antigens were rapidly released from *Populus* pollen, spreading across the surface of the adjacent stigma surface in both 'compatible' and 'incompatible' situations. They further reported no evidence of any reaction between stigma tissues and pollen antisera on gel diffusion plates.

The experiments outlined in this chapter are designed to determine the feasibility of the involvement of pollen diffusate antigens in the incompatibility process of *Populus*. The antigenic content of extracts of diffusates and macerates of pollen of different species (with and without
prior solvent treatment) will be compared. Preliminary trials will be conducted to determine which, if any, of the antigens of *Populus* pollen are enzymic.

**MATERIALS AND METHODS**

(a) **Preparation of antigens**

(i) **Diffusate antigen.**

Viable pollen of *P. bolleana* and *P. nigra* var. *italica* was used in 1.5 g quantities for the preparation of diffusates. Two such samples, one from each species, were then hexane washed as described earlier (Chapter 2), then allowed to dry completely, at room temperature. The hexane prewashed (BH and NH*) and intact pollen samples of each species (B and N*) were then each placed in an isotonic buffer solution for 30 minutes at 4°C. The buffer used was the same as that used for the enzyme studies in Chapter 5, and consisted of 0.025 M tris-HCl buffer to which was added 0.001 M CaCl₂ and 0.5 M mannitol after which the pH was adjusted to 7.9 (after Howlett 1973). The tone\*city of this buffer proved satisfactory for *Populus* pollen, no bursting of the grains having been observed. The method of preparation of pollen diffusates basically followed that of Howlett (1973) yet differed from the 1% saline extraction medium of Knox *et al.* (1972a); furthermore, in this study, the time of immersion of pollen in the extraction medium was shorter than that used in each of the abovementioned studies.

The suspension obtained after 45 minutes was filtered through Whatman No. 1 filter paper, brought to 90% saturation with ammonium

*The abbreviations used are those established in Chapter 2, notably B and N for 'intact' pollens, BH and NH for hexane prewashed pollens and BHN and NHB for 'false coated' pollens. These will also be used throughout the present chapter.*
sulphate, stirred for 1 hour at 4°C then centrifuged at 10,000 r.p.m. for 40 minutes at 4°C in a Sorvall centrifuge with an 'SS34' rotor. The precipitate was then redissolved in 1 ml. of 0.025 M tris-HCl buffer at pH 7.9. A glass chromatography column was packed with Sephadex G25 gel previously swollen for 3 hours in the same tris-HCl buffer in a boiling water bath. The effective dimensions of the column were 50 x 1 cm. The column was thoroughly washed through with precooled (4°C) buffer prior to use, and also similarly washed between each of the runs thereafter. For each run (with B, N, BH and NH) the sample, of approximately 1 ml, was carefully applied to the top of the column. On its elution from the column the voided material was collected with an LKB 'Minirac' 17,000 fraction collector hooked up to a continuously monitoring LKB 'Uvicord' 4,700 recorder set at 280 nm for the recording of the protein content of the eluate. In each case the chart recorded two earlier, lesser, peaks and a final major peak, as measured by their absorbance at 280 nm. The automatic setting on the fraction collector was occasionally overridden to ensure that material from each of these three '280-peaks' was not mixed. Ten test tubes of eluate were collected for each run. Samples of each of the tubes, originally derived from the intact pollens (B and N), were then examined by spectrophotometry, over both the UV and visible range, using a Varian Techtron 635 spectrophotometer. Samples from tubes contributing to the first two 280-peaks of each diffusate eluate were seen to be reasonably distinct spectrophotometry of samples comprising the 'third peak' showed that the 280 reading, in this case, was a slight shoulder on a larger peak. For this reason all the tubes contributing to the first two '280-peaks' of the eluate were pooled while the others (yellow in colour), representing the third peak, were used as a separate antigen in the case of the intact pollen diffusates only. Thus 6 antigens in all were derived from diffusates, namely the early '280-peaks' of each of B, N, BH and NH, and the third
'280-peak' of B and N diffusates. The latter, due to their markedly yellow colour, were denoted BY and NY. Each of these antigen solutions was concentrated to a volume of 2 ml using 'Amicon' macrosolute condensers.

The final protein concentration in these solutions was assessed by measuring their optical density at 280 nm and using a conversion factor of 1.3% absorbance $280$ (approximately) to 1 mg protein per ml of solution. A 0.8 ml aliquot of this solution was then thoroughly mixed with 0.8 ml of Freunds complete adjuvant using 2 ml glass syringes to gradually blend the two, forming an emulsion of the consistency of whipped cream. Freunds complete adjuvant contains mycobacteria and excites the reticuloendothelial system including, in the response, proliferation of macrophages in the animal which it challenges (Clausen, 1972).

(ii) Whole pollen antigen

In a second experiment, antigens were prepared from whole pollen extracts rather than diffusates. Quantities (0.5 g) of hexane prewashed $P. bolleana$ and $P. nigra$ var. $italica$ pollens and intact $P. bolleana$, $P. nigra$ var. $italica$, $P. yumanensis$ and $P. tremuloides$ pollens were individually mixed with 1 ml of 0.025 M tris-HCl, at pH 7.9 and each then immediately macerated in a glass Potter-Elverheim homogenizer. The homogenate was filtered using Whatman No. 1 filter paper and the filtrate further filtered using 'Millipore' microanalysis filter holders fitted with 0.2 µ 'GSWP' cellulose acetate filter discs, and attached to a suction line. Subsequent treatment of the above extracts followed the procedure described earlier for diffusates.

(iii) Antigens used in double diffusion and immunoelectrophoretic trials

In addition to the antigens described above, and used for the preparation of antisera (some being retained for subsequent testing against antisera), a variety of other antigens were prepared. These included the following:
(a) pollen diffusates of species other than those used above. These were prepared in the same way as in (i) but not purified beyond their filtration through Millipore GSWP 0.2 μ cellulose acetate filters. The extra diffusates used included those derived from both intact, and hexane prewashed, pollens.

(b) homogenized whole pollens used 'raw' (without filtration).

(c) homogenized flowers either whole or in part, that is, stigma or ovary, used as in (b).

(d) homogenized leaves of Populus, Salix and those of unrelated families.

(e) solvent washings of pollen of Populus pollens, redissolved in 0.025 M tris-HCl buffer after the solvent had been completely removed by evaporation.

The degree of interaction between the lectin, conconavalin A and both pollen antigens and antisera, was tested on double diffusion plates, using a con A concentration of 1.5 mg per ml of water.

(b) **Immunization Procedure**

Laboratory rabbits were used because of their low cost and ease of handling, and because they have little tendency to form multiple immunoprecipitates (Clausen, 1972). The rabbits were initially challenged with the Freunds-antigen emulsion, half being injected into the webs of each of the hind paws, the remainder, into the muscle behind the shoulder blades (the Trapezius). Booster injections were given, with the same antigen preparation and into the same sites, at 30 and 65 days following initial challenge. The first response was detected (where produced) at between 35 days (whole pollen homogenate) and 75 days (pollen diffusate).
The procedure was modified for the second experiment (whole pollen homogenate) owing to the discomfort suffered by the animals after injections into the webs of the feet, and despite the greater efficiency of injections at this site (Clausen, 1972). Thus, in later experiments, and following the initial challenge, boosters were administered intramuscularly in the thighs and shoulders, and subcutaneously in the back.

(c) Collection of antiserum

The rabbits were restrained in a box specially designed for this purpose, and blood drawn from an incision in the marginal vein of the ear, previously sterilized with 70% ethanol, the accumulation of blood having been stimulated by either heating the ear with the warmth from a desk lamp, or with xylol. Quantities of blood up to 15 ml could safely be taken at each bleeding, rabbits in these experiments being bled at 2 week intervals with a booster injection administered between each sampling.

Blood was allowed to clot in test tubes at room temperature for about an hour, placed in a cold room overnight, then centrifuged to facilitate the removal of the serum. The serum collected was divided into 1 ml quantities, sealed in vials and stored frozen. Serum, once thawed, was used immediately or discarded.

(d) Immunodiffusion

Immunological analyses were made using the double diffusion-in-gel techniques described by Ouchterlony (1968) and Clausen (1972) and known as the 'Ouchterlony technique'. Gels were prepared with the aid of an LKB immuno diffusion kit, the gel itself consisting of 1% agar (Noble) dissolved veronal buffer at pH 8.6, and containing merthiolate, (thiomersal) in a concentration of 1 in 10,000, as a fungicide. Despite initial misgivings about the potentially disrupting effect of merthiolate on enzyme activity (in experiments designed to preserve their activity in the gels) this
fungicide had no deterious effect; merthiolate could not be omitted from the gels as, without it, fungal growth was evident within 30 hours of its exposure to room temperature.

The agar mix could be kept for some weeks under sterile conditions in the cold room, preferably in small batches, and melted immediately before its use - 15 ml of gel being used per glass plate (carefully prelevelled). Wells were cut in the gel, using a template to obtain a hexagonal pattern. Each well just accommodated 20 µl of sample, dispensed with a micropipette.

Initially, undiluted and diluted (1/4, 1/8 and 1/16) antigens were tested against the serum (and vice versa) to determine the optimum concentrations of each for best resolution of precipitin bands. The undiluted serum and antigen were found to produce good results.

Gels were 'run' under conditions of high humidity in an enclosed chamber, usually at room temperature or where maintenance of enzyme activity was required, in the cold room. In most cases the precipitin lines become apparent within the first 24 hours but, as a precaution, gels were kept for a week and examined for the possible formation of new precipitation within this period. Ouchterlony (1968) has observed pattern changes after up to 6 years of storage!

Records of reaction were routinely made after 48 hours, both by drawing the precipitin lines and by photographing the plates themselves. The plates were best examined over a specially designed, but simple, light box, the gel being illuminated by scattered, rather than direct, light.

Photographs were taken with a 35 mm single-lens-reflex camera using 'Recordak' film (Kodak) the film being developed in either 'Emofin' or 'Diafine' ultra-fine grain developers. The antigenicity of a number of substances was tested against the prepared sera, in addition to those originally used in their production. The antigenicity of the following
substances was tested using the Ouchterlony double diffusion technique:

1. diffusates and homogenates of pollen, either intact, or hexane prewashed. These antigens included those extracts used to originally challenge the rabbits. Also used were materials freshly prepared, but not taken beyond the filtration stage in the case of the species described above, including extracts from the species *P. monilifera* var. *aurea*, *P. deltoides* var. *angulata* and *Populus* sp (a Taqamahaca clone).

2. diffusates and homogenates of 'false-coated' pollen (BHN, NHB) ('false coated' as described in Chapter 5).

3. pollen diffusates of *P. boileana* and *P. nigra* var. *italica*, both intact and after solvent treatments, at various times (see results) after moistening.

4. the organic solvent washings of pollen, the solvent having been removed, and the residue redissolved in 0.025 M tris-HCl buffer, pH 7.9.

5. raw extracts, consisting of the buffered homogenates of the stigmata, ovaries or whole flowers of *P. alba*, *P. monilifera* or *P. deltoides*.

6. raw extracts similar to (5) but obtained from buffered homogenates of leaves of *P. alba*, *P. nigra* var. *italica*, *Salix* sp, *Petunia* sp, *Pisum* sp, *Geranium* sp, *Xanthium italicum* and *Xanthium spinosum*.

7. the digestive fluid of *Nepenthes* sp, (a pitcher plant).

In addition the ability of the lectin, concanavalin A (con A), to react with various antigens or antisera was tested. Con A was either placed in the wells at a concentration of 10 mg per ml of water, or incorporated in the agar gel by mixing with the buffer in a concentration of 1.5 mg per ml. Methyl mannoside, which acts as an inhibitor to con A, was incorporated into gels in some trials.
(e) Immunoelectrophoresis

Immunoelectrophoresis was performed as described in Appendix VII.

(f) Staining

For Coomassie blue staining of proteins, gels were washed for 3 hours in 0.9% aqueous saline to remove unreacted protein, washed in water, then dried overnight (filter paper being layered over the top of the gel) the final drying was accomplished in a current of warm air. Gels were immersed in 0.5% Coomassie blue for 1 hour then destained in 2% acetic acid.

Staining for enzymes followed exactly the same procedure adopted for electrophoresis and described in Chapter 5 and Appendix VI. Staining tests were made for acid phosphatase, esterase, peroxidase, leucine aminopeptidase and aminotransferase enzymes.

RESULTS

Abbreviations Used

References to antigens and antisera will be abbreviated as follows:

(i) \(B_1, N_1, B_1H, N_1H, BY_1\) and \(NY_1\) will each refer to antiserum obtained from rabbits challenged with extracts of 30 minute diffusates of \(P. bolleana\) (B) or \(P. nigra\) var. \(italica\) (N) pollens, whether intact (B, N) or hexane prewashed (BH, NH). \(BY\) and \(NY\) antisera were derived from antigens which represented the third '280-peak' (yellow in colour) eluted from the Sephadex column.

(ii) \(B_2, N_2, B_2H, N_2H, T_2\) and \(Y_2\) refer to antigens and antisera derived, originally, from whole pollen homogenates, the original antigen being extracted within 5 minutes of hydration of the pollen.
Antigenicity of pollen diffusates and homogenates

The precipitin bands apparent between diffusate antigens and their corresponding antisera, reveal that *Populus* pollen diffusates may contain 3 major, and at least one further minor, antigens. Qualitative differences between the major antigens of each diffusate are not, by themselves, definitive for each species, section, or even compatibility group, within the genus *Populus*, as is evident in plates 6.I and II.

The effects of solvent pre-treatments on the subsequent antigenicity of pollen diffusates and macerates

Solvent pre-treatments differ in their effect on the antigenicity of subsequently released pollen diffusates, the effect varying with both the antigen, and the pollen species concerned. For example, note that the antigenicity of *P. bolleana* diffusate (Bl) is diminished as a result of hexane prewashing (BlH), despite the fact that the same treatment has no apparent effect on the antigenicity of N diffusate (N1)(fig. 6.I(a)). The converse situation is apparent in fig. 6.IV(a), where antisera are compared with whole macerated pollen diffusates of pollen of *P. nigra* var. *italica*, the latter being shown to have a lower antigenicity where the pollen was previously hexane prewashed. In the latter case hexane prewashing had little effect on the antigenuity of diffusate later released. This is also apparent from other such comparisons, several demonstrating that hexane prewashing had no effect on antigenicity (figs. 6.I(a),(b) and 6.II(a),(b)).

Significantly neither the hexane washings of *P. bolleana* nor *P. nigra* var. *italica* pollen showed any antigenic activity in double diffusion tests against antisera of macerates of the same pollen species.

Clearly hexane washing of pollen does not simply cause loss of antigenicity of pollen diffusates in all cases.

It is further evident from double diffusion tests that the antigenicity
of homogenates of hexane prewashed pollen differs little, either qualitatively or quantitatively, from that of intact pollen (figs. 6.I(c), 6.IV(c)).

'False-coating' of Populus pollen had no apparent qualitative effect on (BHN or NHB) diffusates, although NHB produced slightly less precipitin than diffusates of either N or NH pollens (fig. 6.VI(a)).

Although double diffusion may be used to detect antigenic differences between Populus pollen extracts where at least one of the reactants (antigen or antibody) is diffusate-, rather than homogenate-, derived, such differences may be obscured by complex precipitin patterns in those cases where both the reactants originate from whole pollen homogenates (as evidenced for example in figs. 6.III(c), 6.IV(b),(c) and 6.VII(a)). In such cases immunoelectrophoresis is preferred for its better separation ability (see Appendix VII).

The antigenicity of tissues other than pollen

The results show that many of the antigens found in pollen homogenates are shared with other Populus tissues, as well as with tissues of plants of other genera, or even families. Thus, as shown in figs. 6.V(a), (b), (c) and 6.VII(a), (b), Populus pollen, stigmata, ovaries and leaves have some antigens in common. Similarly this study has been able to demonstrate the equivalence of certain Populus pollen antigens with those of leaves of some species of Petunia, Geranium, Salix and Xanthium which surely shows that Populus pollen antigens are not unique. The following observations were also of note:

(i) antigens of homogenized female flowers of P. tremuloides react with N₂, but not B₂, antisera (6.V(c)).

(ii) Salix (Salicaceae) leaf homogenates produce a slight reaction only, with both B₂ and N₂ antisera (6.VIII(c)).
(iii) *P. nigra* var. *italica* leaf antigens react with antisera developed against pollen of *P. bolleana* but not to those of *P. nigra* var. *italica* pollen itself (fig. 6.V(b)).

The third observation (iii) was of particular interest, as were those of other experiments (results not presented) which showed that, in addition, there was evidence of a precipitin reaction between the leaf homogenates of *Geranium* sp and *Xanthium italicum*, and also *Salix* sp and *P. nigra* var. *italica*. Although generally more diffuse, the latter precipitin reactions were similar to those apparent between antigens and antisera.

The effect of solvent prewashing on the antigenic activity of extracts of whole pollen homogenates of *P. bolleana* and *P. nigra* var. *italica* was one of enhancement, rather than diminution, although there was no sign of any new antigen.

The nature of the antigens

Neither con A nor methyl mannoside (a con A inhibitor) affected the precipitin patterns of the gels in which they were incorporated, although methyl mannoside caused a slight degree of sharpening of the precipitin lines. Direct diffusion tests between con A, and pollen or leaf antigens failed to produce any reaction, nor was there any reaction in similar tests between pollen diffusates and the various dilutions of the digestive fluid of *Nepenthes* pitchers.

Enzyme staining of the gels, after double diffusion, demonstrated that several of the antigens were enzymes; those found in precipitin lines involving diffusate antigens included leucine aminopeptidases, aminotransferases, peroxidases and esterases, with precipitin lines involving homogenate antigens showing some (low) dehydrogenase activity.

The possibility that these results are solely due to the enzyme content of the serum, is discounted, as only some of the precipitin bands
gave a positive staining reaction on each gel - the one exception was a
gel stained for esterase (fig. 6.VIII(a)) in which all the major precipitin
bands gave a positive reaction, and yet in this case there is a meta-
chromatic reaction, some bands staining blue, others brown. It is proposed
that these differences are isozymic, a similar difference in staining
reaction having been noted previously between two different esterase isozymes

Most precipitin bands exhibit either esterase or acid phosphatase
activity. Acid phosphatase activity is particularly prominent in precipitin
bands between *P. bolleana*, B₁ and B₂ antisera and homogenates of B, N, BH,
NH, T and Y pollens - in each case there being an extra acid phosphatase-
positive precipitin band in interactions involving B₂ sera, compared with
B₁ sera.

Many precipitin bands exhibit esterase activity, including those
involving pollen diffusate antigens (fig. 6.VIII(a), (b)). In some cases,
esterase positive regions are evident in regions on gels where there is
no apparent precipitin reaction.

**INTERPRETATION OF RESULTS**

Three major precipitin bands are identifiable on double diffusion
gels where diffusate-derived antisera and antigens are run against each
other. These bands will be named 'a', 'b' and 'c' and are distinguished
both by their Rf values and also in comparisons of their precipitin
reactions to a common serum, the complete fusion of adjacent bands sig-
nifying the presence of identical antigens (Ouchterlony, 1968). Band 'a' is
quite distinctive, being close to the antigen wells in each case. It is
difficult to be categorical whether a particular band is 'b' or 'c', in
some cases, due to their similar Rf's and also the possibility that they
appear to be serologically related antigens, as evidenced by the type of
**TABLE 6.1**

An interpretation of the precipitin patterns obtained on double diffusion plates in comparisons between buffer 'diffusate' derived antisera, and either pollen buffer-'diffusate' or macerate antigens. 'a', 'b', 'c' and 'd' refer to identifiable precipitin bands.

**ANTISERA**

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* The other abbreviations are defined at the beginning of the results section of this chapter.
'spur' formation apparent between the precipitin lines of the two
(Ouchterlony, 1968; Clausen, 1972) (for example fig. 6.III(b)).

An interpretation of the precipitin patterns obtained with diffusate
antisera is presented in table 6.I, this interpretation being based
largely on the results of double diffusion tests (plates 6.I, II, III, IV).

The appearance of 'a' in interactions involving B₁, N₁ and N₁H, but
not in either B₁H or any of the pollen macerates could be explained in
several ways:

(i) 'a' appears de novo in diffusate due to its activation, or
synthesis, following pollen hydration, its production being unaffected by
solvent disturbance of the wall in N, yet dependent on the presence of
an intact pollen wall in B - perhaps due to a requirement for a positive
feedback control which may normally be supplied by hexane soluble wall
materials. De novo synthesis or activation of band 'a' might explain its
appearance in some pollen diffusates, but complete absence in all pollen
macerates tested.

(ii) 'a' is an antigen present within each of the pollens tested.
On hydration of the pollen it is released by all pollens except B₁H. The
absence of 'a' in macerated pollen extracts may be due to masking or
inactivation of the antigen binding sites during the maceration process,
or as a consequence of its interaction with some other component of the
macerate.

(iii) 'a' may be a pollen wall antigen more readily removed from
B by hexane prewashing than from N. Its absence in macerated material
could be explained as in (ii).

Of the three explanations offered, (i) is more favoured. The argument
for (ii) is weakened by the observation of extra permeability of pollen
previously hexane washed, a treatment which is shown to facilitate the release of diffusates (Chapter 5).

Proposal (iii) must remain a possibility in the absence of quantitative data about the relative extent of solvent penetration into each of two species of grain, but it is dubious in view of the apparently similar degree of hexane penetration into each (Chapter 3).

Both (ii) and (iii) depend on the hypothetical inactivation of an antigen-binding site upon the maceration of the pollen. The feasibility of proposal (i) is further supported by the already observed de novo synthesis (or activation) of at least one potential antigen (an esterase isozyme) within the first 5 minutes of moistening of the pollen (Chapter 5). It has already been shown in the chapter that at least some of the antigens released by Populus pollen have esterase activity.

Line 'b' is apparently present in the antisera to diffusates of B and N, but not to those of either B,H or N1,H. It is present in the homogenates of all the pollens tested including B, N, T, and Y whether intact or hexane prewashed.

Two possible explanations for the 'b' results could be that:

(i) (the 'obvious' one), that 'b' is simply wall held, and removed by hexane washing
or
(ii) that 'b' is present within the pollen of each species, its activation or synthesis being enhanced by the presence of a component in the pollen wall, (especially in the case of B pollen), which is removed by hexane washing.

As 'b' is found in macerated hexane pre-washed pollen the first suggestion could be acceptable if some of the 'b' antigen remains in the wall after hexane washing and is not subsequently released during the first 30 minutes following hydration of the pollen.
Antigen 'c', like 'b', is found in the macerates of all the pollens listed in the table, whether these were of intact or hexane pre-washed pollen. 'c' is clearly present in the diffusate antisera B₁ and B₁H, but not in N₁ or N₁H antisera, and though present in all the macerates tested, is present in higher levels in those of B and T pollens. Unlike 'b', the release of 'c' remains unaffected by solvent prewashing.

Another band is apparent in the same region as 'b' and 'c' but apparently distinct from either of these (fig. 6.I(a), (b)). It appears to be associated with interactions involving N₁ and N₁H antisera and N₁, N₁H and B₁ antigens, and not apparent in any macerate interactions, in which respect it appears to behave in a similar fashion to band 'a'.

Each of the above results was obtained whether the antigen or antisera was diffusate derived, although, for instance, it is noticeable that N₂H antisera has a higher antibody titre than has N₂ (figs. 6.III(c) vs 6.IV(c)). It is significant that where diffusate antisera are compared with whole pollen homogenate antigens, quantitative differences are more pronounced than in comparisons of the same antisera with diffusate antigens. For example, N₂, N₂H and Y₂ macerates have a higher 'c' antigenicity than do B₂, B₂H or T₂. Similarly the 'b' antigenicity is lower in T₂ and B₂ homogenates than in N₂, N₂H and Y₂ (figs. 6.II(a), (b), (c)).

Although these quantitative differences conform to compatibility groupings, the qualitative results with 'c' antigen, for example, would place P. deltoides var. angulata (Aigeiros) with P. tremuloides and P. bolleana of the section Leuce.

Where both the antisera and the antigens in a double diffusion test were homogenate derived interpretation was extremely difficult. In view of the obvious difficulties in interpretation of such complex patterns it is thus not surprising that Nasrallah and Wallace (1967b) were unable to
resolve S-genotype-determined patterns in anther homogenates. They observed that anther homogenates of *Brassica* were "strongly antigenic in rabbits and a minimum number of seven precipitin bands were resolved in each genotype" (Nasrallah and Wallace 1967b).

Despite such difficulties in interpretation of whole pollen homogenate data in *Populus* it was possible to detect quantitative differences in interactions between T₂ antiserum and various homogenate antigens. It can be seen (fig. 6.VII(a)) that T₂, B₂ and B₂H had a higher concentration of this particular antigen (apparently 'c') than did Y₂, N₂ or N₂H. Similarly the same gel showed that there was greater precipitin reaction between Y₂ antiserum and the pollen macerate antigens of Y₂, N₂ and N₂H than with those of T₂, B₂ or B₂H (fig. 6.VII(a)).

The immunoelectrophoretic results give better indication of the number of antigenic substances present in pollen macerates than could double diffusion alone (see Appendix VI). It was clear from these results that the solvent prewashing of pollen has not had a universal effect on each of the pollen species, there being neither a uniform decrease, nor increase, in the number of antigens produced. This result conforms with the quantitative enzyme results presented in Chapter 5. One antigen in particular is associated with interactions between antisera and antigens from homogenates of the *Leuce* poplars, T₂, B₂, T₂H and B₂H.

The nature of the antigens

The demonstrated enzyme activity of many of the precipitin bands indicates that among the antigenic components of *Populus* pollen diffusates and macerates, are a number of enzymes. Whether all the antigens are enzymes has not been determined nor could it be, considering the vast range of enzymes feasibly present in pollen. It thus remains possible that most, or even all, of the antigens could be shown to have enzyme activity. It

* No attempt has been made to relate specific enzyme activity to individual antigens as this is beyond the scope of the present work.
is easy enough to demonstrate enzyme activity for a particular protein, yet more difficult to prove that one does not have enzyme activity. Many of the *Populus* pollen antigens are shown to have enzyme activity yet, on the broader scale, the number of enzymes examined were relatively few in number. It is proposed that unless a fairly comprehensive survey was to be made of the enzyme activity of *Populus* (or any) pollen diffusates, and with meaningful quantitative results, it would be difficult to come to any worthwhile conclusion about the relative antigen or enzyme level - let alone attempt to determine the proportions of either in such diffusates.

Many of the antigens in *Populus* pollen are esterases including, apparently, precipitin bands 'b' and 'c'. Esterases may be involved in differences in the functional potential of different *Populus* pollen diffusates, but this has not been conclusively demonstrated in this study; nor have either the present, or earlier, studies been able to demonstrate the involvement of any particular antigen, whether enzymic or not, in the incompatibility mechanism of *Populus*.

The results obtained in the various experiments involving concanavalin A and methyl mannoside (an inhibitor of con A) fail to demonstrate the involvement of con A in the formation of the major precipitin bands on double diffusion gels. That con A may account for some of the minor reactions between homogenate derived antisera and antigens remains an open question. Slight evidence for the involvement of con A may be gained from the clarity of bands in diffusion gels containing methyl mannoside, when compared with those without this con A inhibitor. Although not pursued, the possibility remains that some of the precipitin lines observed directly between pollen and leaf or homogenates, even between those of different leaves alone, could be explicable in terms of the presence of con A or...
other lectins in homogenates of one or the other sample, reacting with mannose or glucose residues of glycoproteins from one of the tissues. Similar reactions have been noted on double diffusion plates between con A and pollen diffusates of grasses (Watson et al., 1974).

DISCUSSION

Each of the *Populus* pollen diffusates has been shown to have a fairly high protein content, up to 10% of the total protein being diffused from the grain within 30 minutes of moistening (Chapter 5 and Standifer, 1967). That these proteins include a number of enzymes has also been demonstrated (Chapter 5). The present chapter has shown that some, at least, of these *Populus* pollen diffusate enzymes are antigenic, the potential antigenicity of a number of enzymes being well documented (Clausen, 1972) including those of pollen diffusates of *Cosmos* (Howlett et al., 1975). On this basis, one might expect to detect more antigens in *Populus* diffusates than are apparent. That the proposed potential antigenicity of *Populus* pollen is not realized may be explicable when it is considered that the degree of antigenicity of a substance depends on its degree of 'foreignness' to the animal challenged, on the ability of the animal to produce antibodies against the substance, and on the available levels of the various antigens. It is thus, as Clausen (1972) emphasizes, largely a matter of luck whether a response is produced to the particular antigen or antigens that one is interested in. It is recognized that the substance(s) involved in the compatibility process of *Populus* may not be antigenic, despite evidence of other studies for the involvement of antigens in self incompatibility of several genera. Even if the substances involved in incompatibility reactions are antigenic, there is no certainty that such antigens will be either the only ones present, or even those which predominate. Furthermore, even if the antigen patterns can be shown to correlate with the compatibility groups, this could still be quite coincidental to the mechanism itself on the antigens need not even be involved.
It is clear that serological data itself may be erratically selective and thus, is most unlikely to reflect the details of the compatibility mechanism. When, however, the present results are taken in conjunction with the earlier results of this study and used mindful of the potential hazards of interpretation, they are proven useful.

The results of the serological study of the pollen of *Populus* demonstrate some degree of correspondence between pollen antigenicity and breeding group, qualitative in diffusate, and both qualitative and quantitative in homogenates of pollen. These results confirm and expand earlier results obtained with *Populus* by Knox *et al.* (1972a). It is apparent from the present study, however, that the antigenicity of the pollen species studied is unreliable by itself as an indicator of its breeding group, in contrast with the very close relationship between the two observed in the self-incompatible genera, *Oenothera* (Mäkinen and Lewis, 1962), and *Petunia* (Linskens, 1960).

Comparisons of precipitin patterns formed following interaction between homogenate antisera and homogenate antigens of *Populus* pollen have proved difficult to interpret. Lewis (1952), in a sense, overcame a similar problem by preabsorption of homogenized pollen of *Oenothera* with sera of non-homologous antigens, a technique tried later, without success, by Mäkinen and Lewis (1962) in double diffusion tests of pollen materials from the same species.

It is perhaps not surprising that Nasrallah and Wallace (1967b) were unable to distinguish between the precipitin patterns of the antisera and antigens of whole anther homogenates from *Brassica* species of different S-genotypes. Their failure to detect such differences, it is proposed, would stem from the confusion inherent in the multiplicity of precipitin lives rather than either uniformity or randomness of the antigenuity of each species. This 'non-appearance' of S-genotype proteins in *Brassica*
pollen was regarded as enigmatic by Heslop-Harrison (1975), considering the already known involvement of pollen proteins in the self-incompatibility system of several species. Heslop-Harrison had allowed for the presence of the S proteins in the anther homogenate and proposed that their antigenic sites were masked, whilst the explanation that Nasrallah and Wallace (1967) offered for their result was that the S antigens could be retained on membranes and not released by the extraction procedure; neither explanation would be necessary if the differences are simply obscured by the complexity of the precipitin pattern, rather than absent.

*Populus* leaves and stigmata are shown, in several cases, to have common antigenic identity with pollen homogenates. That this is not found to be true of the antigens of *Populus* pollen diffusates confirms the same finding by Knox *et al.*, (1972a) in their comparisons of homogenized stigma antigens with pollen diffusate antisera.

That some of the antigens found in *Populus* pollen are more widespread in nature is indicated by the fact that there is not only common antigenicity of homogenates of different pollens, as well as of different tissues of representatives of the *Populus* genus, but also that several of the same antigens are also found in homogenates of leaves of other plant families. This must surely be a cautionary note for serologically based interpretations of affinities between different plants.

This study has determined that many of the antigenic substances of pollen diffusates or homogenates are either esterases or acid phosphatases. Heslop-Harrison (1975), commented on the rapid loss of S-antigens from the intact *Brassica* stigma, as observed by Nasrallah and Wallace (1967), and proposed that S proteins may be present within the pellicle. No attempt has been made to determine whether the pellicle of *Populus* is antigenic, although the presence of esterase activity at the stigma surface (as well as that of other enzymes) has been demonstrated (Chapter 4).
In this study double diffusion techniques have shown that antigens (some, at least, being enzymic), are released from intact *Populus* pollen within 5 minutes of hydration (see also Chapter 5). Similar results were obtained, with *Populus*, by Knox *et al.* (1972a), who were able to show, using immunofluorescence techniques, that antigens were released from the pollen onto the stigma within minutes, and then rapidly spread across the adjacent stigma surface in both compatible and incompatible combinations. Knox *et al.* (1972a) held that the pollen wall was the most likely site of origin the antigens that are rapidly leached from pollen. They allowed for the possibility that materials may be leached from within the grain yet believed the degree of this leaching to be insignificant. It is held that the argument for the necessary pre-existence, in wall sites, of those proteins which are rapidly released from *Populus* pollen on moistening, (be they antigens, enzymes, 'recognition substances' - or all three) has not been conclusively proven in the earlier studies of *Populus*. It seems unlikely that 2 hour diffusates would not contain proteinaceous material derived from inside the grain.

Certainly the proponents of pollen wall proteins have failed to show why such rapid release of proteins could not occur across the plasmalemma within a short time of pollen hydration.

Although immunofluorescence and enzyme histochemical techniques have in some cases (but not all) shown the almost exclusive location of certain enzymes or antigens to be within the pollen wall, it should be noted that published photographs show pollen which has apparently become hydrated in the approximately 30 seconds prior to freezing, or following other pre-treatments. Hydration, in itself, is a dynamic event, involving a rapid movement of materials and obvious changes in structure, and thus the location of diffusible material after hydration need not reflect its location prior to hydration. A further possibility is that the freezing
process itself could actually enhance diffusate protein release resulting, in some cases, in its concentration in the porous matrix of the intine, despite its original location in the cytoplasm of pollen before hydration. That this cannot be discounted as a possible explanation of the observation by Knox et al. (1972a) of a pollen wall location for some *Populus* antigens may be indicated by the comment by Heslop-Harrison et al. (1973) that the practice of adding the antifreeze, dimethyl sulphoxide, to the gelatine embedment (as used in the earlier *Populus* study) was subsequently discontinued because of its enhancement of permeability of pollen loss of proteins.

It could also be argued that the activity of some antigenic or enzymic proteins outside the wall may simply indicate their activation during their passage through the wall possibly by wall held substances. This proposal is discussed later in this section.

The serological results presented in this chapter support the feasibility both of passage of antigens through the plasmalemma, and also of the 'activation' of certain antigens (including enzymes)

(a) by their passage through the plasmalemma

or (b) within the pollen wall

or (c) as a result of *de novo* synthesis or 'activation' within the grain after moistening (with their subsequent passage through the plasmalemma).

Possible support for the internal origin of many of the short term diffusates from pollen could be gained from the finding of Mäkinen and Lewis (1962) that there were neither qualitative nor quantitative differences in antigenicity between the S-proteins of short term diffusates, and macerates. It is also interesting to recall that Lewis (1952) pre-washed *Oenothera* pollen with ether, prior to its maceration, for the preparation of his antigenic extract suggesting that the antigenic differences that he
observed may well have been due to cytoplasmic rather than wall-held materials.

Overall, the spectrum of results obtained both in this study and by other workers, show no proof against the possibility of relatively rapid diffusion of antigenic and/or enzymic proteins across the plasmalemma shortly after moistening and yet there is considerable evidence for this passage.

Solvent pre-washing of *Populus* pollen has been shown, in this chapter, to variably, cause an increase, or a decrease, in antigenicity of either the same antigen in different pollens, or of different antigens in the same pollen; that is, the effect is not uniform, but depends on the antigen and the pollen species involved. This is in agreement with similar results obtained in quantitative enzyme assays of pollen diffusate from both hexane prewashed, and intact pollens - particularly in esterase assays (Chapter 5).

It is possible that antigens may be affected in their antigenic by pollen wall materials whose presence may have one of several effects depending on the situation, including stimulation of synthesis or 'activation' in some cases, or inactivation or depression of synthesis in others. This is a similar proposal to that made to explain the enzyme (particularly the esterase) results of Chapter 5.

Such wall-held materials need not be proteinaceous, and could be, for instance, phenolic or lipoidal. It would be reasonable to propose, for instance, that a cofactor, inhibitor, or even a substrate, to the particular enzyme controlling its ultimate release in an active form by either positive or negative feedback. Feedback (substrate) control of enzyme activity for example has been well reported (Dixon and Webb, 1960; Barman, 1969).

Such mechanisms, whilst remaining speculative, could well explain both the antigen and the enzyme results obtained (including those involving solvent
prewashing of pollen) in *Populus*, and could also explain the *de novo* synthesis of isozymes in *Oenothera* (Mäkinen and Brewbaker, 1967). There is no conclusive evidence from this study that enzymatic proteins constitute a minor fraction only of the diffusate of *Populus* pollen, as has been inferred (Knox *et al.*, 1972) and to determine this conclusively, it is held, would require a fairly exhaustive survey of the diffusate for a large number of known enzymes.

Similarly, there is no evidence that 'recognition substances' either form a major part of the diffusate, or might not be enzymes despite the counterproposal by Knox *et al.* (1972a) for *Populus* Mäkinen and Lewis (1962) for *Oenothera*. Evidence that S-gene antigens can be enzymes was provided by results obtained by Lewis *et al.* (1967) with *Oenothera* pollen. They found a correlation between the S-gene antigenuity and the enzyme (esterase) activity, and proposed that "a large amount of the protein diffusing from the pollen would be esterase and therefore [this] would be the most important antigen "(Lewis *et al.*, 1967). That they could not inactivate the esterase activity with antisera is not surprising when one recalls (both from results in this chapter, and Howlett *et al.*, 1975) that esterase activity is readily demonstrated, and thus preserved, in precipitin bands resulting from just such an antigen-antibody reaction. The results produced by Lewis *et al.* (1967) showed that S gene segregation was evident in diffusion tests between pollen diffusate antiserum and pollen homogenate antigens. Knox and Heslop-Harrison (1971) asserted that S protein must form a major part of the total, otherwise its precipitin band would be lost against the background of reactions involving, "non-specific proteins - including of course, the enzymes" (Knox and Heslop-Harrison, 1971). This argument by itself may not be valid as firstly, the maximum number of bands which can be formed in a diffusion test will be determined by the reactant with the least number of antigens (or antibodies), this being, in the study by Lewis *et al.*, (1967), the pollen diffusate. Thus although the antiserum
may have the potential to interact with numerous antigens, it can only form precipitin bands with those present. This is clearly demonstrated in the *Populus* results of this chapter. Lewis *et al.* (1967) themselves allowed that enzymes could be S-proteins. The second point is that as mentioned earlier, not all the proteins of interest will necessarily by antigenic to the animal challenged.

The low, or trivial, cross-reactivity between *Gladiolus* and *Ambrosia* pollens, despite their common possession of several enzymes, was used as evidence for a possible distinction between the enzymes and antigens involved (Knox *et al.*, 1970). This argument could not be sustained if these enzymes were not antigenic, or had low antigenic. For example, despite the fact that *Populus* pollen diffusate contains at least six different types of enzyme (let alone isoenzymes) it produces only 3 or 4 major antigens. This may suggest that not all the enzymes are antigenic, but allows that all the antigens could be enzymes (this study and Howlett *et al.*, 1975).

Clearly, antigens and enzymes should obviously not be regarded as separate entities as there is good evidence, in many cases, that S proteins, antigens and enzymes may, in many cases be one and the same. It is from this basis that we should re-examine the earlier proposal, for instance, by Lewis (1952) that the S-antigens could simply be enzymes controlling intermediate stages in the synthesis of an incompatibility substance, or the more elaborate, later, proposals of Linskens (1960) and Mäkinen and Lewis (1962) who suggested that the S-gene locus of self incompatible plants involved both an enzyme, and an activator, cistron.

The determination that glycoproteins are present in some pollen diffusates, for example in those of *Iberis* (Heslop-Harrison *et al.*, 1974) and *Cosmos* (Howlett *et al.*, 1975), follows the earlier observation of Mäkinen and Lewis (1962) that the S substance, in their study, was proteinaceous, allowing for the possibility of a small polysaccharide component. It has
also been found that the allergens causing hayfever are mostly proteins and glycoproteins (Augustin and Hayward, 1962; Augustin et al., 1971). Many enzymes are also known to be glycoproteins (Eyler, 1965) including particularly, those with low molecular weights. It is further known that the lower molecular weight enzymes have a relatively high heat resistance (Dixon and Webb, 1960). In addition to their possession of a high antigenicity, many glycoproteins are also known to diffuse readily through intact membranes (Eyler, 1965). Eylar noted that bound carbohydrates are associated with most extracellular proteins, but few of the characteristically intracellular proteins. He proposed that carbohydrates have a general role in the excretion of proteins by acting "as a chemical label, which upon interaction with a membrane receptor or carrier, promotes the transport of the newly synthesized glycoprotein into the extracellular environment" (Eylar, 1965). He further suggested that the carbohydrate need play no functional role in biologically active proteins and that carbohydrate units from the same cell may be identical, or very similar. It may well be that many of the features of pollen diffusates could be attributable to lower molecular weight glycoproteins, some of which could include enzymes. This may help to explain several of the recognized features of diffusates, including their rapid release, their high antigenicity, their relatively high heat stability (in some cases) their enzyme activity (particularly acid phosphatase and esterase) and also, possibly, differences between their antigenicity and that of the pollen contents.

When taken together with the evidence produced in Chapter 5, the results of the present chapter may allow for a reinterpretation of both the nature, and the function, of diffusates, as well as reinforcing the earlier arguments (Chapter 4) for a cytoplasmic, rather than simply a pollen wall, location for the bulk of the components of short term buffer diffusates. The two chapters show that hexane-removeable pollen wall materials may have an effect on both the antigen, and the enzyme, content of pollen
diffusates. It does not seem unreasonable to propose that such (non-protein) wall materials in *Populus* pollen may thus have an indirect role in the events associated with the (in)compatibility mechanism by their effect on the production, by the pollen, of the 'recognition proteins'. This argument shows for the possibility that such 'recognition proteins' are possibly enzymes, some of which may be antigenic.
Plate 6.I

Photographs of double diffusion plates. Precipitin lines are apparent between the wells. The plates are unstained, being illuminated from underneath by indirect lighting.

In this plate, and plates 6.II and 6.VII, the abbreviations used are explained as follows:

- B  P. bolleana
- D  P. deltoides
- M  P. monilifera
- N  P. nigra var. italica
- T  P. tremuloides
- Ta  P.sp (a Tacamahaca)
- Y  P. yunnanensis.

Unless otherwise specified the samples are derived from pollen, hexane prewashed where the suffix, -H, is used.

The subscript '1' refers to antigen or antiserum originally derived from pollen buffer diffusates. The subscript '2' refers to antigen or antiserum originally derived from pollen homogenates.

figs.(a),(b),(c) All plates have had antiserum in their central wells (B₁, N₁ in each case) and antigens in the peripheral wells.
Plate 6.II

Photographs of double diffusion plates.

figs (a), (b) and (c). All plates have had antiserum in their central wells \( B_1, N_1 \) in (a) and (b), and \( B_1H, N_1H \) in (c) and antigens in their peripheral wells.
Plate 6.III

Photographs of double diffusion plates.

figs (a), (b) and (c). All plates have had antiserum in their central wells (B₁H and N₁H in (a) and (b), and B₂H and N₂H in (c)) and antigens in their peripheral wells.
Plate 6.IV

Photographs of double diffusion plates.

fig (a) The central wells (B₂ and N₂) contain antigen (whole pollen homogenate) and the peripheral wells, antisera.

figs (b) and (c). The central wells (B₂ and N₂) contain antisera, the peripheral wells, antigens.

In figs (a) and (b) antisera, and antigens, respectively, are diluted by one quarter and one sixteenth.
Plate 6.V

Photographs of double diffusion plates with antisera in each of the central wells.

fig (a) Additional comparisons are made between the pollen derived antisera and antigens of *P. monilifera* ovary homogenate, and *P. nigra* var. *italica* leaf homogenate.

fig (b) Comparisons also include those with *P. alba*, *Salix sp.* *P. deltoides* var. *angulata* and *P. nigra* var. *italica* leaf homogenate antigens.

fig (c) Comparisons also include those with antigens whole flower homogenates of *P. tremuloides* and *P. alba*; ovary homogenates of *P. deltoides*; stigma homogenates of *P. deltoides* and leaf homogenates of *P. alba*. 
Photographs of double diffusion plates with antisera in each of the central wells.

In each case comparisons are made between serum derived from rabbits challenged with extracts of 30 minute isotonic buffer diffusates and diffusates collected in the same manner; over the same period in (a), and also at times of 5, 15 and 30 minutes after hydration in (b).

As already defined, BH and NH are diffusates from hexane prewashed pollen and BHN and NHB are from 'false coated' pollen.
Photographs of double diffusion plates with antisera in each of the central wells.

Additional comparisons are made, in (b) with leaf homogenates of *P. deltoides* var. *angulata*, *P. nigra* var. *italica*, *P. alba* and *Salix* sp.
Plate 6.VIII

Photographs of double diffusion plates with antisera in each of the central wells.

Both are stained for α esterases using α naphthyl acetate as substrate. Note the metachromatic staining in fig.(a).
CHAPTER 7

GENERAL CONCLUSIONS

Each of the experimental chapters in this thesis has contributed further to an understanding of the nature of the incompatibility mechanism in *Populus*, allowing for the fact that by the breadth of the approach, the study must be regarded in general terms as indicative, rather than definitive. At the same time, it may be possible to extrapolate interpretations of the situation in *Populus* to include those of other genera, particularly where these show similar features in their reproductive mechanism. A constant theme of this study has been the argument for a more flexible interpretation of results.

On the basis of the results obtained in this study the sequence of events leading up to, including, and following pollination is summarized below, for *Populus*.

Changes occur in the pollen wall even as the catkins are elongating, often within days of anthesis. Materials, including lipids, are apparently still being deposited in the pollen wall from within, at this stage, and appear to cause pre-existing wall-held substances to be relocated. During this phase the starch content of the pollen plastids decreases. Dehydration of the pollen is probably initiated on dehiscence of the anther, and is particularly rapid once the pollen is shed continuing to the point where the final water content reaches 8%. At this stage the grain has an irregular shape and appears to be collapsed.

The mature receptive surface of the stigma of each of the *Populus* species studied, is characterized by the presence of low, slightly bullate, papillae and an absence of any copious surface exudate. The *Populus* stigma
is thus described as being of the 'dry' type and tentatively placed within
the stigma 'group II' of Heslop-Harrison et al. (1974). Fairly regular
structures (1-2 µ diameter) are present on the receptive stigma and
distinguish it from the non-receptive surface from which they are absent.
As shown in Chapter 4, these structures are largely composed of long chain
fatty acids and are waxy in nature. They are thus described, with justifi-
cation, as 'wax structures' (after Roggen 1972). In sectional view the
receptive stigma surface is seen to be covered by a 'pellicle', (sensu
Mattsson et al., 1974), whose presence is recorded for Populus for the
first time. A cuticle is just discernable beneath both the wax structures
and the 'pellicle'. Vesicles located largely beneath the cuticle, but also
occasionally apparent within the 'wax structures' over receptive papillae,
appear to be formed by the papilla cells prior to stigma maturation. I can
find no other report of the existence of such vesicles at this location in
this, or other genera. The receptive papillae cell is characterized by its,
usually single, prominent tannin vacuole, only occasionally present in the
non-receptive stigma epidermal cells.

On making contact with the stigma surface the grain becomes hydrated
within a few minutes, imbibing water from the stigma surface, possibly, as
Mattsson et al. (1974) have proposed, from the spongy hydrated 'pellicle'.
As the pollen grain swells, it releases a yellow exudate onto the surface
of the exine and this effects the 'sticking reaction' (Roggen 1972),
whether the grain is compatible with the stigma surface, or not. The
'sticking' may be preferentially associated with the wax structures. The
release of proteins, including materials which are enzymic and/or antigenic,
occurs within minutes of pollen-stigma contact (Chapters 5 and 6). Although
degradation of the 'wax structures' cannot be detected within the first 30
minutes of pollen-stigma contact, these structures can be shown to have
been largely degraded within 3 hours of the initial contact, with freed vesicles now evident in the contact region. There is no apparent 'stigma reaction' following either 'compatible' or 'incompatible' contact: however, within 15 minutes of initial contact the incompatible pollen is shown to have a higher callose content than does compatible pollen. Taking this presence of callose in the incompatible grain as an indication of some deviation of the pollen metabolism from that normally occurring following 'compatible' pollination, it is clear that incompatibility events are initiated within the first 15 minutes of pollen-stigma contact in *Populus*. A further (indirect) indication of probable disruption of pollen metabolism following an 'incompatible contact is the marked difference in the ultra-structural appearance of the incompatible, compared to the compatible pollen grain. The former is markedly vesicular, as are, to a lesser extent, the incompatible pollen tubes outside the stigma.

'Compatible' pollen tubes penetrate the cuticle close to their point of first contact with the stigma surface then pass, between the cuticle and the pecto-cellulosic wall of the papilla, to the cuticular flange region between papillae, after which they travel within the middle lamella between the papilla cells and then through the stigmatoid tissue towards the ovary.

Incompatible pollen tubes, by contrast, generally fail to penetrate the stigma surface at their point of first contact and may wander erratically across the surface. They appear to have a slightly more vesicular cytoplasm than that of compatible pollen as well as a higher callose content and thinner walls, the latter feature probably being a factor involved in the irregular bulging of these pollen tubes. Some pollen tubes do eventually penetrate the stigma surface. There is no apparent difference in fine structure between compatible and incompatible pollen tubes of *Populus* once they have reached the stigmatoid tissue.
It is clear that the incompatibility reaction in *Populus* takes place at the surface, rather than in the style. This conclusion accords well with Brewbaker's (1957, 1959) correlations, as *Populus* pollen have been shown, in this study, to be trinucleate (Chapter 1 and Appendix I) and reported to be difficult to germinate *in vitro*, these features being characteristic of pollens of plants with sporophytic (in)compatibility control. The observation that the *Populus* stigma is of the 'dry' type, further suggests a sporophytic control of (in)compatibility, in accord with additional correlations noted by Heslop-Harrison *et al.* (1974).

An hypothesis is presented which may serve to explain the *Populus* observations made to date, including those of earlier authors. This hypothesis is similar, in some respects, to Christs (1959) 'cutinase' hypothesis, as furthered by Heinen and Linskens (1961), and Linskens and Heinen (1962).

The hypothesis would explain the *Populus* observations in the following way:

1. each of the pollen grains from the plant of a particular genotype forms, during its development, esterase enzyme(s) capable of degrading the stigma 'wax structures' of female flowers belonging to its own breeding group. In addition the grain may contain lesser proportions of enzymes capable of degrading the 'wax structures' of *Populus* species with which it is incompatible. On the basis of this proposal, one would expect to find that the composition of the 'wax structures' of plants within a particular breeding group would have certain features common to that group but different from those with which it is incompatible.

2. the pollen grain, on contacting the stigma, imbibes water from the stigma surface and swells rapidly. During this process of rapid expansion, and whilst the membrane is accommodating to this change in
conformation, materials are released from the grain, including both lipidic substances (some at least, derived from wall sites), and proteins (including the enzymes described in (1)). The proteins apparently do not come from the wall sites. The lipidic components of these pollen exudates are held to be largely responsible for the 'sticking reaction'.

(3) Once an intimate contact has been established between the pollen and the stigma surface, the enzymes released by the pollen begin to degrade the 'wax structures' - efficiently where the pollen is compatible, and less so in the incompatible situation, that is, where most of the pollen enzymes are 'incongruous' to the substrate. It is proposed that by degrading the 'wax structures' the Populus pollen obtains some metabolic stimulus which may act as a metabolic 'switch' or 'activator', possibly of enzymes. These substance(s) may, for example, be hormonal or nutrient in function or act as co-factors for the action of enzymes, and would be either located within, or contribute to, the composition of the wax structures.

(4) Where the pollen gains ready access to the stimulatory substance(s), as in a compatible situation, their development proceeds normally. If, as in an incompatible situation, the stimulus is not obtained, obtained in minimal quantities, or obtained only after some delay, the metabolism of the grain is disrupted, as evidenced by the callose build-up, pollen tube wall deformations and the failure of the incompatible pollen to penetrate the stigma surface efficiently. The stimulus could, in effect, be regarded as having 'programmed' the pollen for subsequent normal metabolism in the compatible situation. The variation in behaviour of incompatible pollen grains on the stigma surface, ranging from the complete inability of some pollen tubes to penetrate the stigma surface, to a behaviour similar to that of compatible pollen tubes, may simply indicate that there exists a degree of physical and/or chemical variation in wax
structures such that in some, the metabolic stimulus is more accessible than in others. Conversely, the same argument could explain the (lesser) variation in compatible pollen tube behaviour.

In passing, one should mention the paper by Koshimizu et al. (1972) who have noted that aliphatic esters extracted from *Tulipa* flowers stimulated the germination of pollen of *Chrysanthemum*. The (known) esters of which the 'wax structures' of *Populus* are largely comprised could well have a similar function. In addition to the 'brassins', or fatty hormones, found by Mitchell and his co-workers, methyl esters of fatty acids are known to be active in stimulating growth (Fukui *et al.* 1958; Fathipour *et al.* 1967). It is thus feasible that lipoidal materials from the 'wax structures' could be involved in the (in)compatibility mechanism of *Populus*, whether they are the breakdown products of the 'wax structures' themselves or simply lipoidal (or other) materials contained within, or by, them.

The hypothesis is also attractive from a teleological standpoint when one considers that the 'wax structures' and 'vesicles' are present on the receptive, but not the non-receptive stigma surfaces of *Populus*, nor does their function appear to be that of effecting the 'sticking reaction'.

On the basis of this hypothesis one would interpret the 'recognition protein' of Knox *et al.* (1972a) or the 'P-factor' of Willing and Pryor (pers. comm.) as being enzymic. It would be in keeping with earlier terminology to describe these enzymes as *recognition enzymes* (or even 'mentor enzymes'). The 'incompatibility barrier' could be interpreted as being either the 'wax structures' which limit the access of the pollen to the 'stimulatory substance'(s), or as the cuticle and/or pecto-cellulosic material which acts as the final barrier to incompatible pollen tube penetration. Although the hypothesis does share certain features with the
'cutinase' hypothesis of Christ (1959), it is necessary to regard the stigma, and more specifically the wax structures, of *Populus* as containing a universal activator (stimulus), readily obtainable by 'compatible' grains only, rather than an activator specific for 'compatible' grains only, yet readily obtainable by grains whether compatible or incompatible.

One could speculatively apply similar interpretations to the results obtained with genera other than *Populus*; Kroh's (1966) pollen transfer results with *Brassica*, for instance, may also indicate that once such a stimulus is obtained, the subsequent behaviour of the pollen is irrevocably determined, or programmed, and further, that such a stimulus, to be effective, must be obtained shortly after initial pollen-stigma contact. In both *Populus* and *Brassica*, obviously, the compatibility events occur within 30 and possibly 15 minutes.

The effectiveness of methods used to overcome *Populus* incompatibility can also be explained in terms of the hypothesis. If the 'recognition proteins' of Knox *et al.* (1972a) are equated with esterase enzymes capable of degrading the wax structures, then it is reasonable to propose that these would be released whether the pollen were intact or previously irradiated, as in the *Populus* studies by Stettler (1968) and Knox *et al.* (1972b). Such enzymes could also survive the protein extraction procedures used by Knox *et al.* (1972a) in their preparation of 'recognition protein' which they added to incompatible pollen to overcome *Populus* incompatibility. In each case the 'recognition' or 'mentor' enzymes would enable a nearby incompatible grain to gain access to the stimulatory substance(s) otherwise unavailable to it. The success obtained by Whitecross and Willing (1975), with solvent stigma pretreatments of *Populus* in overcoming incompatibility...
could be explained in a similar way, but in this case the degradation of the 'wax structures' is effected by solvents rather than enzymes. Too liberal an application of solvent, found by them to be ineffective, would remove the 'stimulatory substance' as well as the 'wax structures' which contain them.

Results reported in Chapter 2, where assessments were made of the behaviour of *Populus* pollens after various pollen and stigma treatments, also tend to support the hypothesis. The prewashing of pollen with isotonic buffer solutions for one minute, or longer, prior to its application to the stigma, would diminish the total quantity of enzyme available to the pollen for its subsequent efforts to gain the required stimulus, even in the case of compatible pollen. As shown in Chapters 4, 5 and 6, the low polarity solvents, used effectively in pollen pretreatments, cannot be shown to remove proteins (including enzymes and antigens) from *Populus* pollen; indeed, in Chapter 5, such treatments were shown to enhance the rate of protein release. Solvent pollen pretreatments may thus be interpreted as enhancing the release of normally less abundant 'congruous' enzymes of incompatible pollen to a level now adequate for the attainment, by the pollen, of the 'universal stimulus'. As discussed in Chapter 2, a similar increase in permeability, but of the plasmalemma, rather than the pollen wall, may explain the success of such treatments as 'electric-aided' pollination, as successfully used by Roggen *et al.* (1972) to overcome the incompatibility barrier of *Brassica*, another genus of the 'dry' stigma group. The success of the 'false coating' treatments of *Populus* pollen in overcoming incompatibility may suggest that the hexane-removeable (largely lipid) compounds of the pollen-wall exert some control over the release of 'recognition enzymes' as proposed in Chapters 5 and 6. Feedback control of such enzyme production by wall-held lipids could be a distinct possibility. This focuses attention on, firstly, the normal location of the materials

under these conditions there may be a change in surface lipid composition and/or structure.
removeable from *Populus* pollen by hexane washing, and secondly, on the question of which of these materials are gametophytically, and which, sporophytically derived. The first question is answered in Chapter 3, whilst the second is largely outside the scope of this project.

The effects of stigma surface treatments on compatible and incompatible pollen of *Populus* (Chapter 2) may similarly be explained in terms of the basic hypothesis presented here. Thus low polarity solvents and detergents break down the 'wax structures', whilst plain buffer, protease and pectin-esterase would be unable to do so. Although lipase may degrade the 'wax structures', the method of its application in this and other experiments (Heslop-Harrison and Heslop-Harrison, 1975), might cause the materials thus exposed to be leached into the reaction medium and thereby effectively lost to any pollen later placed on that stigma surface. Some of the esterases in serum may enhance the breakdown of the 'wax structures' by the pollen, although where the serum is derived from an animal challenged with pollen diffusates (including enzymes), the 'antienzymes' (Chambers 1974) in this serum may diminish the effectiveness of 'recognition enzymes'. The results obtained using serum from both unchallenged and challenged rabbits following the application, in each case, of this serum to the stigma, are consistent with the above analysis. Protein and RNA inhibitors applied to the stigma surface would be taken into the pollen grain as its hydration proceeds and, whilst having no effect on enzymes already present, would inhibit their further production. The further production of 'recognition enzymes' would thus be halted, as also those required for continued growth of, and stigma surface penetration by, the *Populus* pollen, whether compatible or incompatible. The preliminary result which showed that a good seed set may be obtained following incompatible pollination, where the stigma had developed under cold conditions, may indicate that under these conditions there may be a change in surface lipid composition and/or structure.
Apart from demonstrating the effects that each of a number of organic solvents and solvent mixes have on *Populus* pollen, the results obtained in Chapter 3 show that certain of the low polarity solvents, including hexane and diethyl ether, do not enter the pollen cytoplasm within 30 minutes, yet do penetrate both intine and exine. Such solvent treatments are clearly able to extract materials from the pollen wall, but not from its cytoplasm. This result allows for the proposal that pollen wall material may play some part in the incompatibility mechanism of *Populus* although, as proteins cannot be located within such low polarity solvent pollen washings (Chapter 5, 6), one may now question the proposed pollen-wall location for 'recognition proteins' prior to hydration of the pollen. Results obtained in Chapter 3 open the way for more detailed studies of material known to be derived from the pollen wall alone, certainly in *Populus*, and possibly in other genera.

Such a specific analysis, it is maintained, has not previously been possible, considering the obviously rapid relocation of many pollen cytoplasm and wall materials which follows pollen hydration. This hydration obviously occurred during processing of pollen for (for instance) microscopy, pollen wall isolation and diffusate buffer extracts, in previous studies.

The comparison of pollen and stigma surface materials, performed in Chapter 4, has shown that for each of these surfaces in the several *Populus* species studied, the material is largely lipidic and composed of long chain fatty acid esters. The pollen cytoplasm and leaf surface lipids are remarkably uniform in their fatty acid composition from species to species, yet there is a considerable degree of interspecific variation of pollen and stigma surfaces as is predictable on the basis of the hypothesis. Further, the gas-liquid chromatograms of stigma surface esters showed that
some quantitative differences may be correlated with the breeding groups to which each species belongs. This is a significant result as it provides support for the hypothesis that the composition of the stigma surface lipid determines, at least in part, the selectivity of the incompatibility mechanism. There was no evidence of a similar correlation in analyses of material from the pollen surface. Analyses of the stigma surface lipids have demonstrated that they are waxes in the case of *Populus*. Roggen's contention that this was true of those of *Brassica* was disputed by Heslop-Harrison *et al.* (1974).

The other major factors involved in the hypothetical incompatibility mechanism of *Populus*, namely the proposed 'recognition enzymes', are examined in Chapter 5. *Populus* pollen diffusates of 30 minutes duration are shown to contain a number of different enzymes including many (esterases and lipases) appropriate for the degradation of stigma surface lipids. While the total protein released into the diffusate is greater in solvent washed than in intact pollen, the results of quantitative enzyme assays following solvent pretreatments show that many enzymes, and particularly esterases, do not simply reflect the total protein pattern. This result may support the suggestion that pollen wall materials (particularly hexane-soluble components) may exert some control over the enzyme production by the pollen, whether feedback or otherwise. Qualitative (electrophoretic) analyses show that the appearance of several isozymes in 30-minute pollen diffusates, and particularly of esterases, can be correlated with the breeding group to which the species belongs. Attempts, using electrophoretic techniques, to demonstrate feedback control of pollen enzyme production within the first 30 minutes have not been conclusive. This possibility should be further investigated using quantitative techniques.
The results of the serological analyses (Chapter 6) show that many of the antigens released in *Populus* pollen diffusates within 30 minutes are esterases. There has not been a general correlation between precipitin bands appearing on double diffusion gels and the breeding group of the pollens from which each was derived. Immunoelectrophoretic trials (Appendix VI) suggest that this technique, with its better separation of antigens, may be more suited to the resolution of this problem. *Populus* pollen diffusates were shown to vary, both qualitatively and quantitatively in their antigenicity in double diffusion studies. The observed changes in antigenicity of some of the diffusates of hexane prewashed, compared with intact, pollen could suggest that (antigenic) pollen wall proteins have simply been removed, were it not for the fact that proteins cannot be found in such hexane washings (Chapter 4) and secondly, that the material within hexane washings is neither antigenic (Chapter 6) nor enzymic (Chapter 5). Again, the preferred interpretation is that there is a feedback control in the case of certain diffusate antigens (including enzymes) from the pollen of some of the *Populus* species examined.

It must be acknowledged that the hypothesis presented in this chapter as an explanation for the *Populus* incompatibility control mechanism remains speculative at this (preliminary) stage although, clearly, there is considerable evidence provided both by this study, and others, for its support. The further proposal, for a feedback control of pollen diffusate enzyme composition, remains attractive but the evidence is still, as yet, inconclusive.

Evidence is presented throughout the thesis which calls into question a proposed wall location of protein in the pollen of *Populus* prior to its hydration (Knox *et al.* 1972a). The hexane-removeable, wall-held (Chapter 3) materials of *Populus* pollen are shown to be Lowry-, and ninhydrin, negative, to contain no discernable nitrogen, and to be free of enzymic
or antigenic activity. Although there is no certainty that such low polarity solvents would remove bound protein from cellular structures, it is reasonable to expect that they would readily extract protein that is loosely deposited in the pollen wall at a later stage of its development. In this respect the enhancement of permeability of the pollen wall gives some indication of the efficiency of the hexane extraction. It should be recalled that there is an increased, rather than a decreased, quantity of protein released from *Populus* pollen following such an extraction. It is proposed (Chapters 1, 5 and 6) that firstly, it is quite feasible that cytoplasmic pollen proteins may pass across the plasmalemma of an intact grain within minutes of its hydration. Secondly, it is argued that evidence (from microscopy studies) for the presence of proteins in the pollen wall of freeze-sectioned, or fixed, material may be misleading as a guide to their location in the grain prior to its hydration. It is relevant to again quote Dickinson and Lewis (1974) who comment that, "as the pollen grains swell when immersed in an aqueous medium, it is clear that material prepared using an aqueous fixative cannot give an accurate picture of the dry pollen as it is released from the anther".

An important result of the present study, is the demonstrated effect of organic solvents on *Populus* pollen, the observations almost certainly being capable of extrapolation to pollen of other genera. The elucidation of the effects of lower polarity solvents on pollen viability and ultrastructure, and pollen-stigma interactions, has been shown, in this thesis, to contribute to a better understanding of the incompatibility mechanism in *Populus*. Further research into the development, structure, and composition of the pollen wall should prove rewarding.
A logical extension of this research would be the adoption of an approach similar to that of Linskens and Heinen (1962) in further testing the incompatibility hypothesis promoted here. It should be possible to demonstrate, \textit{in vitro}, that enzymes obtained from pollen diffusates are better capable of degrading the surface lipids of stigmata from their own breeding group, than those of groups with which they are incompatible. Clearly the results of this thesis suggest stimulating prospects for future research.
APPENDIX I

Text of a paper submitted to Experientia in November 1975, and currently in press.

Trinucleate Pollen in the Genus Populus

A number of authors have reported that the pollen of Populus is binucleate, apparently basing their conclusions on initial studies of Smith¹ and Nagaraj².

Smith¹ observed division of the generative nucleus within the pollen tube of P. laurifolia. He clearly states that in P. deltoides, P. acuminata and P. adenopoda, the grains are binucleate; only in P. acuminata and P. adenopoda does he report an occasional trinucleate pollen grain. In addition, Smith¹ quotes Chamberlain's³ observations of P. monilifera (in Smith's estimation, probably P. deltoides); "the division of the generative cell which presumably takes place, although I was not so fortunate as to observe it, must occur after the pollen tube begins to form."

Nagaraj² also reported the binucleate nature of pollen of P. deltoides. He comments that "division into separate cells was not observed, but no attempt was made to stain especially for this character. This (the binucleate state) is the condition of pollen at the time of shedding".

Brewbaker⁴ presents a summary of the distribution of binucleate and trinucleate pollen in almost 2,000 species. He records the genus Populus as having binucleate grains. However, Brewbaker did not examine Populus himself and he gives no reference for his source of information. Using Brewbaker's summary, Kirby and Smith⁵ again suggest that the pollen of Populus is binucleate.
This study reports the prevalence of trinucleate pollen grains in clones of *Populus nigra* L. var. "italica", *P. yunnanensis* Dode, *P. deltoides* Marsh. var. *angulata* Ait. and *P. alba* L. var. *bolleana* Lauche. This conclusion is based upon the light microscopic examination of whole pollen after staining with lactopropionic-orcein and upon microspectrophotometric determinations of regions of DNA concentration within newly shed pollen grains.

In each species, trinucleate grains were commonly observed, although sometimes the division of the generative cell was incomplete. Depending upon the species and the developmental state of the pollen at shedding, 10 to 20% of the grains may be binucleate. Therefore, it would appear that the second mitotic division of the pollen nucleus occurs shortly before, or during, pollen shedding. Both of the accompanying figures clearly show the presence of one diffuse vegetative nucleus and two, smaller, generative nuclei.

Further support for our findings may derive from Brewbaker's observations. He suggested a correlation between pollen cytology (bi- or trinucleate grains), type of incompatibility (gametophytic or sporophytic), and the site of incompatibility inhibitors (surface or stylar) in homomorphic incompatibility.

There are several characteristics of *Populus* pollen behaviour that match those proposed by Brewbaker for trinucleate, rather than binucleate pollen. For example, the inhibition of incompatible pollen at the stigma surface is a character of the trinucleate class, as is the difficulty of *Populus* pollen to germinate in artificial media, even at short time after shedding. Furthermore, the *Populus* stigma has no copious exudate, a characteristic of plants with a sporophytic incompatibility system. These features, together with the demonstration of trinucleate pollen, indicate a sporophytic type of incompatibility, a conclusion of importance for poplar breeding.
Zusammenfassung.


**REFERENCES**

1. E.C. Smith, J. Arnold Arboretum 24, 275, (1943)
3. C.J. Chamberlain, Bot. Gaz. 23, 147, (1897)
Pollen grain of *P. nigra* var. *italica* shortly after shedding, fixed in 3% gluteraldehyde in 0.2 M cacodylate buffer and stained with lactopropionic-orcein after Dyer. The hypotonic fixative solution has caused the rupture of the pollen wall, with the release of the protoplast. The single diffuse vegetative nucleus and the two denser staining, smaller, generative nuclei are apparent.

The distribution of DNA in a single, whole, pollen grain of *P. nigra* var. *italica* as determined by microspectroscopy. The Feulgen staining was after Fox, using cold hydrolysis. The apparatus used to measure staining intensity was a Zeiss Microscope Photometer, type OS. This was controlled by a Digital PDP-12 Computer under the direction of an APAMOS 2 programme. The figures in the diagram indicate one-tenth of the absorption at 580 nm.

The clear areas represent staining regions that gave less than 5% transmission. These are areas of DNA concentration; the larger being the vegetative nucleus and the two small areas being the generative nuclei.
APPENDIX II

**POPULUS PROPAGATION TECHNIQUES USED IN THIS STUDY**
(based on those established by Willing pers. comm., 1973)

**Female Flowers**

Approximately 3 months prior to the normal time of flowering, 30-40 cm cuttings were taken from stools of the semi-evergreen black poplar clone 65/27* derived from the cross *P. deltoides* X Chilean evergreen, the 'Persistente' form of *P. nigra* var. *italica* (Pryor, 1969). Ten to twenty such cuttings were maintained in a 20 cm pot containing a soil-vermiculite mix, under an intermittent mist spray until rooting was established. Rooted cuttings were transplanted individually into 20 cm plastic pots which had 2 cm peat moss placed at the bottom, and were then fitted with a coarse sand-soil mix.

About 3 to 4 weeks prior to the normal time of flowering, cuttings 20 to 40 cm long, and bearing female flower buds, were taken from clones (including 60/141, 60/156, 60/160, and 60/166) of the American Eastern Cottonwood, *P. deltoides* marsh (section Aigeiros), and either grafted directly, or with the aid of 'bottle grafts', onto the previously established stock (fig. 2.I(b) and 2.II(b)). Grafts were also made using *P. monilifera* material which was available later in the season.

**Pollen**

Pollen was collected from a number of poplar species including *P. tremuloides*, *P. alba* var. *bolleana*, *P. yunnanensis*, *P. sp* (a Tacamahara

* Botany Department, Australian National University, code. Not registered with the International Poplar Commission.
clone), *P. nigra* var. *italica*, *P. deltoides* var. *angulata* and *P. monilifera* var. *aurea*. In each case branches bearing unopened male flower buds were collected about 1 week prior to the normal time of anthesis, and placed in tubs of water in a glass house (fig. 2.I(a)). Paper was spread on the surface underneath the branches and daily collections of pollen made by gently tapping the branches and collecting the pollen from the paper. Pollen thus collected was immediately transferred to a dessicator and left there for 1 to 2 days at room temperature. Pollen was whilst still fresh (shortly after dessication) or after dessication and storage at -18°C for periods up to 9 months.

**Progeny**

Seed was collected following the dehiscence of the capsule (fig. 2.III(a)), freed of 'cotton' and dessicated overnight. It was either stored at -18°C or sown, within a few days of collection, onto trays containing 'perlite' which was then compacted firmly and watered. Trays were then placed in a 2 cm depth of water under an intermittent (5 seconds every 10 minutes) mist sprayer. Following germination, 'Aquasol' nutrient solution was applied twice weekly for about three weeks, by which time the first true leaf pair had unfolded. At this stage it was possible to discriminate between *Aigeiros X Aigeiros* and *Aigeiros X Leuce* progeny (fig. 2.III(a),(b)). Seedlings were then transplanted to wooden flats containing a soil/peatmoss/sand mixture (2:1:1) and transplanted to the field prior to the spring of the following year.
APPENDIX III

Preparation, including methyl esterification, of pollen and stigma extracts for gas-liquid chromatography and mass spectroscopy.

250 mg of pollen was boiled in 20 ml of 15% KOH in a water bath for 1 hour in order to saponify the lipids. The resulting liquid, containing pollen wall 'ghosts', was extracted with 2 x 50 ml ether washes, and the ether phase, containing non-saponifiable lipids (and possibly sterols) put to one side. The aqueous phase, containing fatty acids and other saponifiable lipids, was adjusted to a pH of 1, using concentrated HCl, washed with 2 x 50 ml quantities of ether and the aqueous phase discarded. The ether fraction was dried over anhydrous Na₂SO₄, the ether removed under vacuum (using a rotary evaporator), and the fatty acids methylated by refluxing with a previously prepared anhydrous methanol-HCl mixture (50 ml methanol, 2 ml concentrated HCl) for one hour in a 50°C water bath. After methylation the remaining sample was dried in a stream of nitrogen gas at approximately 40°C then 1 ml of redistilled hexane added. Samples were stored under nitrogen in sealed vials at subzero temperatures.
APPENDIX IV

Methods used for the quantitative analysis of enzymes in pollen extracts (Chapter 5).

(i) 2.6.1.1. Aspartate aminotransferase* (glutamate-oxalacetic transaminase)

Reaction catalysed

\[ \text{L-aspartate + L-oxoglutarate} \rightarrow \text{oxaloacetate + L-glutamate.} \]

Reaction medium (modified from Hatch and Man 1973):

- Hepes buffer pH 7.5 (50 mM)
- L-oxoglutarate (2.5 mM)
- pyridoxal phosphate (1 mg/ml)
- di-sodium ethylene diamine tetracetate (di-sodium EDTA) (1 mM)
- nicotinamide-adenine dinucleotide (di-sodium salt) (di-sodium NADH) (0.25 mM)
- malate dehydrogenase (6 units)
- aspartate (2.5 mM)

Enzyme was added until a final volume of 1 ml was reached. The reaction was started by the addition of aspartate, and the decrease in optical density over 1 hour read at 340 nm using a 'Varian-Techtron' UV-visible dual beam spectrophotometer, Model 635.

The activity was finally computed as µmole substrate/hr/mg protein, based on the total protein estimations conducted as described in Chapter 5.

* The enzyme nomenclature used in this thesis follows the recommendations of the Commission on Biochemical Nomenclature on the Nomenclature and Classification of Enzymes (revised 1972).
(ii) 3.1.1 Esterases
(carboxylic ester hydrolases)

**Reaction catalysed**

\[
\text{A carboxylic ester} + \text{H}_2\text{O} \rightarrow \text{an alcohol} + \text{a carboxylic acid anion.}
\]

The esterase activity was tested with each of a number of substrates including: napthol as acetate, β napthyl caprylate, α napthyl laurate, β napthyl myristate, α napthyl palmitate, α napthyl stearate and β napthyl oleate, all of which were purchased from Sigma Chemicals.

**Reaction medium** (modification of Koeha, 1970).

- Hepes buffer pH 7.5 (25 mM)
- 0.1 ml of substrate being 1.5% (W/V) of the substrate dissolved in water, 70% acetone, or NN-dimethylacetamide (depending on the solubilities of each).

The sample was added and the reaction allowed to proceed for 1 hour at 25°C with continual shaking - after which 1 ml of 0.2% (W/V) fast blue RR was added. Two minutes later 1 ml of 20% trichloracetic acid (T.C.A.) was added, then after 10 minutes, a further 10 ml of ethyl acetate. The mixture was thoroughly shaken and then allowed to partition out. One ml of the upper (ethyl acetate) phase was withdrawn and its optical density compared (at 430 nm) with that of an ethyl acetate blank using the spectrophotometer. The reagent blank was used in each case to correct for non-enzymic colouration.

A different method was devised for determination of lipase activity based on that of Abe *et al.* (1964) and used originally as a histochemical stain. Abe *et al.* differentiated lipase and esterase by the preferred hydrolysis of long chain fatty acid esters by lipase.
Reaction medium (after Abe et al. 1964)

- Tris buffer pH 7.4 (0.4 M) 5 ml
- sodium taurocholate (46.5 mM) 1 ml
- distilled water 3.9 ml
- 2% naphthol as nonanoate
- in NN dimethylacetamide 0.1 ml

Upon addition of the sample the procedure was as outlined above for esterases, and with the same controls.

(iii) 3.1.3.2 Acid phosphatase

Reaction catalysed

An orthophosphoric monoester + H₂O \[ \rightarrow \]

an alcohol + orthophosphate

Reaction medium (modified after Slack and Hatch 1967)

- citric acid buffer pH 4.8 (50 mM)
- p-nitrophenyl phosphate (di-sodium) (10 mM)

Total volume 1 ml. The sample was added and the increase in optical density at 420 nm (due to released of p-nitrophenyl) recorded.

(iv) 3.1.6.1 Aryl sulphatase

Reaction catalysed

A phenol sulphate + H₂O \[ \rightarrow \]

a phenol + sulphate.

Reaction medium (modified after Koehn, 1970)

The procedure was the same as that for the esterases (given above) with the exception that α-napthyl sulphate (K salt) was used as the substrate. The optical density of the reaction mixture was measured at 430 nm, and appropriate controls used.
3.4.11.1 Aminopeptidase

(leucine aminopeptidase)

Reaction catalysed

\[ \text{Aminoaryl-peptide} + H_2O \rightarrow \text{amino acid} + \text{peptide} \]

Reaction medium (after Goldbarg and Rutenberg 1958)

- Hepes-KOH buffer pH 7.1 (50 mM)
- 2 leucyl \( \beta \) napthylamide (0.1 mg/assay)

Final volume 1 ml. After 1 hour at 25°C the reaction was terminated by the addition of 0.5 ml of 2N HCl, then 0.5 ml sodium nitrite (0.2% W/V) was added and mixed immediately. Exactly 3 minutes later 0.1 ml ammonium sulphamate (0.5% W/V) was rapidly blended with the mixture which was then left for a further 3 minutes. Colour was developed by the addition of 2 ml of 50% (W/V) of N-(1 napthyl) ethylene diamine in 95% ethanol. The optical density at 580 nm was recorded after 45 minutes, the results being calculated against \( \beta \) napthylamine as control.
APPENDIX V

Methods used for the preparation of gels for electrophoresis (after Moran, 1975) (Chapter 5).

Starch gels were made using 13% hydrolysed starch (Connaught Laboratories, Toronto) in a mixture of two buffers (Brewbaker et al. 1968). Buffer '126A' pH 12.2, consisted of 0.05 M lithium hydroxide and 0.2 M boric acid in distilled water, whilst buffer '126B' (pH 8.2) contained 0.01 M citric acid and 0.065 M 'Tris' in distilled water. Ten parts of 126A were mixed with 1 part of 126B to produce a buffer with a final pH of 8.4. Gels were used shortly after preparation.

10% acrylamide gels were prepared by first mixing the following components (to make one gel). The proportions for 7% gels are given in brackets.

- 190 mls Tris-citric buffer, pH 8.7
- 18.05 g monoacrylamide (12.60 g for 7%)
- 0.95 g N,N' methylene bisacrylamide (0.67 g for 7%)
- 0.2 g ammonium persulphate

When mixed, 0.3 mls of dimethyl-amino-propionitrile (DMAPM, Cyanimid, Australia) was added, and the mixture poured into moulds designed to exclude all air from the gel surface during polymerization, which takes place within 1 hour. Although the gels may be stored for several weeks, in all cases they were used shortly after preparation. The methods of preparation were those of Moran (1975).
APPENDIX VI

Reaction media (stains) used for the gel electrophoretic study of enzymes (Chapter 5).

1.1.1.1 Alcohol dehydrogenase (ADH)

Reaction catalysed

\[
\text{alcohol + NAD} \quad \rightarrow \quad \text{aldehyde or ketone + NADH}
\]

Reaction medium (after Shaw and Prasad, 1970)

- nicotine adenine dinucleotide (NAD) 20 mg
- nitro-blue tetrazolium 10 mg
- phenazine methosulphate (PMS) 2 mg
- phosphate buffer (0.2 M) pH 7.0 90 ml
- substrate: ethanol 5 ml

Enzyme activity indicated by blue staining.

1.1.1.37 Malate dehydrogenase (MDH)

Reaction catalysed

\[
\text{L - Malate} + \text{NAD} \quad \rightarrow \quad \text{oxaloacetate} + \text{NADH}
\]

Reaction medium (after Shaw and Prasad, 1970)

- NAD 15 mg
- nitro-blue tetrazolium 20 mg
- PMS 2 mg
- phosphate buffer pH 7.0 (0.2 M) 90 ml
- substrate: sodium malate pH 7.0 (1 M) 10 ml
1.4.1.2 Glutamate dehydrogenase (GDH)

Reaction catalysed

\[
\text{L-glutamate} + \text{H}_2\text{O} + \text{NAD} \rightarrow 2\text{-oxoglutarate} + \text{NH}_3 + \text{NADH}
\]

Reaction medium (Shaw and Prasad, 1970)

As for ADH, but with a substrate of 10 mls of sodium glutamate pH 7.0 (1 M).

Enzyme activity (both MDH AND GDH) indicated by blue staining.

1.11.1.7 Peroxidase

Reaction catalysed

\[
\text{oxidized donor} + \text{H}_2\text{O}_2 \rightarrow \text{oxidized donor} + 2\text{H}_2\text{O}
\]

Reaction medium

- O-dianisidine, 100 mg
- ethanol 5 ml
- sodium acetate buffer pH 5 (0.2 M) 100 ml
- substrate: hydrogen peroxide, 2 ml, added just prior to use.

Enzyme activity indicated by brown staining.

2.6.1.1 Aspartate aminotransferase

(glutamic oxaloacetic transaminase [GOT])

Reaction catalysed

\[
\text{L-aspartate} + 2\text{-oxoglutarate} \rightarrow \text{oxaloacetate} + \text{L-glutamate}
\]

Reaction medium (after Brewbaker et al. 1968)

- A. fast violet B salt 30 mg
- water 2 ml
B. Polyvinyl pyrrolidone (PVP)  250 mg
phosphate buffer pH 7.5 (0.2 M)  5.5 ml
pyridoxyl - 5 - phosphate  0.2 ml
and as substrates: (added to B)
L - aspartate (0.2 M)  1.7 ml
and alpha-keto glutarate (2-oxoketo-glutamate)  0.7 ml
each previously adjusted to pH 7.5 using KOH (1.46 mg/ml). Albumen
was omitted from the recipe of Brewbaker et al. (1968) as Mandryk
(1971) has determined that its omission is not detrimental to
the result. Transaminase activity is indicated by pink colouration.

3.1.1.6 Esterase

Although relatively non-specific (Dixon and Webb, 1960), the action
of esterase in this instance (with napthyl acetate as substrate) is as
follows:

\[ \text{An acetic ester} + \text{H}_2\text{O} \rightarrow \text{an alcohol} + \text{acetate.} \]

Reaction medium

- phosphate buffer pH 7.5 (0.2 M)
- water  40 ml
- fast blue RR salt  100 mg
- substrate: either \( \alpha \) or \( \beta \) napthyl-acetate using 3 ml of a
  1\% solution in 70\% ethanol (or in some cases 1.5 g of each
  of \( \alpha \) and \( \beta \) napthyl acetate combined).

As blue colouration usually indicates the presence of esterase
activity, although in some cases there was a metachronic effect with
different esterase isozymes staining a brown colour, an effect noted
earlier by Grover (1974). It is conceded, however, that the brown stained
region could also be produced where azo dyes have bound non-specifically
to polyphenols and phenolicglycosides in the extracts (as suggested by
Knox (pers. comm.)) since the control for this plate was not run
due to limited availability of materials.
3.1.3.2 Acid phosphatase

Reaction catalysed

An orthophosphoric monoester + $H_2O$ → an alcohol + orthophosphate.

Reaction medium (after Brewbaker et al. 1968)

- Acetate buffer pH 4.0 (0.2 M) 100 ml
- Fast blue RR salt 100 mg
- Polyvinyl pyrrolidone (PVP) 200 mg
- Sodium chloride 1 g

Substrate: α-napthyl acid phosphate, 100 mg - mixed just prior to staining.

Acid phosphatase activity is evidenced by blue staining.

3.4.11.1 Aminopeptidase-cytosol

(leucine aminopeptidase)

Reaction catalysed

Aminoacyl-peptide $+ H_2O$ → amino acid + peptide.

This enzyme has a broad specificity, hydrolysing most L-peptides, and splitting off an N-terminal residue with a free amino group. It also has esterase activity (Commission on Biochemical Nomenclature 1975).

Reaction medium (after Brewbaker, 1968)

- Tris A buffer (0.2 M) 50 ml
- Tris B buffer (0.2 M) 10 ml
- Water 40 ml

Substrate: 1-leucyl-beta-napthylamide hydrochloride 40 mg.
APPENDIX VII

The Immunoelectrophoresis of *Populus* pollen extracts

**Method**

Gels were prepared in the same manner as for the double diffusion, after which 5 holes were cut in each gel using an LKB template. The electrophoresis was performed in an LKB 2117 'Multiphor' tank, equipped with a cooling system, using a current of 33mA, the voltage being 200V, and supplied by an LKB, 3371E D.C. power supply. The distance between the electrode wicks was 7.5 cm, and veronal buffer pH 8.6 was used as the tank buffer. After 2 hours electrophoresis of the antigen, the gel was removed from the system, troughs were cut with the template, and 100 µl of antiserum added to each trough. The gels were then placed in a moist chamber for two days after which the precipitin patterns were then recorded by drawing as for double diffusion, after which 5 holes were cut in each gel using an LKB template. The electrophoresis was performed in an LKB 2117 'Multiphor' tank, equipped with a cooling system, using a current of 33 mA, the voltage being 200 V and supplied by an LKB 3371E D.C. power supply. The distance between electrode wicks was 7.5 cm, and veronal buffer, pH 8.6 was used as the tank buffer.

After 2 hour electrophoreses of the antigen, the gel was removed from the system, troughs cut with the template, and 100 µl of antiserum added to each trough. The gels were then placed in a moist chamber for two days after which the precipitin patterns were recorded as before.

The antigens placed in the wells were each homogenates of pollens made within 10 minutes of their immersion in isotonic buffer. Antisera were those obtained from rabbits challenged with pollen homogenate extracts.
Results and Discussion

The results are presented in fig. A.VII. Clearly there are more antigens in the pollen than could be resolved by simple double diffusion techniques.

Although a proper analysis is not offered of these (preliminary) results, it is already clear that both *P. bollæana* (B) and *P. tremuloïdes* (T) pollens contain an antigen which moves towards the cathode during electrophoresis. This precipitin band is only seen when both the antigen and the antiserum are from pollen of the section *Leuce*. 
Diagram A.VII

Drawings of precipitin lines obtained on immunoelectrophoretic plates. Antigens (*Populus* pollen homogenates) were placed in the wells, and antisera (against *Populus* pollen homogenates) placed in the troughs.

The abbreviations are defined in Chapter 6.
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