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**DEVELOPMENT OF ANTHERS AND POLLEN GRAINS
OF *BRASSICA NAPUS* L.
A HISTOLOGICAL AND HISTOCHEMICAL STUDY**

by

Caroi Lorraine Wenzel

**A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Master of Science**

**Carleton University
Ottawa, Canada
January, 1991**

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Development of Anthers and Pollen Grains
of *Brassica napus* L.
A Histological and Histochemical Study.

submitted by Carol Lorraine Wenzel, B.Sc. (Hons)
in partial fulfilment of the requirements for the
degree of Master of Science.

Thesis supervisor

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Carleton University
January, 1991

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ABSTRACT

The development of anthers and pollen grains of *Brassica napus* is examined, in particular the vacuole and its contents within pollen grains. Anther development is similar to most angiosperms except for the presence of epidermal 'motor' cells which may contribute to pollen dispersal. Vital staining with Toluidine blue O reveals that the large central vacuole in the live grains breaks up into small vesicles around the first mitosis. Vital staining, chromatography of extracts of these grains, and analyses of the specificity of Toluidine blue O for various purified compounds, suggest that these vesicles accumulate phenolic compounds, possibly flavonoids. In fixed and sectioned pollen grains there is a sudden occurrence of heavy cytoplasmic staining and poor nuclear staining which coincides with the onset of accumulation of phenolic compounds within the vesicles. Treatment of pollen grains and other 'model' plant cells with conventional chemical fixatives and freeze substitution procedures suggests that this is an artifact probably caused by mordanting of released vacuole compounds throughout the cell.

ACKNOWLEDGEMENTS

I would like to thank Dr. Margaret E. McCully for her patience and guidance throughout this thesis and to Dr. Wilfred A. Keller for financing most of this work. I would also like to thank Drs. Stephen C. Fry and Richard W. Horobin for their help with the analyses of Toluidine blue O, Drs. John A. Webb and Martin J. Canny for some useful comments on the project, and Dr. David Gianassi for sending me information on flavonoids. Assistance in translation of some German literature was given by Dr. Basil Mogridge and much appreciated technical assistance by Linda J. Sealey. Finally, I would like to thank Ilona Fiser and Linda Languille for help with chromatography and UV spectroscopy, and Dr. Rukmal Abeysekara and Thomas P. Wilson for generously helping with the plates and binding of this thesis.

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CHAPTER 1
GENERAL INTRODUCTION

1 History and economic use of plants of the genus *Brassica*

Brassica napus L. is a member of the Cruciferae, a family of major economic importance including crop plants grown for vegetables, salads, animal feed, oilseed, mustard condiments and industrial purposes. Some of the economically important *Brassica* species include *B. oleracea* L. (eg. cabbage, kale, brussel sprouts, cauliflower, broccoli), *B. napus* L. (rapeseed), *B. rapa* L. (turnip), *B. campestris* L. (eg. field mustard - mostly a weed), *B. juncea* L. (eg. brown mustard), *B. nigra* L. (black mustard), *B. carinata* L. (Abyssinian mustard) and *B. hirta* L. (white mustard) (Bailey, 1951). This study will focus on rapeseed, also called canola.

The name rape is derived from the latin word *rapum*, meaning turnip (Weiss, 1983). Rapeseed may have originated in the Afghano-Pakistan region, other Mediterranean areas, and possibly the Turko-Iranian region. It appears to have been first cultivated in the Eurasian region - possibly in India, China and Japan - around 2000 BC, with cultivation in Europe beginning around the thirteenth century (Weiss, 1983). Originally it was mainly used as a lamp oil (Weiss, 1983). During World War II demand for special lubricating oils arose (Weiss, 1983) resulting in the introduction of *B. napus* into Canada to be used for the production of marine oil and oil for aircraft engines (Runciman and Olson, 1975). *Brassica* oilseed now ranks about fifth in importance behind soya bean, cotton seed, groundnut and sunflower seed (Crisp, 1976; Weiss, 1983). In Canada *B. napus*, soya beans, flaxseed and sunflower seed are the four major oilseed crops (Runciman and Olson, 1975). Canada is the world's leading exporter of rapeseed, exporting mainly to Japan, India and other

Asian countries (Weiss, 1983). France, West Germany and Poland are other main exporters of rapeseed oil (Weiss, 1983).

Today rapeseed oil with high erucic acid (a long, 22 carbon chain, fatty acid) content is used for many industrial purposes such as grease, jet engine lubricants, polyester resins and plastics, flotation agents in potash refining, detergent manufacture, and rubber substitutes, to name a few (Runciman and Olson, 1975). Initially, rapeseed oil's usefulness for human consumption was limited. High erucic acid content in rapeseed can have detrimental effects on the digestive system, reproduction and on cardiac tissue (Beare-Rogers, 1975) and legislation banning the use of oil with high erucic acid content for human consumption has been introduced (Weiss, 1983). The presence of glucosinolates in the seeds and other plant parts renders meal unpalatable and toxic to livestock (Weiss, 1983). Consequently, rapeseed breeders have concentrated on reducing these components for crops intended for human or livestock consumption. In Canada (and elsewhere) rapeseed 'double-zero' cultivars low in glucosinolate and erucic acid have been developed (Downey *et al.*, 1975). Today rapeseed oil is commonly used in animal feeds and margarines. Breeders are now attempting to increase the desirable linoleic fatty acid content of rapeseed while decreasing the undesirable linolenic fatty acid content which decreases the oil's shelf life (Downey *et al.*, 1975).

2 Breeding homozygous lines

Homozygous lines are used in plant breeding to increase the degree of genetic and phenotypic similarity within a crop so that these crops can be used as cultivars or as parents for F_1 and other

hybrid cultivars (Crisp, 1976). Five or six generations of inbreeding by self pollination produces these homozygous lines in crucifers (Crisp, 1976). In contrast to the lengthy traditional breeding methods there is now world-wide interest in the production of homozygous plants from 'haploid' culture of male or female gametophytes. Basically, this involves the culture of pollen (either isolated or within anthers) or ovules in artificial medium in order to induce them to divert from their normal gametophytic to a sporophytic pathway (ie the artificial production of an embryo by unfertilized gametes). The haploid chromosome complement can then be doubled at some stage in the subsequent sporophytic development to produce, hopefully, a completely homozygous plant. The use of haploid culture to obtain homozygous plants in a short period of time is therefore considered to have potential uses in breeding programs (Chase, 1974; Winton and Stettler, 1974; Crisp, 1976; Keller *et al.*, 1987; Foroughi-Wehr and Wenzel, 1989).

The spontaneous formation of haploid embryos (ie one form of apomixis) can occur without the fusion of gametes from, for example, development of an unfertilized egg cell (parthenogenesis), some other cell in the female gametophyte (apogamy), or from a pollen grain (androgenesis) (Juan-Ramon, 1974). (Gametophytes of lower plants represent one case of 'normal' haploid individuals - Juan-Ramon, 1974.) The rate of spontaneous apomixis involving haploid cells in nature in most species is too low to be of use for plant breeding schemes and hence the *in vitro* induction of embryogenesis from gametophytes represents a possible method for large scale production of haploid embryos (Chase, 1974).

Due to the large number of pollen grains within the anther, culture of pollen grains is potentially the best method for production of embryos. There is still relatively little known about what induces pollen grains to undergo embryogenesis. Induction of embryogenesis seems to depend on external factors such as the genotype and growth conditions of the donor plant, and on the culture temperature and medium composition (Keller, 1984; Keller *et al.*, 1987). However, the selection of pollen grains at the appropriate stage in their development seems to be the most important factor. There appears to be an embryogenic 'window' from the early uninucleate to early binucleate stage in pollen grain development in most species tested, during which induction of embryogenesis is optimal (Keller *et al.*, 1987). Bud length is usually used to select the appropriate cytological stage for pollen grain culture. There is some evidence that induction of embryogenesis in cultured pollen grains of some species involves the synthesis of new mRNAs, but more data is required (Raghavan, 1987).

3 Purpose of this study

In vitro induction of embryogenesis from pollen grains or anthers of *Brassica napus* has been achieved at a frequency of about 5% of the total number cultured (eg. Keller and Armstrong, 1977 and 1978; Lichter, 1982; Chuong and Beversdorf, 1985; Fan *et al.*, 1988). To determine why so few pollen grains are induced to form embryos (and hence possibly increase the frequency) and to further characterize the embryogenic 'window' in pollen grain development, it is first essential to understand the gametophytic pathway leading to normal pollen grain development. It is only through knowledge of

the normal pollen grain development in relation to its normal environment within the anther that the alternative embryogenic pathway taken by a few pollen grains when placed in an artificial medium can be understood. This study therefore examines the *in vivo* development of anthers and pollen grains of *Brassica napus*.

4 General anther development

The stamen consists of the anther and filament and most anthers have similar features. Each anther is usually bilobed, with sporogenous tissue from which the pollen grains develop lying within 'locules' in the center of each lobe. There are two or four locules per anther (one or two per lobe respectively) extending the entire length of the anthers - these become cavities at maturity. Surrounding the locules are four specialized cell layers - from the inside to outside, the tapetum, the middle layer(s), the endothecium and the epidermis.

The tapetum is considered essential for the nourishment of the developing pollen grains either directly by the metabolism and subsequent secretion of nutrients, or indirectly as food materials from elsewhere (in the anther or other plant parts) pass through the tapetum into the locules (Echlin, 1971). It is also assumed to be involved in the formation of sporopollenin which is eventually deposited in the outer wall of the pollen grain, the exine, and the synthesis and release of tryphine and pollenkit - substances composed primarily of lipid which are deposited on the surface of pollen grains upon final release of tapetal cell contents into the locule (Bhandari, 1984).

There are two types of tapetum, the secretory/glandular type and the amoeboid/plasmodial type. In the amoeboid type, the

tapetal cell walls lyse and the intact protoplasts become amoeboid and intrude into the locules amongst the developing pollen grains, and/or create a 'periplasmodium' of fused protoplasts along the periphery of the locule (Echlin, 1971; Esau, 1977). Shortly before anthesis (*ie* the period of flowering) the protoplasts dehydrate and deposit their contents onto the pollen grains (Esau, 1977). The secretory tapetum remains relatively intact until its maturity when the walls are lysed and the released cytoplasm is deposited onto the pollen grains (Echlin, 1971; Esau, 1977). The secretory tapetum is said to be short-lived and disappears around the time of pollen grain release from the tetrad (Echlin, 1971; Esau, 1977), but this timing is extremely variable (Bhandari, 1984). Mauseth (1988) distinguishes the two types of tapetum simply as the 'parietal' (secretory) tapetum which remains intact at the periphery of the locules secreting material into the anther sac, and the 'invasive' (amoeboid) tapetum in which the cell walls of the tapetum are lysed, releasing protoplasts into the sac. Only the secretory tapetum produces Ubisch bodies, also termed orbicules, which apparently consist of sporopollenin, the main constituent of the pollen grain exine. The function of Ubisch bodies is uncertain. They are generally considered to be by-products of tapetal sporopollenin synthesis (Echlin, 1971; Heslop-Harrison, 1971) but may facilitate pollen dispersal (dehiscence) by providing a hydrophobic surface within the locule (Echlin, 1971; Gunning and Steer, 1975).

The number of cell layers in the middle layer varies - generally these layers are crushed early in anther development (Esau, 1977). The endothelial cells form a 'fibrous' layer underneath the epidermis

and develop band-like secondary wall thickenings in most anthers predominantly those which have longitudinal dehiscence along the stomium (a specialized region between locules of the same lobe consisting of epidermal cells with primary walls only) (Eames, 1961a; Esau, 1977) although Gerenday and French (1988) have found secondary thickenings in endothelial cells of anthers which dehisce by an apical pore mechanism. The shrinkage of these cells during anthesis is assumed to result in the dehiscence of the anthers (Eames, 1961a; Stanley and Kirby, 1973).

5 General development of angiosperm pollen grains

Pollen grain development occurs throughout the development of the anther. Initially, each of the numerous diploid pollen mother cells in the locules undergoes meiosis to produce four haploid nuclei. Cytokinesis occurs either 'simultaneously' after meiosis 2, or 'successively' after both meiosis 1 and 2, to produce the tetrad of microspores (Esau, 1977). Callose is deposited on the inside of the mother cell walls (which later almost completely disintegrate), separating the microspores from one another except where they are joined by very wide (ie as much as 1.5 μm) cytoplasmic bridges (Esau, 1977). The term microspore is also used to describe uninucleate pollen grains at any stage in their development. Generally after release from the tetrad, the uninucleate pollen grains undergo mitosis to produce a generative and a vegetative nucleus (responsible for the production of the male gametes and the gamete of the pollen tube respectively). The generative nucleus and its associated cytoplasm is always separated, by the formation of a plasma membrane or some type of wall, from the vegetative nucleus

with its associated cytoplasm thus forming two distinct cells before the first mitosis of the pollen grain. The generative cell eventually (either before or after pollen release) divides mitotically to form the two sperm nuclei.

While still within the tetrad, the pollen grains develop their first wall, the primexine (except in regions of the future apertures), which is probably composed of cellulose and is considered the 'template' for later exine formation (Heslop-Harrison, 1971). 'Probacula', which are said to be composed of lipid and protein and resemble in form the mature exine, soon develop in the primexine. Protosporopollenin is deposited on the probacula (Esau, 1977). The mature exine is composed mainly of sporopollenin, which is probably a polymer of carotenoids (Brooks and Shaw, 1971). Deposition of sporopollenin commences within the tetrad with the extracellular polymerization of protosporopollenin on the primexine (Heslop-Harrison, 1971). However, sporopollenin deposition occurs mainly after release of the pollen grains from the tetrad, presumably when sporopollenin precursors from the tapetal cells are secreted into the locules and subsequently polymerize on the exine (Heslop-Harrison, 1971). The exine gradually extends over the aperture regions. The inner wall layer, the intine, is composed mainly of pectin and hemicellulose, and is generally formed (starting in the aperture region but gradually extending around the entire protoplasm) after commencement of sporopollenin deposition on exines of pollen grains still within the tetrad or after being released (Heslop-Harrison, 1971). Finally, debris from the disintegrated tapetum is deposited onto the exine (Heslop-Harrison, 1971; Esau, 1977). The colour of

pollen grains is said to be derived from carotenoids and/or flavonoids; the function of these pigments is still unclear (Stanley and Linskins, 1974).

The cytoplasm of the pollen grain does not have vacuoles when the grains are first released from the tetrads. However a large vacuole is soon formed, pushing the nucleus against the pollen grain wall (Esau, 1977; Knox, 1984). The origin of the vacuole is uncertain, as is true for all plant cells. Vacuole development has been attributed to many different mechanisms including: dilation and/or vesiculation of endoplasmic reticulum cisternae; fusion of vesicles derived from the Golgi; enlargement of pre-existing vacuoles; de novo synthesis of a tonoplast around hydrophilic compounds in the cytoplasm (Gunning and Steer, 1975; Esau, 1977). After the first mitosis of a pollen grain the cytoplasm of the vegetative cell accumulates large amounts of storage reserves - proteins, lipids, polysaccharides (P particles), and/or other carbohydrates (Knox, 1984). At this stage the vegetative cell's cytoplasm becomes very dense (Knox, 1984). Reports on the subsequent development of the vacuole in pollen grains of different angiosperm genera vary (discussed later).

CHAPTER 2
DEVELOPMENT OF ANTHERS AND POLLEN GRAINS
OF *BRASSICA NAPUS* L.

1. INTRODUCTION

Anther, and especially pollen grain, culture of the economically important rapeseed, *Brassica napus*, have gained much popularity as methods for the production of haploid embryos (eg. Keller and Armstrong, 1978; Dunwell *et al.*, 1983 and 1985; Chuong and Beversdorf, 1985; Swanson *et al.*, 1987; Fan *et al.*, 1988; Gland *et al.*, 1988; Aslam *et al.*, 1990). In spite of the attention given to the culture of pollen grains of *B. napus*, however, there have been few studies examining the *in vivo* anther and pollen development in this species. A better understanding of the normal development of anthers and pollen grains of *B. napus* in itself is important but it may also shed light on the alternative embryogenic pathway(s) involved in *Brassica* haploid culture systems.

My study examines the development of anther and pollen grains from the stage at which the pollen grains are released from the tetrad up to pollen grain maturity using primarily conventional histological and histochemical methods of fixed and sectioned cells, and vital staining of live pollen grains. A brief structural study of anther cell development and its dehiscence mechanism is done. However, I primarily examine the development of the vacuole and its contents within the pollen grains and relate these features to cytoplasmic staining patterns using a variety of dyes observed by optical microscopy.

There are a number of studies on various aspects of pollen grain development in *Brassica* species. Grant *et al.* (1986) also did a general optical microscopy study of fixed, sectioned and stained (with Toluidine blue O) tapetum and pollen grains in *B. napus* but

only up to the stage of the first mitosis of the pollen grains. Smith and Scarisbrick (1990) examined the morphology and histology of all stages of the developing floral parts of *B. napus* (including the anthers, although they did not examine anther cell structure and dehiscence mechanisms) and related this development to the general histology of fixed, sectioned and stained (with safranin O and fast green) developing pollen grains. Both Grant *et al.* (1986) and Smith and Scarisbrick (1990) state that just before mitosis the cytoplasm in the pollen grain becomes very darkly stained and appears to be 'avacuolate' in comparison to the presence of a large vacuole and light cytoplasmic staining in less mature pollen grains. Using the fluorochrome fluorescein diacetate (which stains the cytoplasm and presumably outlines the vacuole) on live pollen grains of *B. campestris*, Singh *et al.* (1985) detected the presence of a large vacuole in uninucleate pollen grains, but no evidence of a vacuole(s) in more mature pollen grains was found using this method. Zaki and Dickinson (1990) examined fixed and sectioned late uninucleate pollen grains of *B. napus* treated with stains specific for protein, carbohydrate and lipid and found light cytoplasmic staining and the presence of 'amorphous deposits' within the otherwise unstained large vacuole. In this paper they did not examine all stages of pollen grain development in *B. napus* although they state that during culture of the uninucleate pollen grains the large central vacuole 'disintegrates into smaller organelles', an event they claim occurs *in vivo* as well. Ultrastructural studies on the vegetative cell cytoplasm in fixed and sectioned mature pollen grains were done on *B. oleracea* (Dumas *et al.*, 1985; Elleman and Dickinson, 1986) and *B. napus*

(Charzynska *et al.*, 1989). These all mention the presence of vesicles or other spherical 'bodies' of unknown origin or said to be derived from dictyosomes - the fate of the original large vacuole was not discussed in any of these studies. Cytochemical methods using azo dyes to detect β -galactosidase activity in live (whole) or fixed and sectioned pollen grains of *B. campestris* suggested the presence of this enzyme activity in the intine and peripheral cytoplasm during the early vacuolate period and prior to the generative cell division (Singh and Knox, 1984 and 1985; Singh *et al.*, 1985). In addition, Fan *et al.* (1988) examined nuclear fluorescence (with the DNA binding fluorochrome 4',6-diamidino-2-phenylindole) in whole pollen grains at all stages of development for *B. napus*, and Swanson *et al.* (1990) used Hoechst 33342 DNA stain, Tinapol 5BM as an intine stain, and 3,3'-diethyloxadicarbocyanine iodide, DiOC₂, (normally a mitochondrial stain) as a stain for the exine in whole uninucleate pollen grains of *B. napus* - no mention of the vacuole or cytoplasm is made in either of these two studies.

In general, most authors agree that immature pollen grains of *Brassica* and other angiosperm genera contain a large vacuole. However, reports on the development of this large vacuole after the first mitosis of the pollen grain vary. Many authors state that this vacuole disappears, presumably because it is progressively 'resorbed' into the cytoplasm (Echlin, 1972; Heslop-Harrison, 1972; Vithanage and Knox, 1979; Knox, 1984; Grant *et al.*, 1986; Barnes and Blackmore, 1988; Raghavan, 1988; Smith and Scarisbrick, 1990). Others state (or imply) that the large vacuole breaks up into smaller vesicles (Esau, 1977; Sawhney and Bhadula, 1988; Zaki and Dickinson,

1990). There are numerous studies of mature pollen grains which contain vesicles said to be of unknown origin or thought to originate either from vesiculation of dictyosomes and/or dilated ER cisternae (Schroder, 1985; Elleman and Dickinson, 1986; Lord and Eckard, 1986; Sack *et al.*, 1988; Charzynska *et al.*, 1989; Weber, 1989). This survey demonstrates some of the controversy on the development of the vacuole within pollen grains.

In my study I examine the structure but also the contents of the vacuole(s) in developing live pollen grains of *B. napus* using vital staining, primarily with the dye Toluidine blue O. There are reports in the literature (see review by Stadelmann and Kinzel, 1972) which suggest that staining of live cells with certain dyes such as Toluidine blue O can be used to distinguish vacuoles which do not contain phenolic compounds (ie 'empty' saps) from those vacuoles which do contain phenolics (ie 'full' saps). Chemical analyses of extracts of these developing pollen grains is attempted for identification of the compounds within the vacuoles/vesicles. In addition, conventional chemical fixation methods and a freeze substitution procedure done on mature pollen grains and other 'model' plant cells are used to determine the effects of these protocols on the subsequent cytoplasmic and nuclear staining patterns of fixed and sectioned pollen grains.

2. MATERIAL AND METHODS

2.1 Plant material and growing conditions

The plants used were *B. napus* L. cv Topez 4679. Four seeds were buried approximately one inch deep in each 13 inch fiber pot containing the following soil mixture: 1 part sterilized soil (Central Experimental Farm, Ottawa); 3 parts ASB peat substitute (Greenworld Gardening Products, Pointe Sapin, New Brunswick); 1 part sterilized sand; 1 part perlite (Terra-Lite (Grace Co.) Horticultural Products, Ajax, Ontario); 1 lb (454 g.) of lime added per 30 bushels of mixture. After 1-2 weeks growth the best seedling in each pot was chosen and the others were removed. After 3 weeks of growth, 26 g. of Nutricote (controlled release fertilizer; 14:14:14, nitrogen: phosphoric acid: potash) was scattered on top of the soil in each pot.

Plants were grown in growth cabinets maintained at 70% relative humidity, maximum 300 footcandles fluorescent and incandescent illumination with a 16 hour daylength from 07:00 to 23:00 hours. The seedlings/plants were watered twice a day and given a 20:20:20, N:P:K mixture daily in addition to Hoagland's solution (Type B, full strength) once a week. For the first six weeks of growth, the plants were maintained with a 'light' temperature (ie. 16 hour daylength) of 20°C and a 'dark' temperature (ie. 8 hour night) of 15°C. After approximately six weeks, when the primary inflorescence (from the shoot apex) was just emerging, the plants were transferred to a growth cabinet with a 'light' temperature of 10°C and a 'dark' temperature of 5°C. These growth conditions are identical to those for plants used for pollen/anther culture; the temperature at which donor plants are grown is considered an

important factor for the induction of microspore embryogenesis (p.c., W.A. Keller).

Primary inflorescences (formed at the shoot apex) and lateral inflorescences (formed from lateral buds on peduncles and/or in leaf axils) with varying numbers of buds and open flowers taken from plants of different ages were examined. *B. napus* inflorescences are a raceme, bearing single flowers on pedicels arranged spirally on the peduncle. The oldest buds/flowers are at the base of the inflorescence with new buds being produced continuously at the apex (Fig. 1a). Development of the anthers and pollen grains can thus be followed by examination of buds and flowers at appropriate developmental stages within individual inflorescences (Fig. 1b).

The six stamens within each flower are tetradynamous: there is an outer pair of stamens with short filaments and two inner pairs with longer filaments (Heywood, 1978; see Fig. 1c). The inner pairs of stamens are covered by a thin pair of sepals which overlaps a wider pair of sepals covering the outer pair of stamens (Fig. 1d). Since the sepals develop earlier, even in the young buds when the filaments have not yet elongated, the inner and outer pairs of stamens can be easily differentiated. Although preliminary studies indicated no obvious structural difference among the six anthers only the inner pairs were used for the work done using fixed and embedded tissue, so as to minimize, as much as possible, any developmental heterogeneity amongst anthers (see below).

2.2 Anther development

The external and internal structure of a minimum of ten anthers at various stages of development was studied. External

Figure 1

Fig. 1a: Lateral inflorescence showing the oldest buds/flowers at the base of inflorescence and youngest unopened buds at the apex.

0.7 X.

Fig. 1b: Buds and flowers taken from a primary inflorescence and lined up - oldest flowers at the top and youngest buds on bottom with decreasing maturity of buds/flowers from left to right hand side of each row. 0.6 X.

Fig. 1c: Petals and sepals removed to show stamens and carpel.

From left to right: 4 mm bud; 8 mm bud; early anthesis - filaments elongating and inner anthers turning away from carpel; mid anthesis - anthers curling back at tips; late anthesis - filaments fully elongated, anthers completely dehisced. 1.5 X.

Fig. 1d: Sepals from 8 mm bud. Pair of sepals covering four inner stamens on left side and pair of sepals covering outer two stamens on right. 2.5 X.

Figure 1

Fig. 1e: Large 'motor' epidermal cells on back of basal region of anther. Base of anther beyond bottom of micrograph. 12 X.

Fig. 1f: Large 'motor' epidermal cells in upper portion of anther. Tip of anther beyond top of micrograph. 15 X.

Fig. 1g: Front of anther showing longitudinal slit progressing down furrow between locules of each lobe during anthesis. 8 X.

Fig. 1h: Back of anther showing collapsed 'motor' cells in fully dehiscent anther late in anthesis. 11 X.

Figure 1

features were examined with a stereomicroscope (see section 2.4). Internal features were initially studied using hand-cut sections (using a one-edged razor blade) of fresh material. Subsequently, selected anthers were chemically fixed and embedded in plastic for histological and histochemical examination. In general, anthers were sectioned throughout their entire length.

2.2.1 Preparation of embedded material

Each bud examined (petals removed from those with open flowers) was completely immersed in a drop of 3% glutaraldehyde (J.B. EM Inc., no. JBS-013A) at pH 6.8 in 0.025 M potassium phosphate buffer with or without 0.5-1% Triton X-100 (Sigma Co.) - the drop was placed on dental wax. The anthers were then excised (using tweezers and a razor blade) from the bud lying within the fixative solution. Some anthers were fixed, instead, in 10% aqueous acrolein (Polyscience Ltd., EM grade, no. 00016) containing 0.5% Triton X-100. For both fixation procedures the anthers were either left intact or cut in half lengthwise between the lobes, hence leaving the locules intact. In addition, to facilitate fixative access and plastic infiltration into the locules, some of these anthers were also cut transversely or their locules were punctured with a needle tip along their entire length.

Fixations were done overnight, on ice. The next day the tissue was rinsed (on ice) three times (10 min. each) in either (a) pH 6.8, 0.025 M potassium phosphate buffer for the glutaraldehyde fixation or (b) distilled water for the acrolein fixation. All subsequent processing was identical for both fixative treatments. The tissues were dehydrated with a methyl cellosolve series at 0°C and then

infiltrated with glycol methacrylate monomer mixture as per O'Brien and McCully (1981). The glycol methacrylate monomer mixture contains: 95 ml glycol methacrylate (GMA; Hartung Associates, New Jersey); 5 ml polyethylene glycol 200 (Ruger Chem. Co., Inc., New York); and 0.15 g of the accelerator azobis (2 methyl propionitrile) from Eastman Chemicals. Briefly, the dehydration solvents used were methyl cellosolve, ethanol, propanol, and butanol respectively - two changes (30 min. each) of each solution were done on ice. The last butanol change was warmed to room temperature then replaced with butanol:GMA monomer mixture (1:1) for 30 min. Fresh 100% GMA monomer mixture was added and the tissue left overnight at room temperature in darkness. The next day fresh 100% GMA monomer mixture was added and the tissue then left undisturbed for one week in the dark. Tissue pieces were polymerized in 100% monomer mixture GMA at 60°C or with UV light at room temperature. Oxygen was excluded for both polymerization methods by placing the GMA monomer mixture and tissue into an aluminum weighing dish and (a) putting another dish directly on top of the GMA monomer mixture for those tissues polymerized at 60°C, or (b) exposing to UV light in a sealed plexiglass box, maintaining a constant through-flow of nitrogen gas. Transverse and longitudinal sections were cut about 2 μ m thick (using a 'Porter-Blum' Ultra Microtome MT-1 (Sorvall Inc., Connecticut, USA) and glass knives) throughout the length of the anthers.

2.2.2 Histochemical analyses

Both hand-cut and plastic-embedded sections (on slides for plastic-embedded tissues) were stained with Toluidine blue O and

iodine/potassium-iodide (IKI). All other stains mentioned below were only used for plastic-embedded materials.

2.2.2.1 General cytoplasmic and nuclear staining

Sections were stained for 10-60 sec. with 0.05% Toluidine blue O (C.I. 52040 : Fisher Sci., lot 735481, or; Polysci. Inc., lot 73736) in pH 4.4 potassium benzoate buffer (see O'Brien and McCully, 1981). (For pollen studies some sections were also stained with 0.05% (w/v) Toluidine blue at (a) pH 11, in 1% sodium borate, or (b) pH 1.5, in 1% acetic acid.) The sections were rinsed for about 30 sec. in distilled water and mounted in water for observation using bright-field optics.

2.2.2.2 Carbohydrates

The Periodic Acid Schiff's (PAS) reaction is used as a test for the presence of glycol groups in carbohydrates (Pearse, 1985). Inherent aldehydes in mounted sections were reduced with 1% (w/v) aqueous sodium borohydride for 60 min., rinsed in running water for 15 min., oxidized in 1% periodic acid for 10 min., rinsed in running water for 15 min., treated with modified Schiff's reagent (Fisher Sci., Co.) and finally rinsed in running water for 30 min. After each rinse the slides were placed on a 60°C hot plate to dry and adhere sections to the slide. Sections on the slides were then mounted in water and examined with bright-field or fluorescence optics using green excitation. A positive reaction with PAS gives a pink colour in bright-field and red fluorescence with green excitation (O'Brien and McCully, 1981). Control sections were treated similarly, except that the sodium borohydride blocking and/or the periodic acid oxidation steps were omitted.

The iodine potassium iodide (IKI) stain imparts a bluish-black colour to the amylose of starch, and a yellowish colour to amylopectin and other polysaccharides (Horobin, 1988). Potassium iodide (2 g) was dissolved in 100 ml water and 0.2 g iodine was dissolved into this solution. Fresh hand-cut sections were stained for about 1 min. while plastic-embedded sections were stained for 1-2 hours. Sections were rinsed for ca. 1 min. in running water, mounted in water and observed with bright-field or phase contrast optics (O'Brien and McCully, 1981).

2.2.2.3 Protein stains

Three acidic dyes, naphthol blue black (C.I. 20470, Polysci. Inc., lot 143-4), light green (C.I. 42095, BDH Chem. Ltd., no. 34043) and acid fuchsin (C.I. 42685, Allied Chem. Co.) were used.

Naphthol blue black (1% w/v) was dissolved in 7% (v/v) acetic acid. Plastic-embedded sections were stained with this solution, rinsed briefly in 7% (v/v) acetic acid, air dried and mounted in immersion oil or water (O'Brien and McCully, 1981). With acid fuchsin, plastic-embedded sections were stained with 1% (w/v) aqueous solution, rinsed 2-3 min. in tap water and mounted in water (O'Brien and McCully, 1981). For light green, plastic-embedded sections were stained with 0.1% (w/v) dye solution in 1% (v/v) glacial acetic acid, rinsed briefly in 1% acetic acid, rinsed 2-3 min. in water and finally mounted in water (O'Brien and McCully, 1981). All sections were stained for 30 s - 10 min. at 25°C (for naphthol blue black and acid fuchsin also at 60°C). Bright-field optics were used to examine the stained sections. Positive reactions are as follows: dark

blue with Napthol blue black; pinkish-red with acid fuchsin; green with light green (O'Brien and McCully, 1981).

2.2.2.4 Lipid (Sudan) 'stain'

Plastic-embedded sections were immersed in a saturated solution of Sudan Black B (Mochrome, Edward Gurr Ltd.) in 70% ethanol and left for 5-24 hours. The sections were then rinsed for 1-2 min. with 70% ethanol then mounted in a drop of melted glycerine jelly (O'Brien and McCully, 1981). Bright-field optics were used to observe all sections 'stained' with Sudan dye. Expected colour for a positive reaction with Sudan Black B is bluish-grey to black (O'Brien and McCully, 1981).

2.2.2.5 Callose

Aniline blue dye can detect callose - a positive reaction gives strong yellow fluorescence with blue excitation and yellowish-white fluorescence with UV excitation (O'Brien and McCully, 1981). The aniline blue was prepared at 0.05% (w/v) in pH 8.5, 0.067 M potassium phosphate buffer (prepared using 7 ml of 0.1 M potassium phosphate, monobasic and 98 ml distilled water). Aniline blue used was water soluble, CI 42755 from Fisher Sci. Co. (certification no. EK 11). The plastic-embedded sections of pollen at all developmental stages were immersed in the aniline blue solution for 30 s - 10 min and then examined with UV and blue excitation.

2.3 Pollen grain development

Initially, a considerable amount of effort was spent attempting to find a good, consistent method to stain nuclei at all stages of pollen grain development. In addition, vital staining was used to show the progression of vacuolar development within the pollen grains. These

results suggested that the pollen grains accumulate phenolic compounds within their vacuoles during maturation. Attempts were made to verify this using paper chromatography and UV spectroscopy. Various chemical fixation protocols and a freeze substitution procedure were used to try to retain these compounds *in situ*. Since the grains are small (20-30 μ m diam), different chemical fixation procedures were tested on the retention of compounds within the large, easily visible, vacuoles of 'model' plant cells (see Appendix).

2.3.1 Nuclear staining

Table 1 outlines all of the nuclear staining procedures tried on whole *B. napus* pollen grains at various developmental stages. Each trial was repeated at least twice. In addition, sections (cut with a microtome and glass knives) of the plastic-embedded material (fixed in either (a) 3% glutaraldehyde in pH 6.8, 0.025 M potassium phosphate buffer, or (b) 10% aqueous acrolein) were treated with variations of the Feulgen staining reaction (Table 2). The sections were then examined with fluorescence optics using green excitation.

2.3.2 Vital staining

All vital staining was done by either (a) squashing individual anthers into a drop of dye solution between a coverslip and slide or (b) grinding (with a mortar and pestle) the anthers up in the dye solution, filtering through a 44 μ m nylon mesh and then placing a drop of the stained pollen grains on a slide and covering with a coverslip. Various developmental stages of pollen grains in over 50 inflorescences were examined. Most of these were taken from plants in the growth cabinets maintained at 10°C 'light' temperature and 5°C

Table 1: Staining procedures for whole pollen grains of *B. napus*

Dye¹	Fixation procedure²	Ref.³	Isolated	Anther
DAPI or Hoechst 33258 (2-5µg/ml)	stain unfixed pollen	1	X	
"	Carnoy's fixative (50-95% ethanol: glacial acetic acid: chloroform, 6:3:1)	2	X	X
"	1:1, ethanol:acetic acid	-	X	X
"	2:1, ethanol:acetic acid	-	X	X
"	1:1, ethanol:propanoic acid	-	X	X
"	2:1, ethanol:propanoic acid	-	X	X
"	9:0.5:0.5, 50% ethanol:acetic acid:formalin	-	X	X
"	3% glutaraldehyde in 0.025 M, pH 6.8 potassium phosphate buffer	-	X	X
"	3% paraformaldehyde in B-1 buffer +/- 0.02% NP-40 or 1% DMSO	3	X	X
"	as above preceded with 3% cellulase or 1% glucuronidase or 1% macerage (Sigma Co.)	3	X	
"	3% paraformaldehyde in B-1 buffer ⁴ +/- 0.02% NP-40 for 1 hr → wash 3 x B-1 buffer (10 min. each) → 1 hr 1% Triton X-100 in B-1 buffer → 3 x B-1 buffer (10 min. each) wash	3	X	
"	0°C, 100% methanol	-	X	

Table 1 continued

<u>Dye¹</u>	<u>Fixation procedure²</u>	<u>Ref.³</u>	<u>Isolated</u>	<u>Anther</u>
Feulgen	3:1, ethanol:glacial acetic acid for 1-3 hr 5M HCl, 20°C, 45 min. Schiff's reagent, 1 hr squashed/mounted in 45% acetic acid	4	X	X
"	3% paraformaldehyde in pH 6.8, 0.025 M potassium phosphate buffer, 1 hr +/- 1% Triton X-100 in buffer 1 hr 1% sodium borohydride 1 hr rinse 15 min. in running water 30 min. Schiff reagent rinse 30 min. in running water observe fluorescence (green excitation)	-	X	
ethidium bromide	stain unfixed pollen 30 sec.-10 min. in aqueous 0.01% ethidium bromide mount in water UV fluorescence	5	X	

Note for Table 1:

1. Dyes used: DAPI, 4'6-diamidino-2-phenylindole (Sigma Co.) examined with blue excitation; Hoechst 33258, Sigma Co. examined with blue excitation; for Feulgen reaction used modified Schiff's reagent, Fisher Sci. Co., examined with green excitation; ethidium bromide, BDH Chem. Co. examined with UV excitation.

2. Fixation procedure done with 'isolated' pollen grains or pollen grains 'in anther' (X indicates which method was used). For the 'isolated' pollen grains, the four inner anthers from one bud were ground up with a mortar and pestle into 5 ml of modified B5 medium (Keller and Armstrong, 1977) without growth regulators but with 13% sucrose added, and then centrifuged at 1000 rpm, 3 min. The pollen pellet was washed and centrifuged an additional three times in the modified B5 medium and then a drop of pollen grains was spread over a dried polylysine-coated coverslip (Note: (1) Polylysine used = 1 mg/ml 300,000 mwt poly-L-lysine solution (Sigma); (2) Coverslips immersed in chromerge (made by dissolving 10 g potassium dichromate in 85 ml distilled water and then adding 10 ml concentrated HCl) overnight, washed thoroughly with water, dried and then coated with polylysine solution.) The coverslip with the pollen attached was then immersed in the fixative solution (when one was used) and treated as outlined above.

Table 1 continued:

In addition some intact anthers were immersed in the fixative solution, rinsed three times with pH 6.8, 0.025 M potassium phosphate buffer and then individual anthers were placed on a slide with a drop of the dye solution and squashed with a coverslip.

3. References: (1) Pechan and Keller, 1988; (2) Fan, et al., 1988; (3) personal communication, Dr. Daina Simmonds; (4) Kott et al., 1988a; (5) O'Brien and McCully, 1981; - = no reference.

4. B-1 buffer (pH 7) contains 0.1 M mannitol, 25 mM (pH 7) sodium phosphate buffer, 10% dimethyl sulfoxide (Sigma Co.), and 0.5 mM phenyl methyl sulfonyl fluoride (Sigma Co.) (p.c., D. Simmonds).

Table 2: Variations on Feulgen staining of embedded sections of pollen grains¹

N ²	<u>Hydrolysis in HCl</u>		<u>Schiff Reagent</u>		
	<u>Time (min.)</u>	<u>Temp (°C)</u>	<u>Type³</u>	<u>Conc. %</u>	<u>Time (min.)</u>
1	9-30	60	Schiff	100	45-120
6	9-120	~22	Schiff	100	45-120
1	9, 10, 11	60	acriflavine	10 ⁻³ 5 x 10 ⁻³ 10 ⁻⁵	45-120
6	9-120	~22	acriflavine	10 ⁻³ 5 x 10 ⁻³ 10 ⁻⁵	45-120
1	9, 10, 11	60	auramine	10 ⁻³ 10 ⁻⁴	45-120
1	9, 10, 11	60	corphosphine	10 ⁻³ 10 ⁻⁴	45-120

Note for Table 2

1. Reaction done as follows:

1% sodium borohydride, 30 min. → 15 min. H₂O rinse → HCl hydrolysis → 15 min H₂O rinse → Schiff-type reagent → 30 min. H₂O rinse

2. N = Normality

3. Schiff 'type' reagents used: Schiff = modified Schiff reagent (Fisher Scientific Co.); acriflavine (BDH (Canada) Ltd., lot 35367); auramine O (Fisher Sci. Co., CI 41000); coriphosphine O (Allied Chem. Co., CI 46020, lot no. 0265 029).

'dark' temperature (section 2.1) but some were also taken from plants grown in the greenhouse at *ca.* 20°C. All pollen grains were stained with (a) 0.025 - 0.05% (w/v) Toluidine blue O (CI 52040; Polyscience lot 73736 or Fisher Sci. lot 735481) at pH 11, in 1% (w/v) sodium borate buffer, or (b) 0.1% (w/v) neutral red (Chroma-Gesellschaft Schmid Co., Stuttgart, no. 1B469) at pH 7.8 in 0.025 M potassium phosphate buffer. For pollen in anthers just prior to or during anthesis an additional 0.3 M sorbitol and 0.3 M mannitol solutions were added to the neutral red solution to prevent pollen grains from bursting. The stained pollen grains were examined using bright-field or Nomarski optics.

In addition, various stages of pollen grains from a few inflorescences on plants grown at 10°C/5°C were examined with other 'vital' stains: 0.01 - 0.05% (w/v) acridine orange (Polyscience Inc., no. 0115, lot 138-1) in pH 9, 0.025 M potassium phosphate buffer; 0.1 ppm (w/v) aqueous Rhodamine B (CI 45170, Allied Chem. Co., New York, lot 1624-9). Pollen grains treated with these dye solutions were examined with UV excitation.

Positive reactions for accumulation of dyes in vacuole saps are: neutral red, dull brick red ('empty') and violet red ('full'); Toluidine blue O, violet ('empty') and greenish ('full'); acridine orange, red fluorescence ('empty') and green fluorescence ('full'); rhodamine B, no uptake ('empty') and fluorescing contents ('full') (Stadelmann and Kinzel, 1972). According to Kinzel (1958) and Stadelmann and Kinzel (1972) 'empty' vacuole saps contain organic acids whereas 'full' vacuole saps contain phenolic compounds.

2.3.3 Preparation of embedded material

All pollen used in this study was taken from plants grown at 10°C/5°C. Only the four inner anthers were used. Table 3 outlines the various protocols used for preparation of plastic-embedded pollen grains. At least 50 pollen grains for each treatment were examined.

2.3.4 Histochemical analyses

Pollen grains (at various stages of development) fixed with 3% glutaraldehyde in pH 6.8, 0.025 M potassium phosphate buffer containing 0.5-1% Triton X-100 and dehydrated with the methyl cellosolve series was tested with all histochemical staining techniques outlined in section 2.2.2. For material prepared by all other protocols however, only the Periodic Acid Schiff (PAS) reaction was used and only the more mature pollen grains taken from buds just before anthesis were examined. To locate these mature pollen grains one anther from the inner stamens of each bud was tested with Toluidine blue O pH 11, 0.025% (w/v) 'vital' staining to ensure that the pollen grain vesicles stained turquoise-green. The other 3 inner anthers were then fixed and embedded.

2.3.5 Chromatographic analyses of compounds in the vacuoles/vesicles

Chromatographic analyses were attempted to determine if the pollen grain vacuoles/vesicles accumulate phenolic compounds during maturation, as suggested by previous vital staining results.

2.3.5.1 Pollen grain isolation

One inner anther from each bud was squashed in pH 11, 0.025 M Toluidine blue O so that those buds with pollen grains having only

Table 3 Protocols for preparation of plastic-embedded *B. napus* pollen grains

<u>Fixative</u>¹	<u>Dehydration</u>²
3% glutaraldehyde in pH 6.8 K phos ³ buffer +/- 0.5 or 1% Triton X-100	2 X methyl cellosolve, 2 X ethanol, 2 X propanol, 2 X butanol
1.5% glutaraldehyde in HEPES ⁴ buffer + 0.5% Triton X 100	as above
4% glutaraldehyde/4% form- aldehyde in pH 6.8, 0.025 M K phos buffer + 0.5% Triton X- 100	as above
5-10% acrolein in distilled water +/- 0.5% Triton X-100	as above
4% formaldehyde/4% acrolein in pH 6.8, 0.025 M K phos buffer + 0.5% Triton X-100 + (1% caffeine or + 0.01% malachite green)	acetone - 5, 10, 30, 50, 70, 90 95, 100%
0.5% glutaraldehyde in pH 6.8, 0.025 M K phos buffer + 0.2% caffeine + 0.5% Triton X-100	as above
4% formaldehyde/4% acrolein in pH 6.8, 0.025 M K phos buffer + 0.5% Triton X-100 + (0.01% malachite green and 1% caffeine or 1% caffeine alone)	50:50, methyl cellosolve:buffer 100% methyl cellosolve 50:50, methyl cellosolve:ethanol 100% ethanol 50:50, ethanol:propanol 100% propanol 50:50, propanol:butanol 100% butanol

Note for Table 3:

1. (a) Pollen remaining within anthers were prepared as outlined in section 2.2.1. In addition, some anthers were ground up into the fixative solution (using a mortar and pestle), filtered through a 44 μ m nylon mesh then centrifuged at ca. 500 rpm for 3 min. The pollen in the pellet were then attached to 1 mm² dried, polylysine-coated (0.02% aqueous poly-L-lysine solution, Sigma P8920) millipore 1.2 μ m filters (Millipore Corp., Massachusetts, lot 30545-2).

(b) All fixations were done overnight on ice. The next day the pollen was rinsed (2 x 15 min.) in the same carrier solution as the fixative but without the fixative and Triton X-100.

(c) Fixatives used: glutaraldehyde (J.B. EM. Inc., no. JBS-013A); acrolein (Polyscience Ltd., EM grade, no. 00016); paraformaldehyde (Fisher Sci. Co., T-353, lot 741231).

2. Dehydration was done on ice: solvents were changed every 30 min. The last solution was brought to room temperature and then replaced with 50:50 of this solvent and glycol methacrylate monomer mixture for 30 min. Fresh GMA monomer mixture was then added and left o/n. The next day fresh GMA monomer mixture was added and the tissue left undisturbed for ca. 1 week. Tissue pieces were then polymerized in GMA monomer mixture at 60°C or under UV light in the presence of nitrogen gas.

3. K phos buffer indicates potassium phosphate buffer, 0.025 M at pH 6.8.

4. HEPES buffer was made according to Mersey and McCully (1978).

purple-staining vacuoles were separated from the more mature pollen grains with vacuole contents which stained turquoise-greenish. About 2 g of fresh anthers for each vacuole 'type' were collected for the first five trials but for the sixth trial only 0.2 g of anthers were collected. The anthers were then ground up with a mortar and pestle in about 50 ml of water, filtered through a 44 μ m nylon mesh to collect the pollen grains, and then centrifuged at 1000 rpm for 3 min. to obtain a pollen pellet. For the first four trials only the pollen pellet was examined and the water in which it was isolated was discarded. For the last two trials however, this water was also analyzed separately. All samples were stored overnight at 0°C.

2.3.5.2 Extraction procedure

The next day, (a) the pollen pellet and, for the last 2 trials (b) the residue from the evaporated water sample (into which the pollen had been isolated) were individually added to 50 ml of 100% methanol and shaken for 24 hours at room temperature; methanol is widely used for the extraction of flavonoid compounds (Mabry *et al.*, 1970; Crawford, 1973). The methanol was evaporated and 10 ml of 5% aqueous methanol and 10 ml of diethyl ether were added, mixed, and allowed to stand; the lower aqueous phase contains the flavonoids while chlorophyll separates into the upper (ether) phase (p.c. D. Gianassi). The ether phase was discarded and the aqueous phase was evaporated.

2.3.5.3 Paper chromatography

About 2 ml of 100% methanol were added to the dried extracts, mixed and then centrifuged at 1000 rpm for 3 min. 50-75 μ l of the

extract were applied to 46 x 57 cm Whatman I chromatography sheets (Whatman III sheets were used for trial 1). For the first two trials two dimensional chromatography was done using 3:1:1, tertiary-butyl alcohol:acetic acid:water as the first solvent (long dimension) and 15:85, acetic acid:water as the second solvent (Mabry *et al.*, 1970). The remaining chromatograms were run with only the first solvent. Ascending chromatography was done for the first 4 trials while descending chromatography was used on the last two. The dried chromatograms were examined with long wavelength UV (365 nm) lamps (UVL.21, Blak-Ray, UV Products Inc., California). The first two chromatograms were then exposed to ammonium hydroxide fumes and then viewed again with the UV lamps.

2.3.6 UV spectroscopy of compounds in pollen grain vacuoles

UV spectroscopy was done on the extracted pollen grains from the last two trials (5 and 6) of paper chromatography - the first (trial 5) used about 2 g of fresh anthers and the second (trial 6) used only 0.2 g of anthers of each vacuole 'type' (as characterized by pH 11, 0.025 M Toluidine blue O staining, see section 2.3.5.1). Pollen grains were isolated and both the pollen pellet and the water into which they were isolated were extracted as outlined in sections 2.3.5.1 and 2.3.5.2. The evaporated extracts were diluted 100 fold with absolute methanol and then examined with a spectrophotometer (Cary 219, Varian Co.) scanned at 2 nm/sec. from 250-700 nm.

2.3.7 Freeze substitution

Mature pollen grains with vesicles containing compounds which stain turquoise-green with pH 11, 0.025% (w/v) Toluidine blue

O were freeze-substituted; one of the four inner anthers from buds taken prior to anthesis was squashed in a drop of the Toluidine blue O solution to verify that the pollen grain vesicles had turquoise-green compounds and the other three anthers were then freeze-substituted in the following manner. Some of the anthers were cut into *ca.* 1 mm³ pieces to be subsequently freeze-substituted. Other anthers were ground up with a mortar and pestle in a pH 7.8, 0.1% (w/v) neutral red solution (containing 0.3 M mannitol and 0.3 M sorbitol) and then filtered through a 44 µm nylon mesh to isolate the pollen grains. The stained pollen grains were then attached to dried polylysine-coated (0.02% aqueous poly-L-lysine solution, Sigma P8920) millipore 1.2 µm filters (Millipore Corp., Massachusetts, lot no. 30545-2) cut into *ca.* 1 mm² pieces, before being plunged into the freezing mixture.

The freeze-substitution protocol was done exactly as described in Canny and McCully (1986) except that the infiltration into Spurr's epoxy resin was altered as follows. After warming to room temperature for 24 hrs, the tissue was slowly infiltrated up to 10% resin over the first hour of infiltration, and then changed every two hours with the following solutions, named in order of use: 1:1 resin:ether, 3:1 resin:ether, 100% resin. The cells and tissues in 100% resin were left overnight. This resin was changed once a day for the next four days and finally polymerized at 70°C. (It is worth mentioning that the Spurr's resin precipitates when petroleum ether is used (by mistake) instead of diethyl ether, and that LR White resin precipitates with both petroleum and diethyl ether.)

The unstained pollen grains which were freeze-substituted in the anther were examined with UV excitation to test for autofluorescence (presumably indicative of phenolic compounds). The isolated pollen, which had been stained with neutral red before freeze-substitution, was sectioned (with a microtome using glass knives) and then examined with bright-field and Nomarski optics.

2.4 Microscopy and photomicrography

External features of anthers were examined with an Olympus SZH stereo microscopy system equipped with a coaxial vertical illuminator SZH-1LLC halogen bulb (6V, 20W) and a transformer TL-2 power source. Optical microscopy was done with an Olympus Vanox AHB-LB microscope equipped for bright-field, phase contrast, Nomarski, interference contrast and epifluorescence optics. The fluorescence filters used are outlined in Table 4 (from Olympus Optical Co., Ltd., instruction manual for vertical fluorescence illuminator attachment, model AH-RFL-LB). Olympus C-35AO-2 or C-35AO-4 cameras were used for photomicrography, using the Olympus SZH stereo microscope or Olympus Vanox AHB-LB. In addition, a Meopta monocular microscope with an external light source was set up for dark-field microscopy to view some of the unstained anther and pollen sections. For this microscope a Nikon PFM camera was attached to the monocular tube. For photography of whole inflorescences and buds a Nikon FM2 camera was used.

Photomicrographs were taken with several films. Kodak Ektachrome 200 was used for colour photography - these films were processed at Japan Camera, Ottawa. For black and white photography either Kodak TMAX 100 film exposed for 160 ASA or Kodak Plus X

Table 4: Filters used for Olympus Vanox epifluorescence

<u>Excitation Method</u>	<u>Exciter Filter</u>	<u>Dichroic Mirror</u>	<u>Barrier Filter</u>
UV	U (UG-1)	U(DM 400 + L-420)	L-420 and up
violet	V(BG-3)	V(DM 455 + Y-455)	Y-475 and up
blue	B(BG-12)	B(DM 500 + O-515)	O-530 and up
green	G(iF-545 + BG-36)	G(DM 580 + O-590)	R-610

Pan 125 ASA films were used. All development and printing was at 20°C. For the Plus X Pan, each film was developed in full strength Ilford Microphen developer (Ilford Ltd., Mobberly Cheshire) for 5 min. For TMAX 100, each film was developed in Kodak TMAX developer (Eastman Kodak Co., Rochester, New York) diluted 1:4, developer:water for 10 min. Both Plus X Pan and TMAX 100 films were then rinsed with running water for 1 min. then fixed in Kodak fixer - 5 min. for Plus X Pan film and 8 min. for TMAX 100 film. The films were slowly agitated every 30 sec. during development and

fixing. After fixing, the films were rinsed in running water for 30 min.

All printing of black and white micrographs was done on Ilfobrom single weight, glossy nos. 1-5 papers (Ilford Inc., New Jersey, USA) using Kodak Dektol developer (Kodak Canada Inc.) diluted 1 part developer to 2 parts water. Prints were then briefly rinsed in running water, fixed for 5 min. in full strength Kodak fixer, and then rinsed for 1-2 hours in running water.

3. RESULTS

3.1 Anther development

In general, anthers of *B. napus* are similar in anatomy to those of many other angiosperms (Figs. 2a and b). Each anther has four locules (containing sporogenous cells), two in each lobe of the anther with a vascular bundle from the filament running up the back of the anther between the lobes. The anther consists of the tapetum, middle layer, endothecium, epidermis and some connective tissue in the central portion of the lobes surrounding the vascular bundles.

3.1.1 External features

No significant changes in external anther structure occur until the beginning of anthesis (period of flowering) when the filaments begin to elongate. The filaments of the two inner pairs of stamens elongate more than those of the outer pair (Fig. 1c). Simultaneously, the four inner anthers rotate ca. 95° from having the 'front' of the anther (with the longitudinal slit) facing the carpel to having the two anthers of each pair 'back-to-back' (with their vascular bundle region facing each other - see Fig. 1c). During anthesis the tips of the anthers curl backwards (Fig. 1c) as a vertical slit progresses basally between the two locules of each lobe (Fig. 1g). Separation at the stomium (Figs. 2a; 3f and g) between two locules in each lobe results in the longitudinal slit which opens up the locules for pollen dispersal (dehiscence).

On the back of the anthers (adjacent to the vascular bundle) are distinct epidermal cells which are larger than other epidermal cells (Figs. 1e and f; 2a and b; 3a); they enlarge considerably (to protrude away from the vascular bundle) just prior to anthesis.

Fig. 2a Transverse section of *B. napus* anther with only one lobe fully drawn and labelled

Fig. 2b: Whole mount of *B. napus* stamen viewed from the side

Fig. 3a: Whole mount of anther during early anthesis. 'Motor' cell at top (asterisk) is much larger than other epidermal cells below it in the micrograph. Nomarski. 250 X.

Fig. 3b: Whole mount of anther during early anthesis. Most anther epidermal cells are not aligned in regular files of cells. Nomarski. 420 X.

Fig. 3c: Squash of an anther during late anthesis - the tapetum is gone and the endothelial cells with regular secondary wall thickenings are facing the locule. Arrows delimit the periphery of one cell. Nomarski. 700 X.

Fig. 3d: Anther during late anthesis. Anther was gently squashed between a slide and coverslip to show the secondary wall thickenings in cells of connective tissue. Arrows delimit periphery of one cell. Nomarski. 590 X.

Fig. 3e: Transverse section of a glutaraldehyde-fixed anther with mid- to late binucleate pollen grains. Ubisch bodies in tapetum (T) are very refractile. Dark field. 480 X.

Fig. 3f: Transverse section of a glutaraldehyde-fixed anther at the same stage as 3e and stained with PAS reaction for carbohydrates. Starch granules (dark spots) are present in most anther cells. The vascular bundle (vb) and stomium (arrow) are labelled. Bright field. 120 X.

Fig. 3g: Transverse section of a slightly more mature anther than in 3f showing fewer starch granules in cytoplasm of anther cells. The vascular bundle (vb) and stomium (arrow) are labelled. PAS staining. Bright field. 140 X.

Figure 3

While most other epidermal cells of the anther are not aligned in any specific order (Fig. 3b) these large cells are located in a distinct strip along the full length of the back of the anthers, adjacent to the vascular bundles (Figs. 1e and f; 2a and b). Those cells in the lower (proximal) portion of the strip (Fig. 1e) are larger and protrude from the anther proper more than the cells nearer the tip of the anther (Fig. 1f). These large cells appear 'motor-like' in function since they lose turgor and eventually collapse as the anther matures. The collapse of the cells in the upper portion of the anther appears to cause the anther to curl back at the tip. The collapse of the larger cells in the lower portion of the anther appears to spread open the locules, hence maximizing pollen dispersal (Fig. 1h). These 'motor' cells are best studied in fresh anthers. All epidermal and 'motor' cells at all stages of anther development collapsed when 10% acrolein fixative was used for the embedded anthers (no similar artifact was observed when 3% glutaraldehyde was used as a fixative).

It is worth mentioning that the epidermal cells (not including the 'motor' cells) have vacuoles with greenish contents when fresh tissue is stained with 0.025-0.05% (w/v), pH 11 Toluidine blue.

3.1.2 Structure of anther cell layers

3.1.2.1 Endothecium and connective tissue

Cell walls of both the endothelial layer and the connective tissue develop secondary wall thickenings late in the development of the anther. The endothelial cells develop regular widely spaced annular thickenings (Fig. 3c). The endothelial cells 'wrap' circumferentially around the locules, generally in a direction perpendicular to the anther's longitudinal axis. The cells of the

connective tissue vary in size and develop considerably different secondary wall thickenings than the endothelial cells (Fig. 3d). In general, secondary wall thickenings of cells at the tip and base of the anthers occurs slightly later than in cells in middle regions of the same anthers (no data collected) suggesting that anther development is not synchronous throughout its length.

Young cells of the epidermis, endothecium and connective tissues have starch granules within their cytoplasm (Fig. 3f) which eventually disappear with anther maturation. The sequence of starch loss was not closely followed but it was noted that in general starch loss begins in the central connective tissue and disappears last in endothelial and connective cells in the regions where the two locules join at the stomium (Fig. 3g). Almost no starch was present in mature anthers examined late in anthesis.

Most endothelial cells, appear to senesce during anthesis as judged by (a) the absence of cytoplasm and nuclei in sections of plastic-embedded anthers, (b) absence of nuclear fluorescence of DAPI-treated fresh anthers, and (c) absence of vacuolar staining with 0.025%, pH 11 Toluidine blue (vacuoles in live endothelial cells have purplish contents).

3.1.2.2 Tapetum and middle layer

Both the tapetum and middle layer are eventually lost during anther maturation. Observations of sectioned material shows that the single middle layer becomes crushed or disappears shortly before or just after the cell walls of the endothecium develop secondary thickenings (Fig. 4e). The tapetum initially has thin but clearly defined anticlinal and periclinal cell walls (which don't stain

Figure 4. All micrographs are transverse sections from glutaraldehyde-fixed anthers except 4f which is a longitudinal section.

Fig. 4a: Early uninucleate pollen stained with Toluidine blue O at pH 4.4. Protoplasm has pulled away from wall. The exine (large black arrow), nucleus (small black arrow) and nucleolus (small white arrow) in one pollen grain are shown. Bright field. 650X.

Fig. 4b: Late uninucleate pollen stained with Toluidine blue O, pH 4.4. The nucleus (small white arrow) and nucleolus (small dark arrow) are labelled in one of the grains. Pollen have large vacuole(s) with contents that mostly don't stain except for a few aggregates within some pollen vacuoles (large black arrow). Tapetum (T) has many vesicles, and walls that don't stain (large white arrows). Bright field. 1000 X.

Fig. 4c: Late uninucleate pollen stained with Naphthol blue black protein stain. Nucleus (large white arrow) with a large nucleolus (asterisk) are labelled in one grain. The large vacuole(s) mostly don't stain except for a few aggregates inside (black arrows). Bright field. 1200 X.

Fig. 4d: Binucleate pollen stained with Naphthol blue black. The generative nucleus with its dark nucleolus is surrounded by some cytoplasm and, all of this is encircled by many tiny unstained vesicles (large white arrows). The nucleoli (asterisks) in the vegetative and generative nuclei are labelled in one grain. The nucleus of one generative cell is shown by a small white arrow. No large vacuole(s) are visible. Bright field. 950X.

Fig. 4e: Late binucleate pollen stained with Naphthol blue black. Little cytoplasmic detail is obvious - there are some vesicles seen but no large vacuole(s). The nuclei and nucleoli are almost indistinguishable from background cytoplasmic staining. Both layers in the pollen walls are stained. . Endothelial cells (En), disintegrating middle layer (M), and tapetum (T) containing may small vacuoles are also shown. Bright field. 730 X.

Fig. 4f: Trinucleate pollen grain stained with Toluidine blue O at pH 4.4. Cytoplasm shows even, solid staining. No large vacuole(s) or nuclei are visible. Tapetum and middle layer have disappeared. Bright field. 330 X.

Fig. 4g: Late uninucleate pollen grain stained with PAS. Light staining of pollen cytoplasm, intine and exine. Tapetal cytoplasm (T) and walls (arrows) have stained. Bright field. 1150X.

Fig. 4h: Binucleate pollen grain stained with PAS. Dark spots present in pollen cytoplasm (especially around vegetative nucleus). Intine and exine lightly stained. Ubisch bodies in tapetal cytoplasm (T) are refractile (arrows). Bright field. 1000 X.

Fig. 4i: Trinucleate pollen grain stained with PAS. Dark staining throughout cytoplasm and even darker staining of intine (arrow). The vague light circular area in some of the pollen grains is the center of the vegetative nucleus. Exine lightly stained. Tapetum and middle layers have gone leaving the endothecium (En) as the innermost layer. Bright field. 500 X.

Figure 4

with Toluidine blue or protein stains - see Fig. 4b) but do stain very lightly with the PAS reaction for carbohydrates (Fig. 4g). These cell walls become less defined with anther maturation (compare Figs. 4b and 4e) presumably because the walls are being digested at this stage. Just before or during anthesis the tapetum disappears entirely (Fig. 4f and i). The tapetum could only be examined in anthers with intact locules uncut during preparation since any damage to the locules resulted in the tapetum being pulled away from the other anther tissue.

The tapetum contains many vesicles (Figs. 4b and e). Histochemical studies of GMA-embedded anthers show that staining for general basophilia (ie. affinity for basic dyes), proteins and carbohydrates in the tapetal cytoplasm decreases with anther maturation. (No lipid staining was observed - only the cuticle of epidermal cells showed positive Sudan Black B 'staining'.) The histochemical results, however, need to be interpreted with caution since they may be due to artifacts created as a result of tissue preparation (see below). In addition, tiny, highly refractile particles, which do not appear to stain with any of the dyes tested, are present in the cytoplasm of the tapetal cells, in general just preceding or following endothelial cell wall secondary thickening (Figs. 3e and 4h).

3.2 Pollen development

3.2.1 Nuclear staining of whole and sectioned pollen grains

No reliable method was found to consistently stain *B. napus* pollen grains throughout their development. In general, nuclear

staining of pollen grains which had been isolated and washed before fixing and staining was better than in those pollen grains squashed from fixed/unfixed anthers probably because of better fixative and stain access to the isolated and washed pollen grains. Also, for most methods (except those using unfixed pollen grains) there does not seem to be much difficulty in nuclear staining of the tetrad period nor the uninucleate pollen grains (with a central nucleus) just released from the tetrad. However, as soon as the unstained pollen become distinctly trilobed and yellowish, nuclear staining becomes difficult and unreliable. With some methods (eg. (a) DAPI-treated pollen grains which had been isolated and prefixed in Carnoy's fixative or 0°C, 100% methanol, or (b) Feulgen-treated isolated pollen grains prefixed with 3% paraformaldehyde - see Table 1) nuclear staining of the most mature tricolpate pollen nuclei was frequently quite good, although still unreliable.

Even with these technical difficulties, from those pollen grains with good nuclear staining the observed general progression was from the uninucleate grains which undergo a mitotic division to produce binucleate grains (containing a vegetative and generative nucleus each) and finally the trinucleate pollen grains with two sperm nuclei (produced by mitotic division of the generative nucleus) and the larger vegetative nucleus (see Fig. 5).

Tables 5 and 6 give the results for some inflorescences in which the nuclear staining of the pollen grains was good enough (ie. nuclei of at least 80% of the grains per anther were detected) to follow their cytology at different developmental stages. Of interest, are the observations that (a) there are stages when the nuclei in either the

Figures 5a-e are of isolated pollen grains of *B. napus* that had been fixed in 3% paraformaldehyde, treated with the Feulgen method (Table 2) and viewed with epifluorescence optics using green excitation. These micrographs were taken of pollen from the few inflorescences in which good nuclear staining in the developing pollen was obtained. However, Fig. 5f is more representative of the extent of pollen nuclear fluorescence observed, especially at this stage of pollen development. Fig. 5f is of DAPI-induced (blue excitation) fluorescence of pollen, squashed from an anther previously fixed in Carnoy's fixative.

Fig. 5a. Pollen still in tetrads. A few microspore mother cells are seen and one, lower right, has just undergone its first meiotic division. 800X.

Fig. 5b. Mostly early uninucleate pollen. 700X.

Fig. 5c. Mostly late uninucleate pollen. 600X.

Fig. 5d. Binucleate pollen. Large white arrow denotes the vegetative nucleus and a small white arrow shows the smaller, brighter generative nucleus in one of the grains. 500X.

Fig. 5e. Arrows denote the vegetative nucleus in all grains. From left to right, the grains have 2 sperm nuclei, a generative cell in metaphase, and a generative nucleus not yet undergoing mitosis. 800X.

Fig. 5f. Probably uninucleate pollen. A few pollen mother cells and some anther debris are fluorescing (bottom right). 350X.

Figure 5

Table 5: Plants used for pollen grain cytology

<u>Plant</u>	<u>Age</u> <u>(days)</u>	<u>Inflorescence</u>	<u>Total #</u>	<u>Buds/Flowers</u>	<u># Open Flowers</u>
1 a	66	primary		66	4
1 b	66	lateral		31	4
1 c	66	lateral		40	2
2	80	primary		64	8
3	80	primary		65	13
4	91	primary		56	6
5	100	primary		68	20

Table 6: Cytological stage of pollen grains at various bud lengths and the onset of secondary wall thickening in endothelial and connective tissue cells¹

Bud length (mm) for various stages of pollen (a-k)
and when secondary wall thickening started (l)³

Plant ²	a	b	c	d	e	f	g	h	i	j	k	l
1a	1.5- 2.5	2- 2.5	-	2.5	2.5	3	4- 4.5	4.5- 6	-	8	8- open	6
1b	0.5- 1.5	1.5	-	2.25	2.25	2.25- 4.5	3.25	-	4.5- 6.5	8.5	8.5- open	7
1c	0.5- 1.75	1.75	-	2	-	2.5- 3.25	3.25	4.5	4.5- 5.5	5.5- 7.5	open	6.5
2	0.75- 2	2	2	2- 2.5	2.5	3	3- 4	4	4.75	6- 7	7- open	6
3	1- 1.5	2	2	2- 2.5	2.5	3- 4.25	2.5- 4.25	4.5	5.5- 6.5	6- 8	8- open	6.5
4	0.5- 1.5	1- 2	-	2- 2.25	-	2.75- 3.5	3	4.25	-	-	6- open	-
5	1.5	2- 2.25	-	2.5	-	2.5- 4	2.5- 4	4	4- 6	6- 8.5	6- open	7

Note for Table 6:

1. For cytology, pollen was squashed from anthers (fixed in Carnoy's fixative) and treated with DAPI as outlined in section 2.3.1. Hand sections of fresh material stained with Toluidine blue O, pH 4.4 were used to determine the onset of secondary wall thickenings in endothelial and connective tissue cells.
2. Plants used: see Table 5.
3. Descriptions of a-l:
 - a. Pretetrad; DAPI-negative walls; mother cell nuclei DAPI-positive
 - b. Tetrad; DAPI-negative walls; pollen nuclei DAPI-positive
 - c. Pollen recently released from tetrad: round; thin exine; bright small, often speckled, centrally-located nucleus
 - d. Bright DAPI-positive intine; lobed shape; most pollen (uninucleate) have centrally-located nucleus although some are in the lateral position.
 - e. Lobed (uninucleate) pollen with DAPI-negative walls and bright DAPI-positive nuclei either centrally or laterally located.
 - f. Lobed (uninucleate) pollen with DAPI-negative walls and nuclei
 - g. Lobed (uninucleate) pollen with DAPI-negative walls and faint, diffuse DAPI-positive nucleus laterally-located.
 - h. Rounded-lobed binucleate pollen; small, condensed generative nucleus (laterally-located) and larger, diffuse, centrally-located vegetative nucleus (both DAPI-positive); DAPI-negative walls.
 - i. Small generative nucleus (laterally-located) and no visible vegetative nucleus; DAPI-negative walls; roundish pollen
 - j. Two small laterally-located sperm nuclei and no visible vegetative nucleus (roundish pollen); DAPI-negative walls.
 - k. Oblong microspore with two sperm nuclei and vegetative nucleus DAPI-negative walls.
 - l. Secondary wall thickening of endothelial and connective tissue cells of anther.

uninucleate grains or the vegetative nucleus in bi- and trinucleate grains do not stain using most methods, (b) often more than one pollen cytological stage is present in each anther, and (c) bud length does indicate the general but not the exact grain cytological stage.

With the embedded material the best protocol was with $10^{-3}\%$ acriflavine for 45 min. following a 1 N HCl (ca. 10 min.) or 6 N HCl (ca. 30 min.) hydrolysis step. However, even with this method as with the other staining procedures the nuclei in the late uninucleate pollen grains and the vegetative nuclei in the bi- and trinucleate pollen grains often did not fluoresce well.

3.2.2 Histological and histochemical analyses of plastic-embedded pollen grains

With the fixation and embedding procedures used, the protoplasm of all early uninucleate and some late uninucleate pollen grains was pulled away from the walls as indicated by a clear region, between the protoplasm and walls, which does not stain with any of the dyes tested (see Fig. 4a). It is worth noting that staining fresh pollen with pH 4.4, high concentration 0.05% (w/v) Toluidine blue O is damaging to the pollen grains and results in the cytoplasm (which stains purple) pulling completely away from the walls of the early uninucleate pollen to form a round mass in the center of the grain whereas in many of the late uninucleate pollen grains the plasma membrane usually remains attached at the 'tips' of the lobes while separating from the walls in other regions with this treatment. The protoplasm was never observed pulled away from the walls in either the embedded or the fresh (stained with Toluidine blue O as above) binucleate and trinucleate pollen grains.

Nuclei in pollen grains up to and including the early to mid-binucleate stages can be observed when sections are stained with Toluidine blue O or any of the protein stains (Figs. 4b-d). With Toluidine blue O (at all pHs tested) the nucleus stains bluish-green and the nucleolus stains purplish. With the protein stains both the nucleus and nucleolus stain (dark blue with Naphthol blue black, green with light green, pinkish with acid fuchsin), the nucleolus staining darker than the nucleus (Figs. 4c and d). In pollen grains at late binucleate and trinucleate stages the nuclei (especially the vegetative nucleus) are not readily visible when stained with Toluidine blue O or protein stains (Figs. 4e and f) although often with pH 11 Toluidine blue O the two sperm nuclei in the generative cell stain very dark purplish so that they can be seen against the dark cytoplasmic staining (not shown). As mentioned in section 3.2.1, no reliable Feulgen nuclear DNA staining method of sections of embedded pollen grains was found.

The cytoplasm of pollen grains at all developmental stages examined stains purplish with Toluidine blue O (at all pH levels), gives a positive reaction with protein stains (see above), but stains very little with the Sudan lipid 'stain'. In late binucleate and trinucleate pollen grains the Toluidine blue O and protein staining is quite heavy throughout the cytoplasm (Figs. 4e and f) as compared to earlier stages (Figs. 4b-d).

The PAS reaction for carbohydrates produces interesting results. All parts of the uninucleate pollen grain stain only very pale pink (Fig. 4g) - it is difficult to judge whether or not the contents of the vacuoles stain as well (true also for pollen up to the mid-

binucleate stage). Often at the mid- and sometimes at the late binucleate stage dark pink spots are found in the pollen grain cytoplasm (Fig. 4h). These spots often stain bluish-black with iodine potassium iodide reagent for starch while the rest of the cytoplasm and walls stain yellowish. (With IKI the general yellowish cytoplasmic and wall staining does not vary significantly for all pollen developmental stages although the cytoplasmic staining does become darker with increasing pollen maturity.) In the trinucleate and often late binucleate pollen the entire protoplasm stains a dark pink with the PAS reaction showing no selective staining pattern (Fig. 4i). For the trinucleate pollen, staining sections with Schiff's reagent alone results in a light pink staining of the cytoplasm, intine and exine. For the immature pollen grains this treatment results in negligible cytoplasmic and intine staining and light exine staining. Also for all stages of pollen grains, treatment with sodium borohydride for 1 hour, rinsing and then staining with Schiff's reagent for 30 min. produces no staining.

The state of the vacuole in the early uninucleate pollen grains cannot be assessed since the protoplasm has probably pulled away from the walls. In the late uninucleate pollen grains there is usually one large vacuole (or two to three smaller ones) with contents that for the most part, do not stain with any of the dyes tested although some contained deposits of unknown composition (see Figs. 4b and c). In the early to mid-binucleate pollen this large vacuole is no longer observed and there are some smaller vacuoles throughout the vegetative 'cell' cytoplasm but in particular there are small vacuoles/vesicles delimiting the generative nucleus and some

cytoplasm (Fig. 4d). At this stage the generative cell is probably in the process of separating itself from the vegetative cell - these tiny vesicles do not stain with any of the dyes tested including aniline blue dye for callose. In late binucleate and trinucleate pollen there is no evidence of the large vacuole(s) (Figs. 4e and f) but there are often tiny vesicle-like spots, which often don't stain, visible at high magnification (Fig. 4e).

At all developmental stages the exine stains greenish with Toluidine blue O (all pH levels), gives positive staining with protein stains (ie dark blue with Naphthol blue black, green with light green, pinkish with acid fuchsin), is black with Sudan black B lipid 'staining', pink with PAS carbohydrate reaction, and gives negative staining with aniline blue for callose. The staining pattern of the intine at all developmental stages (except early uninucleate pollen grains when it does not seem to be present) was not possible to determine with Toluidine blue O or Sudan black B since it is difficult to distinguish it from the exine and the cytoplasm. However, the intine does give positive staining with the protein stains tested (Fig. 4e) and negative with aniline blue for callose. With the PAS reaction very little pink staining of the intine occurs in the uninucleate pollen grains but the amount of staining gradually increases until, in the mature trinucleate pollen grains, the intine stains very dark pink and can be easily differentiated from the exine and cytoplasm (Figs. 4g-i).

For all stages of pollen development the exine and cytoplasm in the unstained, embedded and sectioned grains produced significant autofluorescence for all excitation wavelengths, in particular blue excitation. This interferes with histochemical analyses using

fluorochromes such as aniline blue and fluorescent Feulgen reagents. Treatment of sections with 1% sodium borohydride reduces but does not eliminate the autofluorescence.

3.2.3 Vital staining

Toluidine blue (pH 11) staining shows that each young pollen grain has one to three vacuoles with purple contents (Fig. 6a) and older pollen grains have no large vacuoles but many tiny vesicles with greenish contents (Fig. 6e). Table 7 outlines the gradual transition in vacuolar staining patterns of developing (live) pollen grains with Toluidine blue O. The other dyes tested, ie neutral red, acridine orange and rhodamine B stained the walls of young pollen grains (those which have purple vacuole contents with Toluidine blue O) too heavily to allow observation of the vacuole staining patterns. This was probably due to the presence of dissociated cationic dye molecules (even in the dye solutions at their pK_a , 50% of the molecules are ionic) - the cationic dye molecules would bind to anionic groups on the exine. Toluidine blue O also stains the exine (green) but the vacuoles can still be observed since they have purple contents. For those pollen grains with vesicles coloured green with Toluidine blue O, the other dyes used 'coloured' the same vesicles as follows: neutral red, dull reddish (Figs. 6f and i); 0.01% (w/v) acridine orange, mostly greenish-yellow fluorescence (Fig. 6g) except for reddish-orange fluorescence in vesicles of pollen grains at the outer edge of the squash which were exposed to higher dye concentrations than those in the center of the squash; 0.05% (w/v) acridine orange, reddish-orange fluorescence; rhodamine B, pinkish-orange fluorescence (Fig. 6h).

Figures 6a-e are of live pollen grains after vital staining with 0.025 %, pH 11 Toluidine blue O. Figures 6a-d are of late uninucleate pollen and Fig. 6e is of late binucleate-trinucleate pollen.

Fig. 6a: Nuclei (small arrows) stain bluish-green and vacuoles (large arrows) stain purplish. Bright field. 1000X.

Fig. 6b-d: Same two pollen grains observed with increasing time in the dye solution. The vacuole in the pollen grain at the bottom of the micrograph bursts first (6c) followed soon after by the vacuole in the pollen at top (6d). Bright field. 750X.

Fig. 6e: The vesicles have accumulated the dye but individual vesicles are not visible at this magnification. Bright field. 500X.

Figures 6f-l are of late binucleate-trinucleate pollen grains showing accumulation of various dyes within the many vesicles. The individual vesicles are not seen in figures 6f-h at these low magnifications.

Fig. 6f: Live pollen grains after vital staining with neutral red.
Bright field. 230X.

Fig. 6g: Live pollen grains after vital staining with 0.01% acridine orange. UV excitation. 230X.

Fig. 6h: Live pollen grains after vital staining with rhodamine B. UV excitation. 230 X.

Fig. 6i: Live pollen grain after vital staining with neutral red.
Higher magnification shows the presence of individual vesicles.
Bright field. 1350X.

Figs. 6j-l: Sections of pollen grains prestained with neutral red and then freeze substituted. Figs. j and k show vesicles of various sizes that have retained the neutral red (the two larger ones in Fig. 6k may instead be neutral red that leaked out of vesicles and stained nuclei). Fig. 6l shows a burst pollen grain at top with its exine (small black arrow) and protoplast (white asterisk) which has retained neutral red in vesicles. The neutral red has leaked out of the vesicles in the other pollen grain (black asterisk) to stain the cytoplasm.
Nomarski. 990X.

Figure 6

Table 7 Vital staining of *B. napus* pollen grains with 0.025-0.05% Toluidine blue O pH 11

Staining pattern	Bud length (mm) ¹ in inflorescences 1-7 ²						
	1	2	3	4	5	6	7
Pollen grains just released from tetrad. Exine pale blue. Some tiny purple aggregates in vacuole with otherwise unstained contents. Nucleus greenish-blue.	NO ³	2-3	2	NO	1.5	2.75	NO
Very light purple or unstained vacuole contents with tiny purple aggregates dispersed within vacuole. Nucleus stains greenish-blue. Exine greenish.	2.5-2.75	NO	3.5	NO	NO	NO	NO
One large (or 2-3 smaller) vacuole(s) stained purple often with tiny purple aggregates within vacuole. Nucleus (or nuclei) greenish. Exine greenish.	3.25-5.5	3.5-5.5	4-5.5	4	4-5	4	3.25-4.25

Table 7 continued

<u>Staining pattern</u>	<u>Bud length (mm)¹ in inflorescences 1-7²</u>						
	1	2	3	4	5	6	7
"Transition" period when vacuole is breaking up into elongated 'tube-like' vacuoles and spherical vesicles. Some vesicles with greenish and others with purplish contents. Exine greenish. Nuclei not visible.	5.75-6.25	6.5-7	6-7	6	5	4.5	5.5
Pollen grains have many tiny vesicles with turquoise-green contents. Exine pale greenish. No sign of original large vacuole or nuclei. Exine pale greenish.	6.75-A	8.25-A	8-A	6-A	A	6.25-A	5.5-A

Note for Table 7:

1. A = open flowers/anthesis
2. Both lateral and primary inflorescences examined.
3. NO = not observed due to (a) most lateral inflorescences have fewer buds than primary inflorescences and there is a large developmental 'gap' between consecutive buds or (b) for inflorescence 4 only buds > 4mm in length were examined.

All stages of pollen eventually burst in all staining solutions tested. With Toluidine blue O, the large vacuole(s) in uninucleate-binucleate pollen grains, which have purple contents, burst within minutes of exposure to the dye solution (Figs. 6b-d). Most older pollen grains with greenish vacuole contents after staining with Toluidine blue O burst after 10-30 min (true also for pollen grains stained with acridine orange or rhodamine B at this stage). Adding 0.3 M sorbitol and 0.3 M mannitol to the neutral red solution prevented older pollen grains from bursting for about 30-60 min.

3.2.4 Freeze substitution of pollen grains

Freeze substitution of mature pollen grains which had previously been vital stained with neutral red (to show dull red staining of vesicle contents) resulted in retention of vesicle staining in only about 20% of all pollen grains (see also Appendix A). Sections of those embedded pollen grains which had retained neutral red showed the presence of many small vesicles of various sizes (Figs. 6j and k). Sections of pollen grains which had lost their neutral red staining during the freeze substitution procedure showed that either (a) these pollen grains had burst, losing their protoplasm (sometimes the protoplasts retained the vesicle staining), or (b) the neutral red had leaked out of the vesicles staining cytoplasm, nuclei (?) and walls (Fig. 6l).

3.2.5 Chromatography and UV spectroscopy of pollen grain vacuole contents

The compound(s) travelled very little with the 85% aqueous acetic acid solvent and hence all results outlined below are with 3:1:1 tertiary butyl alcohol: acetic acid: water solvent. The

chromatography results for the first four trials (using the methanol extracts) showed that extracts from older pollen grains with vesicles coloured greenish with Toluidine blue O had a large fluorescent region ranging from R_f 0.6-0.8 which usually had a yellow fluorescent spot at R_f 0.7-0.8 and a blue fluorescent spot at R_f 0.6-0.8 (Fig. 7a). Ammonia fumes intensified fluorescence of these spots. For these same trials the methanol extracts of younger pollen grains (with purplish vacuole contents with Toluidine blue O) had negligible or no fluorescence (Fig. 7a). When 50 μ l of the methanol extracts from these younger and older pollen grains were spotted onto a Whatman 1 chromatography paper, dried, and subsequently stained with pH 4.4, 0.05% (w/v) Toluidine blue O, the extract of the older pollen grains stained greenish whereas the extract of the younger pollen grains did not stain (Fig. 7b). For the last two chromatography trials (trial 5 and 6) in which both the methanol extract (of the pollen pellet) and the water sample in which the pollen were isolated were analyzed, the results varied. For trial 5 (using 2 g anthers) no fluorescence was observed for the methanol extracts but for the water extracts both vacuole 'types' showed a fluorescent blue spot at R_f 0.7. For trial 6 (using 0.2 g anthers) no fluorescence was observed for any of the extracts probably because not enough material was present.

UV spectroscopy of the last two trials (ie 5 and 6) also gave inconsistent results (Table 8). Basically, for each trial no significant difference was noted between pollen grains with different vacuole 'types' but there was considerable variation between the two trials.

Figure 7. Methanol extracts of pollen with green vacuoles (g) and purple vacuoles (p) after vital staining with pH 11, 0.025-0.05% (w/v) Toluidine blue O.

Fig. 7a: Paper chromatography using 3:1:1, tertiary-butyl alcohol: acetic acid: water and viewed with UV excitation. Pollen with green vacuoles has significantly more fluorescence than pollen with purple vacuoles. Line at top is solvent front. Labels (p and g) are placed over origin of chromatogram. About 0.2 X.

Fig. 7b: Extracts spotted onto Whatman 1 filter paper, dried and subsequently stained with pH 4.4, 0.05% (w/v) Toluidine blue O. Pollen with greenish vacuoles (g) have greenish outer ring (central portion unstained due to diffusion) and pollen with purplish vacuoles (p) show no staining. Filter paper itself stained significantly bluish. 1.5 X.

Figure 7

Table 8: UV spectroscopy of pollen extracts

Trial	(g) anthers	λ (nm) of absorption maxima of water extract		λ (nm) of absorption maxima of methanol extract	
		P¹	G¹	P¹	G¹
5	2.0	260-270	260-270	290-310	290-310
6	0.2	<250	<250	<250	<250

Note for Table 8:

1. P = purple, and G = greenish contents of pollen grain vacuoles and vesicles when stained with 0.025% (w/v) Toluidine blue O, pH 11.

3.3 Summary of results

Table 9 summarizes the results of this study on development of anthers and pollen grains in *B. napus*.

Table 9: Summary of results

Approx. bud length (mm) ¹	Stamen characteristics	General pollen characteristics	Vital staining of pollen with Toluidine blue O, at 0.025-0.05%, pH 11
<1.5	all cell layers intact; starch throughout	pollen mother cells up to tetrad stage	not detected
1.5-2.5	as above	early uninucleate, just released from tetrad: thin exine, central nucleus	tiny purple aggregates in otherwise unstained large vacuole
2.5-3.5	as above	late uninucleate: 1-3 large vacuoles; intine now visible; nucleus in lateral position next to walls	purple contents in the large vacuole(s) often containing tiny purple aggregates
3.5-5	middle layer disint- egrating; tapetal cells have Ubisch bodies and their walls are disint- egrating	early-mid binucleate: many small vacuoles especially around generative 'cell'; some small pink spots in cytoplasm after staining with PAS	transition period when large vacuoles are breaking up into many small vesicles; difficult to stain - some vesicles purplish, others greenish

Table 2: continued

Approx. bud length (mm) ¹	Stamen characteristics	General pollen characteristics	Vital staining of pollen with Toluidine blue O, at 0.025-0.05% pH 11
5-7	as above but also endothelial and con- nective tissue cell wall thickening and starch disappearing from most connective tissue and endothelial cells	late binucleate: no large vacuoles present but many small vesicles; heavy cyto- plasmic staining; nuclei not easily visible in sectioned cells; some pink spots in cytoplasm with PAS staining; intine easily visible	as above
7-9	tapetum disappearing or gone; endothelial and connective tissue cell wall thickening complete; 'motor' cells beginning to expand; starch almost all gone	trinucleate pollen: no large vacuoles; heavy cytoplasmic staining and hence difficult to see nuclei; solid PAS pink staining of cytoplasm and dark pink intine	many greenish vesicles
early anthesis ²	filaments extend; inner anthers starting to rotate; 'motor' cells fully expand; endo- thelial and connective cells dying	as above	as above

Table 9: continued

Approx. bud length (mm) ¹	Stamen characteristics	General pollen characteristics	Vital staining of pollen with Toluidine blue O, at 0.025-0.05%, pH 11
late anthesis	longitudinal slit progresses down anther as 'motor' cells collapse at the tip of the anthers first causing it to curl back; later collapse of 'motor' cells later in the basal part of the anther causes locules to spread open (dehiscence completed); most anther cells die	as above	as above

Note for Table 9:

1. Bud length is only an approximation and may vary significantly amongst inflorescences.
2. Anthesis is period of flower opening.

4. DISCUSSION

4.1 Anther development

Development of anthers of *B. napus* is similar to that of most angiosperms. The tapetum is probably the secretory type since it remains in a relatively intact layer surrounding the developing pollen grains until it is released into the locules, and Ubisch bodies develop in its cytoplasm. Both Grant *et al.* (1986) and Smith and Scarisbrick (1990) have also observed that the tapetal cells in *B. napus* persist along the periphery of the locules, up to the stage of the first mitosis of the pollen grains, but later become 'disorganized'. Smith and Scarisbrick (1990) also examined more mature anthers and found that the tapetum had 'disintegrated', presumably meaning that it was no longer present. Neither of these two studies reported the presence of Ubisch bodies, but this is not surprising since these refractile bodies are only visible in electron microscopy studies, or unstained sections viewed with bright or dark field optics, or in optical microscopy of sections treated with the PAS reaction. The tapetal cell walls are probably composed of some kind of non-carboxylated (or methylated) polysaccharides, eg hemicelluloses or methylated pectins, since they stain positively with the PAS reaction for glycol groups but are unstained by Toluidine blue O (a pink colour would be seen for carboxylated compounds). The histochemistry of the cytoplasm of this layer (as in the pollen grains) needs to be interpreted with caution as many changes in tissue composition and/or structure could occur during tissue preparation for microscopy. Some examples include the following: extraction of lipids usually occurs unless they are cross-linked (via C=C

instead graze through several different files. This is an important consideration if one wants to measure the lengths of these cells. For example, Gould and Lord (1988) attempted to relate the length of epidermal cells to the anther growth rates in *Lilium*. Unfortunately their measurements of cell lengths are wrong since those cells are also not aligned in files.

4.2 Pollen grain development

The most significant findings of this study are that (a) the vacuole(s) in the pollen grains appear to break down into many small vesicles during pollen grain maturation and may be accumulating phenolic compounds, (b) the contents of the vacuoles/vesicles in pollen grains are usually lost during preparation for microscopy, and (c) no reliable nuclear staining method for all stages of pollen grain development was found.

4.2.1 Development of the vacuole(s)

Vital staining of live pollen grains with Toluidine blue O shows a large vacuole in immature pollen grains which begins to break up into 2-4 smaller vacuoles prior to the first mitosis. Subsequently these vacuoles fragment even more so that the mature pollen grains contain many small vacuoles/vesicles. The other vital stains tested (ie neutral red, acridine orange and rhodamine B) stain the exine so much that the vacuole(s) is obscured in the immature grains, but this wall staining decreases with development so that many tiny vesicles are seen in the mature grains. Chemically fixed, sectioned and stained pollen grains show no apparent vacuole in the early uninucleate pollen grains (whose protoplasm appears to have pulled away from the walls during preparation for microscopy), one or more

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large vacuole(s) in the late uninucleate pollen grains, no large vacuole(s) but many small ones especially around the generative cell in the mid-binucleate grains, and very dense cytoplasm with few obvious small vesicles in the mature pollen grains. In these mature grains the dark cytoplasmic staining is probably impeding visualization of the vesicles (see section 4.2.3). Many tiny vesicles that have retained neutral red dye molecules are seen in freeze substituted mature pollen grains (immature grains were not examined with this procedure). All of these results suggest that the large vacuole in immature pollen grains breaks up into many small vesicles as the grains mature.

There are numerous examples of large vacuoles breaking up into smaller vacuoles in other plant cells. For example, the large vacuole in cells of the vascular cambium of trees during the growing season, fragments into many small vacuoles in the winter and subsequently coalesces into large vacuoles the following spring (Bailey, 1954; Robards and Kidwai, 1969). Dehydration of many cells and plasmolysis of tulip epidermal cells results in the vesiculation of the large vacuole, and this change is reversible when cells are returned to their 'normal' environmental conditions (Guillermond *et al.*, 1933). During the dehydration stage of development in protein-storing seeds the large vacuole in cells of the cotyledons is reported to break up into smaller vacuoles, forming the protein bodies/aleurone grains (Guillermond *et al.*, 1933; Millerd, 1975; Hara-Nishimara, 1987; Muntz, 1989) although the endoplasmic reticulum and/or Golgi bodies have also been implicated in the formation of protein bodies (Harris, 1979; Oparka and Harris, 1982;

Torrent *et al.*, 1989). During imbibition and subsequent germination, the protein bodies are reported to fuse to reform a large vacuole (Gifford and Bewley, 1983).

4.2.2 Analyses of contents of vacuoles/vesicles

Evidence that the vesicles in maturing pollen grains accumulate phenolic compounds is based primarily on vital staining results with Toluidine blue O. Vital staining of the developing pollen grains of *B. napus* shows that the contents of the 1-3 vacuoles in the immature grains stain purplish whereas the many vesicles in the mature grains have greenish contents with this dye. According to Kinzel (1958) and Stadelmann and Kinzel (1972) vacuoles with 'empty' vacuole saps containing organic acids stain purple with Toluidine blue O in contrast to the greenish colour obtained with this dye in 'full' vacuole saps thought to contain phenolic compounds such as flavone glycosides or tannins. Hence the colours obtained after the vital staining of *B. napus* pollen grains with Toluidine blue O suggest that vacuoles in immature pollen grains have 'empty' saps whereas more mature pollen grain vesicles have 'full' saps containing phenolic compounds. However, the specificity of Toluidine blue O for organic and phenolic compounds has not been thoroughly investigated. For this reason a more in-depth analysis of the dye's staining patterns with various purified compounds is examined (see chapter 3).

In spite of the heavy wall staining which prevents observations of vacuoles in the immature pollen grains, results with the other vital stains tested - ie acridine orange, neutral red, rhodamine B - show that these dyes also accumulate in the vesicle saps (which stain green with Toluidine blue O) in the mature pollen grains (although

the colour observed, for example with acridine orange, depends upon the dye concentration used).

Chromatography results from the first four trials done showed that fluorescent spots were obtained from methanol extracts of mature pollen grains thought to contain phenolics whereas negligible fluorescence was obtained from methanol extracts of immature pollen grains which presumably do not have any phenolics.

Toluidine blue O staining of these extracts dried onto chromatography paper gave greenish staining from the mature pollen grains but no staining for the immature pollen grains. These results do indicate a significant difference in the contents of the vacuoles in immature and mature grains with a shift to phenolic accumulation with maturity. There is always the possibility that the immature pollen grains may have burst during isolation and lost phenolic compounds into the water in which they were isolated from the anthers but the staining reaction suggests this was not the case. Although preliminary evidence suggests that some phenolic compounds may stain pinkish with Toluidine blue O, the purplish colour observed in the vacuoles of the immature pollen grains is mostly produced by carboxylated polysaccharides (see chapter 3).

Chromatography and UV spectroscopy of the two subsequent trials did not show any significant difference between the immature and mature pollen grain extracts within any given trial but did differ significantly from each other and from the first four trials. The fact that different plants grown in different growth cabinets were used for the last two trials may have resulted in this variability. Also, flavonoids are known to be unstable with time and in the presence of

light, oxygen and high temperature (Stanley and Linskins, 1974). Without knowing this information, the pollen extracts used for these unsuccessful trials were isolated and stored for varying times in the presence of light and oxygen. Hence it is quite likely that the original compounds may have changed composition before being examined. It is of course impossible to say if the pollen grain vacuole/vesicle contents were isolated in the complete absence of anther debris (insoluble, eg tapetal cells, or soluble - recall that the anther epidermal cell vacuoles also stain greenish with Toluidine blue O vital staining) and other components of the pollen grain cytoplasm. However, although more data is required before the compounds within the pollen grain vacuoles/vesicles at different stages of development can be chemically identified the results from the first four trials do suggest that compounds, probably phenolic, are present in the mature pollen grains but not the immature ones.

4.2.3 Retention of vacuole/vesicle contents

Sections of chemically fixed and embedded pollen grains stained with Toluidine blue O (at all pH levels tested) or protein stains give light cytoplasmic staining and good nuclear staining in immature grains, but suddenly cytoplasmic staining becomes very dark and non-specific obscuring nuclear staining in more mature pollen grains (soon after the first mitosis). Grant *et al.* (1986) and Smith and Scarisbrick (1990) observed the same staining patterns in sections of pollen grains of *B. napus* - see for example figures 12-14 in Grant *et al.* (1986). The occurrence of this sudden very heavy cytoplasmic staining which histochemically has been interpreted as being due to large accumulations of RNA and protein in the

cytoplasm (p.c. M.E. McCully), coincides with the onset of green staining with Toluidine blue O of vesicle compounds in live pollen grains. These results suggest that the compounds (presumably phenolics) responsible for the Toluidine blue O green staining in the vesicles of live mature pollen grains leak out of the vesicles during preparation for microscopy. The mordanting of these compounds to others (probably proteins) in the cytoplasm and on the nucleus probably results in this heavy, staining in mature pollen grains. Presumably the compounds in the vacuoles of younger pollen grains also leak out during preparation for microscopy, but they do not affect the staining patterns as much as phenolic compounds. There are reports showing the difficulty in retaining phenolic compounds in vacuoles. This results in the loss of these compounds from the vacuoles and subsequent adhesion to cell proteins hence giving heavy staining of the cytoplasm and nucleus which obscures ultrastructural detail (Mueller and Greenwood, 1978; O'Brien and McCully, 1981).

The PAS reaction is of particular interest. The small pink spots which occur in the otherwise faintly stained cytoplasm of some pollen grains (usually around the period of the first mitosis) may be starch since many of these also stain bluish-black with the IKI reaction for starch. The fact that the immature pollen grains have very faint cytoplasmic staining with the PAS reaction but suddenly the mature pollen grains all have very dark pink staining throughout the entire cytoplasm and in the intine, may also be due to an artifact caused by phenol release from the vacuoles and not the sudden presence of large amounts of polysaccharides with 1,2 glycol

groups. The onset of this solid pink PAS cytoplasmic staining coincides with the occurrence of green staining of vesicle contents of mature pollen grains vital stained with Toluidine blue O, thus the dark PAS staining in the mature pollen grain cytoplasm and intine is likely due to the staining of the phenolic compounds and/or their carbohydrate moieties which have leaked out of the vesicles and bound to cytoplasmic and nuclear proteins. Geier (1980) showed that plant vacuoles containing phenolic glycosides and/or non-glycosidic phenols such as flavan-3,4-diols give a positive reaction, ie stain pink, with the PAS test. The fact that Toluidine blue O staining of sections of fixed and embedded pollen grains gives dark purplish cytoplasmic staining at all stages of development is difficult to explain. It is possible that the phenolics leaked out of the vesicles and became mordanted throughout the cytoplasm and (a) do not stain green in this form with Toluidine blue O, and/or (b) only the carbohydrate moieties of the phenolic compounds stain with the PAS reaction and (if carboxylated) with Toluidine blue O in the fixed and embedded material.

Freeze substitution of mature pollen grains previously vital stained with neutral red results in the loss of the neutral red from the vesicles and subsequent staining of the cytoplasm and/or bursting in about 80-90% of the pollen grains. This anhydrous freeze substitution procedure will only retain compounds that are insoluble in the dry solvents used (Canny and McCully, 1986), thus the phenolic compounds must be soluble in these solvents. No vesicles are seen in sections of freeze-substituted unstained pollen grains. Chemical fixation results in the loss of phenolic compounds

from the vacuoles (Mueller and Greenwood, 1978), the loss of many solutes from plant cells (Coetzee and van der Merwe, 1984) and the destruction of membrane permeability (Harper, 1986). Exposure of live mature pollen grains (prestained with neutral red) of *B. napus* to glutaraldehyde or paraformaldehyde fixatives also results in the loss of compounds, in this case neutral red, from the vesicles with subsequent staining of the cytoplasm and/or bursting of the pollen grains. All of these results indicate that the procedures used to fix and embed the mature pollen grains were not capable of retaining contents within the vesicles.

In addition, exposure of other plant cells to different chemical fixatives and/or freeze substitution usually results in the release of contents from cell vacuoles (see Appendix), providing evidence that this is a common phenomenon. It is therefore an important artifact to consider in the interpretation of histochemical studies of most plant cells.

4.2.4 Pollen nuclear staining

The fact that the nuclear staining in pollen grains at most developmental stages is very difficult and unreliable is probably due to a combination of poor dye access into whole pollen grains, mordanting of nuclei by leaking vacuole contents which prevent their staining, and/or other, unknown factors. Probably, staining of whole pollen grains is impeded by the pollen grain walls since nuclear staining becomes reduced/negligible as soon as the unstained pollen grain exine becomes lobed in form - indeed most of the dyes tested stained the exine heavily. Release of the vacuole contents into the cytoplasm most likely also impedes nuclear staining of whole

pollen grains. The reason why pollen grains prefixed in Carnoy's fixative or 100% methanol often give fairly good nuclear staining (with DAPI) in late trinucleate pollen (but inconsistent results at most other developmental stages) may be because (a) at this stage pollen expand considerably perhaps therefore increasing wall permeability during fixation and subsequent staining, and (b) the alcohol in each fixative solution probably extracts phenolic compounds (as well as most cytoplasmic contents). Although Fan *et al.* (1988) concede that the nuclei of pollen grains in *Brassica* species are difficult to stain, other studies such as Singh *et al.* (1985), Kott *et al.* (1988a) and Pechan and Keller (1988) reported good nuclear staining in pollen grains of *B. napus* using methods which I found to be poor and unreliable.

The poor nuclear staining in sectioned, fixed and embedded pollen grains at most stages of development obviously can not be due to wall impermeability. As mentioned earlier, there is little difficulty staining the nuclei of the immature pollen with Toluidine blue O and protein stains but the nuclei are obscured (probably since they are mordanted with released phenolic compounds) in the mature pollen grains at the stage when Toluidine blue O vital staining indicates that the vesicles are accumulating phenolic compounds (ie the vesicle contents stain greenish). Grant *et al.* (1986) reported similar staining patterns in sections of fixed and stained grains of *B. napus*. This suggests that release of vesicle contents during preparation for microscopy does in fact inhibit nuclear staining. The poor nuclear staining (especially of the vegetative nuclei) of fixed and embedded

pollen grains with the Feulgen method may also be due to the release of phenolic or other compounds from the vacuoles/vesicles.

4.2.5 Possible physiological significance of vacuole contents

It is most likely that the vesicles in the pollen grains accumulate phenolic compounds, possibly flavonoids, during grain maturation. Flavonoids are said to be pigments in pollen grain exines which may be providing protection from UV light and/or attracting insects (see reviews by Stanley and Linskins, 1974 and Mascarenhas, 1990). The presence of the flavonoid kaempferol was reported in pollen of *B. oleracea* (Stanley and Linskins, 1974). It is possible that the flavonoids detected by these authors were not originally from the exines as they suggested, but instead were extracted from the vesicles within the pollen grains.

Flavonoids could be playing an important role in pollen-stigma compatibility. Phenolic 'signals' may be involved in a variety of plant developmental processes (Lynn and Chang, 1990). For example, plant flavonoids induce expression of nodulation genes in *Rhizobium*, indicating an important role of these flavonoids in the symbiotic interaction between the plant and microorganisms (Firmin *et al.*, 1986; Kent Peters *et al.*, 1986; Redmond *et al.*, 1986). Perhaps flavonoid compounds present within the vesicles of pollen grains could play an important role in recognition processes at the stigma-pollen interface.

The fact that the large vacuole begins to break up and accumulate (probably) phenolic compounds immediately after the period in pollen development in *B. napus* considered to be in the

embryogenic 'window' may have implications for pollen grain culture since, for example, many gametophytic biochemical processes are probably initiated during this time. Sangwan and Camefort (1983) found that the embryogenic pollen grains of *Datura* show an electron-opaque deposit on their tonoplast which cytochemical tests indicated to be tannins. However, Zaki and Dickinson (1990) found no correlation between electron opaque material lining the tonoplast of all cultured late uninucleate and early binucleate pollen grains of *B. napus* with the grains' embryogenic potential. Kott *et al.* (1988b) reported that when young, potentially embryogenic, pollen grains of *B. napus* were cultured in the presence of older pollen grains or a supernatant derived from previously cultured older pollen grains, levels of embryogenesis were reduced and abnormalities in embryo morphology increased. The 'toxin' they claim is released from the older pollen grains could be the phenolic compounds I believe are present in the vesicles.

CHAPTER 3
SPECIFICITY OF TOLUIDINE BLUE O

1 INTRODUCTION

Unlike many dyes, Toluidine blue O has not been widely used in the textile industry (Lillie, 1969). Instead this dye (in aqueous solution) has been primarily used in histochemical applications as a bacterial stain, a nuclear stain (eg. in blood cells) or for general tissue metachromatic staining (red-purple) in, for example, animal Nissl granules, cartilage matrix, acid mucins and mast cells (Lillie, 1965 and 1969). O'Brien *et al.* (1964) were the first to use aqueous solutions of Toluidine blue O to stain plant tissues. It is now the stain most widely used by plant histologists and pathologists to study the chemical composition of plant cell walls and their reactions to pathogens. It imparts red, purple, blue and/or green colours to tissue sections.

According to current dogma, Toluidine blue O and other metachromatic dyes¹ stain many polyanionic compounds (containing carboxyl, phosphate or sulphate groups) purplish (Kelly, 1956; Pearse, 1985). The cationic dye molecules (Fig. 8) are thought to bind ionically to anionic compounds and the resulting pink or purple colour is said to require (a) the stacking of dye molecules with each

¹A dye which gives two or more colours upon staining different compounds is defined as a metachromatic dye (Horobin, 1988). A metachromatic colour is defined as being significantly different from the 'normal' orthochromatic dye colour (Pearse, 1985) but this definition can become confusing when dye concentration is considered. The metachromatic colour has also been defined as the colour resulting from a shift in the dye's absorption spectrum towards shorter wavelengths (Pearse, 1985). For example this would mean a change from blue to purple with Toluidine blue O but the term green ('negative') metachromasia has also been used for this dye (Kinzel, 1958; Ramalingam and Ravindranath, 1970). For this report the use of the terms metachromatic and orthochromatic colours will be avoided.

Fig. 8. Structure of Toluidine blue O derived from Lillie (1969) and Anonymous (1971).

Fig. 9. Possible mechanism of the 'ionic' reaction of Toluidine blue O which produces the purplish colour with polygalacturonic acid as an example of an anionic substrate. Diagram is illustrating an interpretation of Pearse (1985).

other, (b) the presence of water molecules between stacked dye molecules and (c) a minimum substrate surface charge density (Fig. 9).

The dye stacking phenomenon is assumed to be a common property of 'metachromatic' dyes (Michaelis, 1947; Sylven, 1954; Schubert and Levine, 1955; Kelly, 1956; Schubert and Hamerman, 1956; Pearse, 1985). This assumption arose primarily from observations that 'metachromatic' colours are produced in dye solutions at high concentrations whereas more dilute solutions give the 'normal' orthochromatic colour. Furthermore, absorption spectra of many metachromatic dyes do not obey Beer's law which states that for any given wavelength the absorption is directly proportional to dye concentration. Instead, many metachromatic dyes show a shift in the absorption maximum towards shorter wavelengths with increasing concentration. For example, Michaelis (1947) reported that Toluidine blue O solutions changed from blue to purple with increasing dye concentration. He reported the presence of an ' α ' band (absorbance at 630 nm) a ' β ' band (absorbance ca. 590 nm) and a ' γ ' band (absorbance at 520-540 nm) in solutions of increasing dye concentration - the ' γ ' band also appeared in the presence of certain anionic compounds. Respectively, α , β , and γ absorption bands were assumed to be monomeric, dimeric and higher molecular aggregates of the dye (Michaelis, 1947). The fact that alcoholic solutions of many metachromatic dyes obeyed Beer's law and gave only the α (monomeric) absorption band at all concentrations tested was taken as further evidence that dye stacking (and the presence of water)

was necessary for production of the metachromasia (Michaelis, 1947; Sylven, 1954; Kelly, 1956; Schubert and Hamerman, 1956).

According to Horobin (1982) other studies including ultra-filtration through membranes or gel-filtration media, osmotic phenomena, conductivity measurements and diffusion effects have also been used to study dye aggregation.

There is little known about the green/blue colour produced by Toluidine blue O staining. The green colour has been presumed to result from the staining of nucleic acids (Michaelis, 1947; Feder and Wolf, 1965), plant lignins (eg. O'Brien *et al.* , 1964), and other polyphenols (eg. Theunissen and Jordaan, 1990) including flavonoids or tannins (Kinzel, 1958; Stadelmann and Kinzel, 1972). However, to my knowledge, with the exception of Ramalingam and Ravindranath (1970) who observed green staining of some synthetic phenolic compounds after staining with Toluidine blue O there has been no published study examining the types of compounds or chemical reactions which give the greenish/bluish colour after staining with Toluidine blue O. The greenish/bluish colour is assumed to be due to either ionic interactions between nucleic acid phosphate groups and cationic dye molecules or hydrophobic interactions between dye benzene groups and substrate aromatic compounds (p.c. , M.E. McCully).

In this study I examined the specificity of the Toluidine blue O dye for various plant cell components, in particular those of the cell wall. This work focused on plant cell wall material since we are most familiar with the dye's staining properties of cell walls in tissue sections and there was a good source of isolated wall components

available. Toluidine blue O staining reactions of cell walls in fresh tissue sections were compared to those in model systems in which purified cell components were stained with the dye. The dye is thought to stain the anionic uronic acids in pectin and possibly hemicelluloses of the cell walls a purple colour in contrast to the greenish colour it stains lignified cell walls such as the secondary thickening of xylem cells (p.c., M.E. McCully).

There have been a number of reports stating that most commercial dyes are 'impure' (Ball and Jackson, 1953; Kelly, 1956; Smith and McCully, 1978; Horobin, 1980; Horobin, 1982; Proctor and Horobin, 1985;). Therefore in addition, I attempted to 'purify' Toluidine blue O samples in order to (a) determine if the different staining colours observed in tissue sections were due to one or more dye components and (b) establish a preparative dye purification method which would eliminate inconsistencies between different dye batches and sources.

2 MATERIAL AND METHODS

2.1 Analyses of the pink/purple staining reaction

2.1.1 Analyses of dye solutions

Changes in the absorption spectra and transmitted colour of Toluidine blue O solutions after the addition of various purified compounds were analyzed. This approach provided a model system potentially capable of distinguishing between different interactions of the dye with individual types of molecules and the use of absorption spectra eliminated the variability in subjective visual observations of dye colours.

A spectrophotometer (DU-64 Beckman) was used to examine the absorption spectra of Toluidine blue O dye solutions made up in 50 mM acetate buffer at pH 4.5. Toluidine blue O (CI 52040, Polyscience Ltd., lot 86864) was always used except for the few experiments done with Toluidine blue O (CI 52040, Fisher Scientific Ltd., lot 895981). The dyes were 'unpurified' ie., they were taken straight from the bottle.

Dye solutions of various concentrations were examined upon addition of various concentrations of (a) sodium chloride and (b) selected carbohydrates (see Tables 10 and 11) in the presence or absence of sodium chloride. (Previous experiments with tissue sections had shown that the purple colour was lost from cell walls of sections placed in salt solutions (see below): the effect of salts on the dye itself and on dye-carbohydrate complexes was therefore examined.) For these experiments the required amount of carbohydrate and/or salt was added to the dye solution to give the final concentration used. To test the effect of temperature, a waterbath (Grant Ltd. 6, Grant Instruments (Cambridge) Ltd., Barrington, Cambridge, England) maintained the specimen in the spectrophotometer at approximately 5°C. Spectra of ice-cold 0.1 mM Toluidine blue O (plus pectin or polygalacturonic acid) solutions were compared with spectra of these solutions at room temperature (about 24°C). For the temperature experiments, the dye and carbohydrate solutions were made up separately in 50 mM acetate buffer, pH 4.5, both at double their final concentrations, and placed on ice for at least 15 minutes. Equal volumes of each were then mixed to give a 0.1 mM Toluidine blue O solution adjusted to pH 4.5 with sodium

Table 10: Compounds tested with Toluidine blue O solutions

<u>Compound</u>	<u>Source</u>
carboxymethyl-cellulose	Sigma No. C-8758, lot 55F-0608, sodium salt
cellulose	(1) Sigma No. S-3755, Sigmacell, Type 100 and (2) Sigma No. S-3755, lot 76F-0720
chitosan	Sigma No. C-0792, lot 89F-0383, practical grade from crab shells
chondroitin sulfate B	Sigma No. C-2413, lot 119F-0680, sodium salt
dextran	Sigma No. D-7265, lot 44F-0174, avg mwt 249,000, Industrial grade
D-galacturonic acid	Sigma No. G-2125, lot 42F-010B, monohydrate
D-glucuronic acid	Sigma No. G-9000, lot 102F-0807, grade 1
gum arabic	From <i>Acacia senegal</i> , isolated by Dr. D.M.W.Anderson, Edinburgh University, U.K.
laminarin	Sigma No. L-9643, lot 78F-3798, ca. 95%, from <i>Laminaria digitata</i>
mannan	Sigma No. M-3640, lot 92F-3901, from yeast
methyl cellulose	Sigma No. M-7140, lot 57F-0522
pectin (apple)	Sigma No. P-2157, lot 26F-0409 containing ca. 80% galacturonic acid and ca. 7.1% methoxy content (without sucrose or other sugars)
pectin (citrus)	Sigma No. P-9135, lot 42F-0369, containing ca. 75.1% galacturonic acid and 7.25% methoxy content

Table 10 continued

<u>Compound</u>	<u>Source</u>
polygalacturonic acid	Sigma No. P-1879, lot 71F-3857 or lot 117C-3878, grade 2, ca. 85-90% (a commercial preparation of de-esterified pectin), sodium salt
ribonucleic acid	Sigma No. R-6625, lot 118F-0073, type 6, from <i>Torula</i> yeast

Table 11: Structures of compounds tested with Toluidine blue O solutions

<u>Compound</u>	<u>Monomer(s)¹</u>	<u>Structure²</u>	<u>Ref.³</u>
carboxy-methyl-cellulose	Mixture of D-Glc and D-CMGlc, β -(1 $\rightarrow$ 4) linked		1
cellulose	β -(1 $\rightarrow$ 4)-D-Glc		1
chitosan	β -(1 $\rightarrow$ 4)-D-amino-Glc		2
chondroitin sulfate B	IdA, NAC, β -(1 $\rightarrow$ 4) linked		2

Table 11 continued

<u>Compound</u>	<u>Monomer(s)</u>	<u>Structure</u>	<u>Ref.</u>
dextran	α -(1 $\rightarrow$ 6)-D-Glc		2
galacturonic acid	D-GalA		1
glucuronic acid	D-GlcA		1
gum arabic ⁴	D-Gal, L-Ara, D-GlcA, L-Rha, D-4-O-CH ₃ -GlcA, protein	complex	3
laminarin	β -(1 $\rightarrow$ 3)-D-Glc with some β -(1 $\rightarrow$ 6)-D-Glc		2
mannan	β -(1 $\rightarrow$ 4)-D-Man with 25-35% β -D-Glc		1
methyl cellulose	CH ₃ -O-D-Glc, D-Glc, mixture. β -(1 $\rightarrow$ 4) linked		2

Table 11 continued

<u>Compound</u>	<u>Monomer(s)</u>	<u>Structure</u>	<u>Ref.</u>
pectin	α -D-(1 $\rightarrow$ 4) homo-GalA (some methyl-esterified GalA) 'smooth blocks' with 'hairy blocks' containing Rha, Gal, Ara		1
polygalacturonic acid	α -(1 $\rightarrow$ 4)-D-GalA		1
RNA	sugar (ribose)-phosphate backbone, base (adenine, guanine, cytosine, uracil)		4

Note for Table 11:

1. Monomers are: Glc = glucose; CMGlc = carboxymethyl-glucose; IdA = iduronic acid; NAC = N-acetylchondrosamine (sulphated on C4); GalA = galacturonic acid; GlcA = glucuronic acid; Gal = galactose; Ara = arabinose; Rha = rhamnose; Man = mannose;
2. Structure shown: dashed brackets = repetition of same or different monomers in heterogeneous polymer; solid brackets = repetition of same monomers in polymer
3. References used are (1) Fry (1988); (2) Anonymous (1983); (3) Street and Anderson (1983); (4) McMurray (1984).
4. Gum arabic is an arabinogalactan or arabinogalactan-protein (p.c., S.C. Fry) with a highly branched structure which was too complex to draw.

hydroxide when necessary. All experiments were repeated a minimum of three times.

Various concentrations of selected isolated compounds, primarily carbohydrates (see Tables 10 and 11), were dissolved in 50 mM acetate buffer at pH 4.5, placed on ice for about 15 min. and then diluted with ice-cold, 0.2 mM Toluidine blue O (CI 52040, Polyscience Ltd., lot 86864) dye solution to give a final 0.1 mM dye solution at pH 4.5. The colour of the cold solutions was observed and recorded and then the solutions were warmed to room temperature (ca 24°C) and their colour was again recorded. The colours observed were those of the solution drawn up a pasteur pipette and compared to the Toluidine blue O solution of the same concentration. These colours were then matched, as closely as possible, with those of the ISCC-NBS Centroid Colour Charts, Standard Sample No. 2106 (Kelly, 1958). All compounds were tested at least twice.

2.1.2 Analyses of tissue sections

Hand sections, primarily of pea (*Pisum sativum* L.), potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon* sp.) mature stems were used for these experiments. Fresh hand sections were placed into dye solutions of varying concentrations (0.005 mM, 0.025 mM, and 0.1 mM) and allowed to stain for up to one week so as to examine the effect of dye concentration on the tissue staining pattern. The sections were placed in either (a) ca. 100 ml. of the dye solution, unstirred or (b) ca. 500 ml. dye solution with constant agitation. In addition, prestained (30-60 sec. in 1.0 mM dye solution) fresh hand sections were placed in 1.0 M sodium chloride or calcium chloride solutions for up to one week in order to observe the effect of

salt on the tissue staining pattern. Only Toluidine blue O (CI 52040, Polyscience Ltd., lot 86864) was used for these studies.

2.2 Analyses of the blue/green staining

2.2.1 Toluidine blue O staining of compounds spotted on to chromatography paper.

Many aromatic compounds are not very soluble in aqueous solutions and hence various compounds (mostly aromatic) -see Table 12 - were dissolved in 100% methanol to give a 0.1 M final concentration of the tested compound in solution. Exceptions to this method were (a) thiamine hydrochloride and sinapic acid were dissolved in 80% methanol, (b) L-phenylalanine and L-tyrosine were dissolved in ammonia and (c) tannic acid, whose molecular weight is unknown, was made up to 0.1% and 1.0% (w/v) in 100% methanol. For all compounds tested 10 μ l of solution was applied as a single spot on Whatman 1 chromatography paper strips (3 spots/strip) and blow dried with warm air. The strips were then dipped through a 1.0 mM Polyscience Toluidine blue O (CI 52040, lot 86864) dye solution and the colours of each spot recorded immediately as well as after the chromatography paper had dried. Each compound was tested at least three times.

1.0 mM dye solution was used to produce an immediate colour change. With lower concentrations the colour change took longer and the compounds diffused too much if left in the dye solution for extended periods of time. The chromatography paper was dipped through the dye solution instead of having the dye solution sprayed onto the paper so as to avoid possible inconsistencies due to uneven

Table 12: Aromatic and other 'ringed' compounds tested with Toluidine blue Q

<u>Compound</u>	<u>Source</u>
benzoic acid	Aldrich Chem. Co., no. 10,947-9
benzylmalonic acid	Aldrich Chem. Co., no. 10,077-3, 99% pure
m-bromobenzoic acid	Aldrich Chem. Co., no. 18,629-5, 99% pure
(+) catechin	Sigma no. C-1251, lot 71F-0214
cinnamic acid	B.D.H. Lab. Chem. Ltd., no. 0299260
o-coumaric acid	Sigma no. C-4400, lot 112F-0018
p-coumaric acid	Sigma no. C-9008, lot 79C-0188
coumarin	Sigma no. C-4261, lot 72F-0795
(-) epicatechin	Sigma no. E-1753, lot 51F-0548
ferulic acid	Sigma no. F-3500, lot 121F-0552
gallic acid	Sigma no. G-7384
guaicol	Sigma no. G-5502, lot 42F-0673
p-hydroxybenzoic acid	Aldrich Chem. Co., no. H2,005-9
methyl 4-hydroxybenzoate	B.D.H. Chem. Ltd., Prod 29231, 9682402E
L-phenylalanine	Sigma no. P-2126, lot 43F-0228
m-phenylphenol	Kodak no. 3166, lot B6N
syringaldehyde	From Dr. Stephen C. Fry, no. 101
tannic acid	Sigma no. T-0125, lot 33F-0682

<u>Compound</u>	<u>Table 12: continued</u> <u>Source</u>
thiamine hydro- chloride	Sigma no. T-4625, lot 18F-0521
L-tyrosine	Sigma no. T-3754, lot 34C-0160

spraying. However, the dipping method may result in diffusion of the compound being tested into the dye solution.

2.2.2 Analyses of tissue sections

In addition, hand sections of mature pea, tomato and potato stems were immersed in (a) dye solutions of various concentrations, (b) calcium chloride and sodium chloride salt solutions (as described in section 1.2.1.2), and (c) 100% acetone and 80% liquid phenol to observe the effects on the staining patterns for at least three trials each.

2.3 Analyses of Toluidine blue O purity.

2.3.1 Analytical purification

In collaboration with Dr. R. Horobin (Sheffield University, U.K.) the purity of various sources of Toluidine blue O was analyzed using various thin layer chromatography (TLC) systems (see Tables 13-15).

2.3.2 Analyses of the 'major' component.

A silica gel TLC plate was streaked along its origin with 10 mM Toluidine blue O (CI 52040, Polyscience Ltd., lot 86864) - see Table 20, code 'a'. The major component was scraped off the plate and then placed in a plastic pipette tip with glass wool at the bottom to trap the silica gel crystals. Methanol was added to elute the dye off of the silica crystals and the eluate was then evaporated leaving the dye crystals behind. The quantity of dye recovered was too small to weigh but after addition of 1 ml of pH 4.5, 50 mM acetate buffer the solution was estimated to be about 0.01 - 0.05 mM as judged by the visual colour of the solution. Hand sections of pea, tomato and potato stems were placed in this solution to determine if this one

Table 13 Dyes used for chromatography

<u>Code</u>	<u>Dye used</u>
1	Toluidine blue O. CI 52040. Polyscience Inc., Warrington, PA. Lot no. 86864, Cat. no. 1234. Certified by the Biological Stains Commission - certification no. BaU-6. % dye content?
2	Toluidine blue. CI 52040. Certistain. 3562240J. Prod 34077. BDH Chemicals Ltd., Poole, England; Gurr microscopy materials. 61% minimum dye content.
3	Toluidine blue O. CI 52040. Sigma Chemical Co., St. Louis, U.S.A. No. T-3260. Lot no. 37C-0217. 5-GM. ca. 85% minimum dye content.
4	Toluidine blue O - certified. CI 52040. (Basic blue 17). Sigma Chemical Co., St. Louis, U.S.A. No. T-0394. Lot no. 57F-4414. Certified by the Biological Stains Commission - certification no. AcU-8. 91% dye content.
5	Azure C. AX 1895. Matheson Coleman and Bell, Division of the Matheson Co. Inc., Ohio, U.S.A. Certified by the Biological Stains Commission for use in histology - certification no. 4. 71% total dye content.

Table 14: TLC plates or chromatography paper used

<u>Code</u>	<u>Plate/paper used</u>
A	Eastman chromatogram sheet. 13181. Silica gel TLC plate with fluorescent indicator.
B	Uniplate RPSF TLC plate. Analtech Inc., Newark, D.E. Lot no. KABX, Cat. no. 52511.
C	Whatman KC18F Reversed Phase TLC plate. 200 μ m thick. lot no. 002685, Cat. no. 4803-600. Whatman Chemical Separation Inc., Clifton, N.J.
D	Diphenyl Reversed Phase TLC plate. 250 μ m thick. Lot no. 002686, Cat. no. 4814-600. Whatman Chemical Separation Inc., Clifton, N.J.
E	Eastman 6064 Cellulose TLC plate without fluorescent indicator. No. 5351.
F	Whatman 1 chromatography paper. Cat. no. 3001 878. Whatman International Ltd., Maidstone, England.

Table 15: Chromatography systems used

<u>Code</u>	<u>Plate/ Paper</u>	<u>Solvents used</u>	<u>Solvent front (mm)</u>
a	A	85:10:5, 95% ethanol: acetone: water	55-83
b	B	9:1, methanol: water + 0.76 M sodium acetate	98
c	C	"	73
d	D	"	74
e	E	35:35:30, isobutanol: acetone:0.2 M hydrochloric acid	91
f	F	4:1:5, n-butanol: acetic acid: water, upper phase	145
g	F	9:1, methanol: water + 0.76 M sodium acetate	135
h	F	35:35:30, isobutanol: acetone: 0.2M hydrochloric acid	
		trial 1	125
		trial 2	157
i	F	35:35:30, n-butanol: acetone: 0.2 M hydrochloric acid	
		trial 1	155
		trial 2	159
		trial 3	370
j	F	35:35:30, n-butanol: acetone: 0.2 M tri-fluoroacetic acid	173
k	F	35:35:30, isobutanol: acetone: 0.2 M tri-fluoroacetic acid	173
l	F	12:3:5, n-butanol: acetic acid: water	205
m	F	10:5:6, ethyl acetate: acetic acid: water	161

Table 15 continued

<u>(mm)</u>	<u>Plate/ paper</u>	<u>Solvents used</u>	<u>Code front</u>
n	F	8:2:1, ethyl acetate: pyridine: water	155
o	F	9:3:3, ethyl acetate: pyridine: water	182
p	F	10:5:6, ethyl acetate: pyridine: water	169
q	F	19:5:3:9, ethyl acetate: pyridine: acetic acid: water	175
r	F	4:3:4, n-butanol: pyridine: water	
		trial 1	185
		trial 2	330
s	F	80 % phenol (w/w)	197
t	F	85:10:5, 95% ethanol: acetone: water	158
u	F	0.2 M aqueous hydrochloric acid	197
v	F	0.2 M aqueous ammonia	185

Note for Table 15:

1. Plate/paper used - see Table 14.

component would produce both purple and green staining in cell walls.

2.3.3 Preparative purification.

Various paper chromatography solvent systems were examined for their potential use in the 'large-scale' isolation of the major component of Polyscience Toluidine blue O (CI 52040, lot 86864) - see Tables 13-15 - as this method is cheap and relatively easy to use for the isolation of large quantities of dye. (The Silica Gel TLC plates were to be used to determine the 'purity' of the large scale isolation procedure.) However, a suitable system could not be found as judged by the fact that usually only one component was isolated for each solvent. Therefore in addition, several trials of column chromatography using (a) silica gel (Sigma No. S-0507, chromatographic grade, 230-400 mesh), (b) a glass column (ca. 1" X 30") with a (c) 85:10:5, 95% EtOH:acetone:water ($\pm$ 1-2% glacial acetic acid) solvent system were run (in darkness) in an attempt to 'purify' 0.5-1 ml of 5-10 mM Toluidine blue O aqueous solutions. Some of the 'major' components from these columns were ran on Silica Gel TLC plates with an 85:10:5, 95% ethanol:acetone:water solvent.

3 RESULTS AND DISCUSSION

3.1 The pink/purple staining reaction

3.1.1 Analyses of dye solutions

3.1.1.1 Dye concentration

The pH of dye solutions ranging from 0.005 mM to 1 mM of Toluidine blue O (Polyscience) in 50 mM acetate buffer, pH 4.5, did not differ significantly from that of the buffer alone. Visually, these solutions change from being blue at dilute concentrations (less than about 0.1 mM) to progressively more purple with increasing concentration. Absorption spectra of dye solutions (≤ 0.125 mM) reveal the presence of two peaks, at about 590 and 635 nm, with the maximum absorption peak changing from 635 nm to 590 nm with increasing concentration, (see Figs. 10-13). The same results were obtained using Fisher Toluidine blue O. Concentrations greater than 0.125 mM Toluidine blue O exceeded the absorption limit of the spectrophotometer. A rough estimation of the absorbance spectrum of a concentrated (1.0 mM) dye solution loaded into a capillary tube to shorten the path length gave an absorption maximum at about 580 nm with a shoulder at about 640 nm (one trial only, using Polyscience dye, Fig. 14).

These results suggest that dye aggregation occurs as the concentration increases. The absorption peaks correspond to the α (monomer, 635 nm) and β (dimer, 590 nm) bands reported by Michaelis (1947). Hence at concentrations ≤ 0.005 mM the monomeric dye form (α band, 635 nm) may be predominant whereas with increasing concentration more of the dye is present in the dimeric form (β band, 590 nm). Since concentrations above 0.125

Fig. 10 0.005 mM Toluidine blue O at 24°C

Fig. 11 0.025 mM Toluidine blue O at 24°C

Fig. 12 0.075 mM Toluidine blue O at 24°C

Fig. 13 0.1 mM Toluidine blue O at 24°C

Fig. 14 1.0 mM Toluidine blue O at 24°C

Fig. 15 0.025 mM Toluidine blue O at 24°C

mM could not be effectively examined, it is not possible to say whether or not a γ band (indicative of higher molecular aggregates) exists in the most concentrated, purple dye solutions. However, the one trial done at 1.0 mM suggests that the trend towards shorter wavelengths with increasing dye concentration continues.

Dye concentrations of 0.005 mM to 0.125 mM also had two absorbance peaks in the UV range; a major peak at 290 nm and a secondary peak at about 250 nm, which were probably due to the aromatic nature of the dye molecules (Fig. 15). Due to time constraints this study focused on the absorption spectra in the visible range.

3.1.1.2 Effect of some carbohydrates on the absorption spectra of Toluidine blue O solutions

Results obtained from these experiments are outlined in Table 16. The monosaccharides glucuronic acid (GlcA) and galacturonic acid (Gala) remained blue at most of the dye concentrations tested. However, high monosaccharide concentrations (1.0 M) became greenish when mixed with the dye solution (Table 16). At high concentrations the GlcA and Gala solutions themselves become yellowish probably due to impurities (p.c., S.C. Fry). It is probable that the shift to the greenish colour at high concentrations of monosaccharides is due to the combination of the yellow impurities and the blue dye solutions, instead of a change in the dye-sugar interaction at the molecular level. (The same is probably true for the greenish colours observed with high (0.5-1.0%) concentrations of pectin mixed with 0.005 mM or 0.025 mM dye solutions - the 0.5%

Table 16: Changes in the absorbance spectra and colours of Toluidine blue O solutions (at room temperature) with addition of some carbohydrates (CHO)

CHO ¹	%CHO	mM T.B. ²	Colour ³	λ (nm) of Absorbance Maxima ⁴		
				1°	2°	3°
GlcA and GalA	0.0	0.005	v.l.g.B.	635	595	-
	0.001	"	"	"	"	-
	0.1	"	"	"	"	-
	1.0	"	y.gr.	"	"	-
as above	0.0	0.025	pale br.B.	635	595	-
	0.001	"	"	"	"	-
	0.1	"	"	"	"	-
	1.0	"	br.g.B.	"	"	-
as above	0.0	0.1	s.B.	595	635	-
	1.0	"	m.g.B.	635	595	-
pectin (apple or citrus) or PGA	0.0	0.005	v.l.g.B.	635	595	-
	0.01	"	"	"	"	-
	0.1	"	"	"	"	-
	0.5	"	l.g.B.	"	-	-
	1.0	"	l.g.B to y.gr.	"	-	-

Table 16 continued

<u>Maxima⁴</u> <u>CHO¹</u>	<u>%CHO</u>	<u>mM T.B.²</u>	<u>Colour³</u>	<u>λ (nm) of Absorbance</u>		
				<u>1°</u>	<u>2°</u>	<u>3°</u>
as above	0.0	0.025	pale br.B.	635	595	-
	0.01	"	"	"	"	-
	0.1	"	"	"	"	-
	0.5	"	l.B.	"	"	-
	1.0	"	l.B. to v.l.g.B.	"	"	-
as above ⁵	0.0	0.1	s.B.	595	635	-
	0.01	"	dark s.B.	"	"	-
	0.1	"	s.p.B. to dull s.V.	"	550	635
	0.5	"	s.V.	550	595	550
	1.0	"	s.p.B. to dull s.V.	595	635	550

Note for Table 16:

1. GlcA is glucuronic acid; GalA is galacturonic acid; pectin (derived from apple or citrus fruits); PGA is polygalacturonic acid. Different lots of each were tested (see Table 10) with no obvious difference noted between lots.
2. Concentration of Toluidine blue 0 (Polyscience Ltd., CI 52040, lot 86864) used.
3. Colour of the solutions was matched with the ISCC-NBS Centroid Colour Charts (Kelly, 1958). v.l.g.B. = very light green blue (#171); y.gr. = yellow green; pale br.B. = pale brilliant blue (#177); br.g.B. = brilliant green blue (#168); s.B. = soft blue (#178); m.g.B. = medium green blue (#173); l.g.B. = light green blue (#172); l.B. = light blue (#181); s.p.B. = soft purplish blue (#196); s.V. = soft violet (#207).
4. Primary (1°), secondary (2°) and tertiary (3°) are three absorbance maxima occurring at different wavelengths where the maximum absorbance of primary > secondary > tertiary. The secondary and sometimes tertiary peaks often appeared as 'shoulders' on the absorption spectrum, ie they did not form a distinct absorbance peak of their own.

Table 16 continued

5. The PGA produced the violet colour at 0.1% PGA with soft purplish blue colours being produced at 0.5% and 1% PGA in contrast to the pectins which produced the violet colour at 0.5% pectin. At 0.1% Toluidine blue, the apple pectin did not produce as consistent results as the citrus pectin.

and 1.0% pectin solutions themselves were yellowish.) The 0.005 mM and 0.025 mM Toluidine blue O solutions showed no change in their absorption spectra after addition of the GlcA or GalA, other than background absorbance due to the monosaccharide solutions (Figs. 16 and 17). The absorbance maximum of the 0.1 mM dye solution shifted towards longer wavelengths (ie. from 595 nm to 635 nm) at high GlcA and GalA concentrations (Fig. 18).

These results suggest that for the dye-monosaccharide combinations tested the Toluidine blue O dye molecules are unable to form the dye aggregates necessary to produce the purplish colour. Most likely these monosaccharides, although anionic, are too dispersed in the solution to provide the minimum surface density required for the dye molecules to stack with one another. The reason for the change in the absorption maximum towards longer wavelengths (ie. from the 'dimeric' to the 'monomeric' dye forms) with the 0.1 mM Toluidine blue O solution containing 1.0 M GalA or GlcA is unclear at this time.

Unlike the monomers, the polysaccharides (pectin and polygalacturonic acid, PGA) did produce a purple/violet colour for certain dye-carbohydrate ratios with the 0.1 mM dye solution. When a significant colour change to violet did occur, a third peak at about 550-560 nm was observed (Fig. 19). The peak at 550-560 nm corresponded to the γ peak reported by Michaelis (1947) and thought to represent extensive aggregation of dye molecules. At appropriate concentrations the polysaccharides probably have an adequate surface charge density within and possibly between molecules which allows the dye cations to stack with each other, producing the

Fig. 16 0.005 mM Toluidine blue O + 1M glucuronic acid at 24°C

Fig. 17 1.0 M glucuronic acid at 24°C

Fig. 18 0.1 mM Toluidine blue O + 1M glucuronic acid at 24°C

Fig. 19 0.1 mM Toluidine blue O + 0.5% citrus pectin at 24°C

purplish colour in the 0.1 mM dye solution. The fact that neither the 0.005 mM nor the 0.025 mM dye solutions became purplish for any of the polysaccharides tested may suggest that the monomeric form of the dye (α band, 635 nm) which is the predominant dye form for both concentrations (see Figs. 10 and 11), is unable to form dye aggregates even if adequate surface anionic charges are present. Possibly the purplish colour is produced only when an adequate amount of the dimeric form (β band, ca. 590 nm) of the dye is present - ie. at about ≥ 0.1 mM Toluidine blue O.

The apple pectin and polygalacturonic acid, unlike the citrus pectin, gave inconsistent results with respect to the visual colour of the solutions, the concentration of polysaccharides required to produce a purplish colour and the appearance of the absorption peak produced at 550-560 nm - the absorption at this wavelength often appeared as a shoulder in the spectrum (Fig. 20) instead of a distinct peak for 'intermediate' colour shifts from blue to purple (eg. for soft purplish blue colours). The reason for these inconsistencies is discussed below.

3.1.1.3 Effect of temperature

As noted above, the apple pectin and polygalacturonic acids produced inconsistent results when added to the 0.1 mM Toluidine blue O solutions. After eliminating some possible sources of error such as inconsistencies in weighing and mixing, the effect of temperature was tested following observations by Dr. Hans Edelmann (Edinburgh University, U.K.) on temperature effects with his own work.

Fig. 20 0.1 mM Toluidine blue O +
0.5% apple pectin at 24°C

Fig. 21 0.1 mM Toluidine blue O at 5°C

Cold temperature enhanced the colour shift from blue to purple. Visually, the cold (ice temperature) Toluidine blue O - polysaccharide solutions produced a much brighter purple colour than the same solutions at room temperature. The colour shift from blue to purple was reversible. Cold solutions (bright purple) became more bluish in colour when warmed up to room temperature, and these warmed solutions returned to the bright purplish colours when replaced on ice for about 15 minutes. Kelly (1956) cites studies stating that metachromasia is dependent upon temperature.

Colour dependence on temperature caused problems with spectrophotometric analysis of cold samples since the spectrophotometer itself caused the solutions to warm up and change their colour. Absorption spectra of cold solutions maintained at about 5°C showed that (a) more dimeric dye molecules were present in 0.1 mM dye solution at 5°C (Fig. 21) than at 24°C (Fig. 13) and (b) the cold dye-polysaccharide solutions had an enhanced absorption at 550-560 nm and no observable peak at 590 nm (Fig. 22) as compared to the solutions at room temperature (Fig. 19) and a small peak at about 635 nm. No difference was noted between the spectra of the apple pectin, citrus pectin and polygalacturonic acid (in 0.1 mM dye solutions maintained at ca. 5°C) but visually, the dye solution containing apple pectin appeared slightly bluish in comparison to the other two solutions.

After warming to room temperature the resulting spectra of apple pectin and polygalacturonic acid solutions were inconsistent with respect to the colour produced and the absorption spectra (as described above) whereas the warmed citrus pectin gave more

Fig. 22 0.1 mM Toluidine blue O +
0.5% citrus pectin at 5°C

consistent results (although its absorption maximum at 550 nm sometimes varied in height). These results suggest that, at room temperature, for apple pectin and polygalacturonic acid either (a) the dye-polysaccharide complex is unstable, or (b) the polysaccharides themselves are unstable. Citrus pectin or its complex with the dye may be more stable at room temperature than the other two polysaccharides tested.

3.1.1.4 Survey of colours produced with Toluidine blue O solutions when mixed with selected compounds.

Table 17 outlines the colours produced in cold and warm solutions of dye-polysaccharide solutions. According to theory, the purplish colour should only be produced with compounds containing carboxyl, sulfate or phosphate groups (Pearse, 1985). Hence it is not surprising that citrus and apple pectin, polygalacturonic acid, carboxymethyl-cellulose and chondroitin sulfate B produced purplish colours in cold solutions (the warm solutions of some of the polysaccharides became bluer possibly due to some of the temperature effects discussed above). Gum arabic contains some glucuronic acid residues but these are widely dispersed throughout the compound (Street and Anderson, 1983) and hence may not allow the dye molecules to stack with each other to give the purplish colour. The RNA may not have been dissolved enough in the dye solution to give reliable results.

No colour change was expected with the other compounds since they do not have the anionic functional groups mentioned above. The fact that laminarin (composed of glucose units) stained purplish

Table 17: Colours produced after addition of various compounds to 0.1 mM Toluidine blue O solutions at different temperatures

<u>Compound</u>	<u>%(w/v)</u>	<u>Colour observed¹</u>	
		<u>0°C</u>	<u>24°C</u>
carboxymethyl cellulose	0.05, 0.1 0.5	bright m.P. duller m.P.	s.V. duller s.V.
cellulose and cotton wool	not soluble	N.D. ²	pale blue
chitosan	not soluble	N.D.	unstained or bluish
chondroitin sulfate B	0.1, 1.0	very bright m.P.	bright m.P.
dextran	0.1, 0.5, 1.0	s.B.	s.B.
gum arabic	0.1, 0.5	s.B.	s.B.
laminarin	0.05, 0.1, 0.5, 1.0	bright m.P.	bright m.P.
mannan	0.1, 0.5, 1.0	s.B.	s.B.
apple pectin	0.1, 0.5 1.0	bright m.P. duller m.P.	s.p.B. or s.V. duller "

Table 17 continued

<u>Compound</u>	<u>% (w/v)</u>	<u>Colour observed</u>	<u>°C</u>	<u>°C</u>
citrus pectin	0.1, 0.5		bright m.P.	s.V.
	1.0		duller m.P.	duller s.V.
polygalacturonic acid	0.1, 0.5		bright m.P.	s.p.B or s.V.
	1.0		duller m.P.	duller "
ribonucleic acid	0.05, 0.1, 0.5		s.B.	s.B.
	1.0 (not soluble)		N.D.	N.D.

Note for Table 17:

- Colours matched to those in the ISCC-NBS Centroid colour charts.
s.B. = soft blue #178; s.p.B. = soft purplish blue # 196; m.P = medium purple #223; s.V. = soft violet #207.
- N.D. = not done

with Toluidine blue O may suggest that this compound was contaminated with 'Toluidine blue O - positive compounds' or that the laminarin does, in fact, stain purplish with Toluidine blue O (for reasons unknown). Cellulose and chitosan (both insoluble in the dye solution) stained bluish with Toluidine blue O - no colour change was expected. These compounds could be (a) physically trapping dye molecules thus only appearing to be stained, (b) oxidised and possibly interacting with the cationic dye, or (c) contaminated with some other compounds.

3.1.1.5 Effect of salts

If the purplish colour is, in part, due to the ionic binding of the cationic dye molecule with an anionic substrate molecule, then a salt should compete with the dye molecules and, if effective, should replace the dye molecules. Indeed, at the appropriate salt concentration, the purplish colour seen with the apple or citrus pectin or with polygalacturonic acid (in 0.1 mM dye solution at room temperature) is converted to the original blue colour of the dye solution. Spectrophotometric analysis of these solutions shows that the addition of the appropriate concentration of salt destroys the absorbance at 550-560 nm which represents the dye aggregation in the purple solutions (see Figs. 23-25). This could indicate that the salt affects either the ionic interaction between the dye and polysaccharide or it may also affect the dye molecules themselves leading to their dissociation from the substrate.

The salts tested did affect the dye molecules themselves. At high sodium chloride concentrations (eg. about 5 M) an increase in the dimeric absorption band (ca. 590 nm) in 0.005 and 0.025 mM

Fig. 23 0.1 mM Toluidine blue O + 0.1 M sodium chloride at 24°C

Fig. 24 0.1 mM Toluidine blue O + 0.5% citrus pectin at 24°C

Fig. 25 0.1 mM Toluidine blue O + 0.5% citrus
pectin + 0.1 M sodium chloride at 24°C

Fig. 26 0.005 mM Toluidine blue O + 5 M
sodium chloride at 24°C

Fig. 27 0.025 mM Toluidine blue O + 5 M
sodium chloride at 24°C

Fig. 28 0.1 mM Toluidine blue O + 5 M
sodium chloride at 24°C

dye solutions was observed (Figs. 26 and 27) and in the 0.1 mM dye solution it produces a purplish colour as well as an absorption peak at about 575 nm (Fig. 28). Evidently, the presence of anionic substrates is not essential for dye aggregation to occur since addition of salt alone can produce a purplish colour with Toluidine blue O. Figure 29a summarizes the effects of salt on Toluidine blue O. Again, Kelly (1956) cites reports of similar phenomena occurring upon addition of salts to metachromatic dyes. It is possible that the salt enhances hydrogen bonding between dye molecules (p.c., S. Fry).

3.1.2 Analyses of tissue sections

3.1.2.1 Effect of dye concentration

At 0.005 mM dye concentration, only the green lignified(?) secondary walls and middle lamellae of the xylem (elements, vessels and fibers) and the lignified(?) middle lamellae of phloem fibers was observed even after staining for a week. No purple staining of primary walls was observed with these tissues although preliminary results of other tissues, eg. ferns and begonias, showed purple staining in some cells after about 3-4 days in the dye solution. With both 0.025 mM and 0.1 mM dye solutions purple staining of primary cell walls occurred after green staining of lignified(?) walls was observed - green staining appeared within 15 min. whereas purple staining usually appeared after 30-60 min. with 0.1 mM dye solutions and only after 1-2 days with the 0.025 mM. These results suggest that the monomeric dye form (α absorption peak at 635 nm) is capable of producing the green staining in these tissues whereas only the dimeric dye form (β absorption peak at ca. 590 nm) produces the purple staining. The dimeric form is absent in the

Figure 29: Staining of 'model' systems with Toluidine blue O

Fig. 29a: Toluidine blue O solutions in glass pipettes at room temperature. The solutions are: (1) 0.1 mM Toluidine blue O; (2) 0.1 mM Toluidine blue O + 0.5% citrus pectin; (3) 0.1 mM Toluidine blue O + 0.5% citrus pectin + 0.1 M sodium chloride; (4) 0.1 mM Toluidine blue O + 0.1 M sodium chloride; (5) 0.1 mM Toluidine blue O + 5 M sodium chloride. Addition of pectin to Toluidine blue O solution gives a violet colour (tube 2) which returns to the original blue colour (or close to it) upon addition of sodium chloride (tube 3). High concentrations of salt such as in tube 5 also produces a purplish dye solution. 1X.

Fig. 29b: 0.5% aqueous citrus pectin (left side) and 1.0 M ferulic acid (right side) dissolved in methanol were spotted onto nitrocellulose filters, dried, and then stained with 0.05% Toluidine blue O, pH 4.4. The pectin stains purplish while the aromatic ferulic acid stains bluish with this dye. 2X.

0.005 mM dye solution and hence only green staining occurs at this concentration. Purple staining occurs in the 0.025 mM and 0.1 mM solutions which have more of the 'dimeric' dye form (at 0.025 mM there is less of the dimeric dye than at 0.1 mM and hence purple staining takes longer at 0.025 mM). At this time it is not possible to determine whether the dimeric dye form also produces green staining.

An interesting phenomenon occurs with phloem fibers. As mentioned above, initially the middle lamellae of the phloem fibers stain the same greenish colour (as observed by eye) as the lignified(?) xylem cells. However, as soon as the purple staining of the primary walls of other cells, eg. collenchyma and parenchyma cells, begins the secondary cell walls of the phloem fibers also begin to turn purple. Eventually the green staining of the (lignified?) phloem middle lamellae can no longer be observed and the entire cell wall appears purple. With additional time, the entire wall becomes the 'normal' bluish colour generally associated with phloem fibers observed after staining with Toluidine blue O, pH 4.4. The reasons for these results are not yet clear but it is possible that only the middle lamellae are lignified whereas the secondary (inner) cell wall is mainly composed of as yet unknown polysaccharides. This would contrast with the res.'s from O'Brien *et al.* (1964) who found that these walls also stain with the phloroglucinol test which is considered indicative of lignification. More work is needed to determine what is happening with these cell walls.

3.1.2.2 Effect of salts

Prestained tissue sections quickly (within 15 minutes) lost their purple colour once they were placed in either the calcium chloride or sodium chloride salt solutions. As stated in section 3.1.1.5 this could be the result of cationic competition between the salt and dye molecules in which the salt ions effectively removed the dye molecules from anionic substrate compounds. It is worth mentioning that preliminary observations of other plant tissues such as fern fronds showed that purple staining in some cells was not removed with these salt treatments (suggesting that these cell walls contain compounds which have a 'stronger' binding capacity to the dye molecules?). In contrast, the green colour of the lignified(?) cell walls was not altered after treatment with the salt solutions suggesting that, unlike the purple colour, green staining is not due to an ionic interaction of the dye with the substrate compounds. However, the bluish phloem fibers changed to purplish and then eventually this purple colour was lost, leaving only the green middle lamellae stained (ie. the opposite of what happened when tissue sections were placed in 0.025 mM or 0.1 mM dye solutions and observed with time). These results can not be explained at this time.

3.2 Analyses of the blue/green staining

3.2.1 Staining of various compounds on chromatography paper

Table 18 shows that, contrary to previous assumptions, not all aromatic compounds stain greenish or bluish with Toluidine blue O - some even stained pinkish! However, these results could have been influenced by (a) presence of impurities in the compound stocks, (b)

Table 18 Colour of compounds spotted on to chromatography paper that was dipped into a 1.0 mM Toluidine blue O solution and then air dried

<u>Compound</u>	<u>Structure¹</u>	<u>Colour of compound²</u>	
		<u>Wet</u>	<u>Dry</u>
benzoic acid		D; N.C. to + turq.gr. and some + pink	N.C. to + turq.bl.
p-hydroxy- benzoic acid		D; N.C. to + turq.gr.	+++ pink
methyl 4- hydroxy- benzoate		++ blue-green	++ bluish inside, +++ bluish- white outer ring (++ D)
m-bromo- benzoic acid		+ pinkish-white	+ light blue with + pink outer ring

Table 18 continued

<u>Compound</u>	<u>Structure</u>	<u>Wet</u>	<u>Colour of compound</u>	<u>Dry</u>
gallic acid		D, +++ pink		+++ pink inside, +++ brown outside
tannic acid	<p data-bbox="857 1236 915 1493">R = Not all -OH may be esterified to R</p>	0.1%: ++ dark purple 1.0%: + turq.gr.	0.1%: +++ p.B., with some ++ y.gr. inside 1.0%: +++ y.gr.	
cinnamic acid		++ whitish	+++ whitish (one was +++ turq.bl.)	
p-coumaric acid		+ greenish	++ turq.gr. with another ++ pink spot diffused	

Table 18 continued

<u>Compound</u>	<u>Structure</u>	<u>Wet</u>	<u>Colour of compound</u>	
			<u>Dry</u>	
o-coumaric acid		+ purplish	++ pink diffused ring around N.C. spot	
ferulic acid		+++ turq.gr.	++ dark turq.gr. with + pink outer ring	
benzylmalonic acid		D; + purplish-white	D; + pink	
syringaldehyde		+++ turq.gr.	+++ light turq.gr.	
(+) catechin		D; ++ green	+++ dark green inside, +++ y.br. outside	

Table 18 continued

<u>Compound</u>	<u>Structure</u>	<u>Colour of compound</u>	
		<u>Wet</u>	<u>Dry</u>
(-) epicatechin	cis form of (+) catechin	++++ turq. gr.	+++ dark turq. gr inside, +++ y.br. outside
coumarin		++ turq. gr.	+ bluish-white
guaiacol		+ greenish	N.C.
m-phenylphenol		D; +++ turq. gr.	+++ turq. bl. inside with very diffused +++ bluish- white outside
thiamine hydrochloride		N.C. to + turq. gr.	N.C.

Table 18 continued

<u>Compound</u>	<u>Structure</u>	<u>Colour of compound</u>	
		<u>Wet</u>	<u>Dry</u>
L-phenylalanine		N.C.	N.C.
L-tyrosine		N.C.	N.C.

Note for Table 18:

- Structures of compounds obtained from Anonymous (1983)
 - Abbreviations for colours of spots: turq.gr. = turquoise green; turq.bl. = turquoise blue; p.B. = purplish blue; y.gr. = yellowish-green; y.br. = yellowish brown.
- D = Diffusion of compound occurred. N.C., +, ++, +++ are subjective estimations of (a) colour intensity of spot or (b) amount of diffusion of compound, where ++ < +++ and N.C. indicates no change from background staining.

concentration of the compounds tested -other concentrations might have produced greenish staining, (c) variable diffusion among compounds when passed through the dye solution and (d) blue background staining of the chromatography paper which interferes with the staining colours of the compounds themselves. The colours of the compounds on the dry chromatography paper may be influenced by oxidation of the compounds. Figure 29b shows the bluish staining of ferulic acid compared to the purplish colour of citrus pectin, both of which were spotted onto nitrocellulose filters and stained with Toluidine blue O.

3.2.2 Analyses of tissue sections

As discussed in section 3.1.2, unlike purple staining, green staining is (a) produced in 0.005 mM dye solutions suggesting that predominantly monomeric and possibly dimeric forms of the dye produce the green staining and (b) this staining is not affected by salt solutions, with the exception of phloem fiber cell walls (which may not be completely lignified).

The green staining was not removed when sections are immersed in acetone whereas the purple staining was removed within about 15 min. However, within a few minutes the purple colour and then, after about 15 min., green staining was removed from tissue sections immersed in phenol solution. The loss of purple staining with both acetone and phenol is probably due to the ring structures of the dye molecules having a greater affinity for the organic solvents than for the anionic substrate molecules. Phenol may be more effective than acetone at removing the hydrophobically(?)-'bound' dye molecules from the greenish stained

substrate compounds. The possibility exists that the acetone and phenol could be (a) extracting substrate compounds to which the dye is attached (this could be tested by washing out the phenol with methanol and then water to see if the lignin(?) can be re-stained), or (b) interacting with the dye molecules themselves to produce these results.

3.3 Toluidine blue O purity

3.3.1 Analytical purification

Results from the thin layer chromatography experiments suggest that (a) the sources of Toluidine blue O tested consist of one major (blue) and two or three minor components (one purple, the other(s) bluish), and (b) the Polyscience dye used for my experiments was one of the 'purest' sources (see Table 19). Minor components may represent demethylated dye compounds (p.c., R. Horobin).

3.3.2 Analysis of the 'major' component of Toluidine blue O

With the pea, tomato and potato stem tissues, the lignified(?) xylem and phloem fiber cell walls stained greenish (within minutes) and about 5-20 minutes later the primary cell walls (especially in the pea epidermis) started to stain purplish. These results indicate that this isolated major component was capable of differentially staining the tissues examined.

3.3.3 Preparative purification

A 'suitable' method could not be found as judged by the fact that only one component was isolated with the paper chromatography systems attempted whereas 4 to 12 components

were separated using the column chromatography methods. When some of the 'major' components from these columns were run on silica gel TLC plates each component again separated into two or three others.

Table 19: Chromatography of dyes

Chr. System ¹	Dye used ²	Dye mM ³	Major component(s)			Minor component(s)				
			Rf	Colour ⁴	I ⁵	S ⁶	Rf	Colour	I	S
a	17	0.001	-	-	-	-	-	-	-	-
a	1	0.01	-	-	-	-	-	-	-	-
a	1	0.05	0.35	b.	1	1	-	-	-	-
a	1	0.1	0.35	"	1	1	-	-	-	-
a	1	1.0	0.27	"	2	3	0.57	b.	1	1
a	1	5	0.22	"	3	4	0.08	b.gr.	1	1
							0.58	b.	1	2
a	1	10	0.22	"	4	4	0.08	b.gr.	1	1
							0.62	b.	2	3
							0.78	p.	1	2
a	18	10	0.33	"	5	5	0.11	b.gr.	1	1
							0.51	b.	2	2
							0.74	p.	1	2
a	1	50	0.19	"	5	5	0.41	b.	2	3
							0.59	p.	3	2

Table 19 continued

Chr. system	Dye used	Dye mM	Major component(s)			Minor component(s)				
			Rf	Colour	I	S	Rf	Colour	I	S
a	2	10	0.21	"	5	5	0.09	b.gr.	1	1
							0.49	b.	2	4
a	3	10	0.17	"	5	5	0.0	p.	1	1
							0.04	b.gr.	1	1
							0.5	p.	5	4
a	4	10	0.14	"	5	5	0.0	b.gr.	1	1
							0.43	b.	4	3
							0.56	p.	1	2
b	1	10	0.51	"	4	4	0.70	b.	1	2
							0.77	p.b.	1	1
b	2	10	0.55	"	4	3	0.72	b.	1	2
b	3	10	0.52	"	4	4	0.0	b.p.	1	1
							0.37	b.gr.	1	1
							0.70	b.	3	2
							0.77	p.	4	2
b	4	10	0.47	b.	4	5	0.69	b.	3	3
							0.77	p.b.	2	2

Table 19 continued

Chr. system	Dye used	Dye mM	<u>Major component(s)</u>		<u>Minor component(s)</u>	
			Rf	Colour	I	S
c	1	10	0.37	"	5	3
						3
c	2	10	0.37	"	5	3
						3
c	3	10	0.37	"	5	3
						3
c	4	10	0.37	"	5	3
						3
c	5	10	0.38	"	4	2
						2
d	1	10	0.59	b.	5	2
						2
d	2	10	0.60	"	5	2
						2

Table 19 continued

Chr. system	Dye used	Dye mM	Major component(s)			Minor component(s)				
			R _f	Colour	I	S	R _f	Colour	I	S
d	3	10	0.60	"	5	2	0.0	p.	1	1
							0.70	b.	1	1
							0.8	p.	3	2
d	4	10	0.61	"	5	2	0.70	b.	3	2
							0.78	p.	1	1
e	1	10	0.52	p.b.	4	5	-	-	-	-
e	2	10	0.53	"	4	5	-	-	-	-
e	3	10	0.55	"	4	5	0.90	p.	1	1
e	4	10	0.53	"	4	5	-	-	-	-
f	1	10 ⁹	0.63	p.b.	5	5	-	-	-	-
f	1	10 ⁹	0.59	"	5	5	-	-	-	-
g	1	10	0.74	p.b.	5	5	-	-	-	-

Table 19 continued

Chr. system	Dye used	Dye mM	Major component(s)			Minor component(s)				
			Rf	Colour	I	S	Rf	Colour	I	S
h (trial 1)	1	10	0.38	p.b.	5	5	0.56	b.	3	3
							0.68	p.	1	2
h (trial 2)	1	10	0.60	p.b.	5	5	0.76 ¹⁰	b.	3	2
							0.80 ¹⁰	p.b.	3	2
							0.91	p.	1	2
i (trial 1)	1	10	0.55	p.b.	5	5	0.67 ¹⁰	p.b.	4	2
							0.71 ¹⁰	"	4	2
							0.81	p.	1	2
i (trial 2)	1	10	0.60	"	5	4	0.67 ¹⁰	b.	1	1
							0.70 ¹⁰	"	1	1
							0.79	p.	1	2
i (trial 3)	1	10	0.76	"	5	5	-	-	-	-
j	1	10	0.81	"	5	4	0.92	p.	2	2
							1.0	b.gr.	2	1
k	1	10	0.80	"	5	4	0.89	p.	2	2

Table 19 continued

Chr. system	Dye used	Dye mM	Major component(s)			Minor component(s)				
			Rf	Colour	I	S	Rf	Colour	I	S
l	1	10	0.82	"	5	5	-	-	-	-
m	1	10	0.81	p.b.	5	4	-	-	-	-
n	1	10	0.0	p.	5	1	-	-	-	-
o	1	10	0.32	b.	4	5	0.18	b.	4	5
p	1	10	0.65	p.b.	5	4	0.53	b.	3	4
q	1	10	0.09	p.b.	5	5	-	-	-	-
r (trial 1)	1	10	1	p.b.	5	4	-	-	-	-
r (trial 2)	1	10	0.91	p.b.	5	4	-	-	-	-
s	1	10	0.96	b.	5	5	-	-	-	-
t	1	10	0.15	p.b.	4	5	-	-	-	-
u	1	10	0.15	p.b.	5	5	-	-	-	-
v	1	10	0.0	p.b.	5	1	-	-	-	-

Note for Table 19:

1. Chr. system = chromatography system used, see Tables 14 and 15.
2. Dye used. See Table 13.
3. Concentration of dye. For TLC plates A-E, 5 μ l of the dye (dissolved in 100 % MeOH) were applied. For paper F, 10 μ l of the dye (dissolved in MeOH) were applied unless stated otherwise. The dye solutions were centrifuged before using to eliminate streaking of the dye crystals at the origin.
4. Colour of spots as judged by eye. b. = blue; p. = purple; b.gr. = blue-green; p.b. = purplish blue; b.p. = bluish purple. The purple colour always faded rapidly as the chromatogram dried.
5. I = subjective estimation of intensity of spot where $I < 5$.
6. S = subjective estimation of degree of streaking of spot where $I < 5$.
7. For chromatography system 'a', dye 'I', different concentrations were used to determine whether certain components were more dominant at particular concentrations.
8. For chromatography system 'a', dye 'I', one silica gel TLC plate was streaked along the origin and the major component was subsequently isolated and used to stain plant tissues (see 2.4.2).
9. For chromatography system 'f', the volume of dye solution spotted on the paper chromatogram was 5 μ l for 9a and 15 μ l for 9b.
10. For chromatography systems 'h' (trial 2) and 'i' (trials 1 and 2), the two 'spots' were separated by a yellow line - this may be a single dye spot with a yellow 'waist'.

4 CONCLUSIONS

Model systems which attempt to analyze compounds in conditions other than those present *in vivo* (or in fixed and embedded sections) require careful interpretation, and it is recognized that much more work is required to ascertain how accurately they represent the *in vivo* situation. The current study has attempted to analyze the specificity of Toluidine blue O primarily for plant cell wall compounds using both model systems and plant tissue sections and although this has proven more complex than anticipated some useful information has been obtained.

The purplish staining obtained with Toluidine blue O in this study was explained in terms of dye aggregation/dissociation phenomena, with the assumption that the dye's absorption maxima are indeed indicative of monomeric, dimeric and higher molecular aggregates of the dye as suggested in the literature for Toluidine blue O and other metachromatic dyes (eg. Michaelis, 1947; Kelly, 1956; Pearse, 1985). In the current study experiments done with tissue sections and dye solutions (to which carbohydrates were added) suggested that anything that enhances dye aggregation such as high dye concentrations, large anionic molecules, high salt concentrations or low temperatures will produce/enhance the purplish colour. The presence of purple staining therefore requires careful interpretation since it is not indicative of specific compounds although it may be valuable in predicting tissue composition.

In contrast to the purple staining reaction, the green staining of aromatic compounds observed with Toluidine blue O is (a) not removed from tissue sections with salt (and hence is probably not

the result of ionic binding between dye and substrate molecules as is thought to be the case with the purple staining), and (b) is produced in very dilute dye concentrations thought to contain predominantly monomeric dye molecules - dimeric (or higher aggregates) dye molecules were concluded to be necessary for production of purple colouration with Toluidine blue O. Surprisingly, not all aromatic compounds tested stained green - the influence of concentration and diffusion of these compounds in the 'model' system tested needs to be further examined before conclusions can be drawn. Nevertheless, the pilot study done does indicate that some aromatic compounds such as ferulic acid, syringaldehyde and epicatechin (a flavonoid) definitely stain green with Toluidine blue O.

At this point, it is not possible to determine if the various methods used to 'purify' the dye (ie. TLC, paper and column chromatography) are in fact separating impurities from the major component or are just degrading the dye molecules. Different solvents/methods may just vary in their ability to degrade the dye. Furthermore, if the dye molecules do exist as monomers, dimers and higher molecular aggregates, it is probably not possible, or at least certainly not easy, to separate and distinguish one from another.

CHAPTER 4
GENERAL CONCLUSIONS

CONCLUSIONS

This project focused on the structure and especially the contents of the vacuoles in developing pollen grains of *Brassica napus*. The key observation in this study is that vital staining live pollen grains with Toluidine blue O shows that immature grains have a large vacuole with purple contents and this vacuole gradually breaks up (soon after the first mitosis) forming many small vesicles which have greenish contents in the mature pollen grains. According to Kinzel (1958) and Stadelmann and Kinzel (1972) these observations would indicate that the vacuoles with purple contents in the immature pollen grains have 'empty' saps containing organic acids, while the many vesicles with greenish contents in the mature pollen grains have 'full' saps containing phenolic compounds such as flavone glycosides or tannins.

Most plant histochemists assume that phenolic compounds such as lignin stain greenish and carboxylated polysaccharides such as pectin stain purplish with Toluidine blue O. However, the specificity of the staining reactions of this dye, in particular with compounds producing greenish and bluish colours, has not been critically evaluated. In my study I attempted to further characterize the contents of the vacuoles in developing pollen grains by chemically analyzing extracts of these grains and by examining the specificity of the staining reactions of Toluidine blue O for a variety of purified compounds. Basically, both studies do suggest that the pollen grains may be accumulating phenolic compounds, but more data are needed before the contents of the developing vacuoles can be positively identified. Nevertheless, it is hypothesized that these compounds are flavonoids and that they may be important signals in pollen-stigma compatibility processes or could be involved in defense mechanisms

against insects. They probably also influence the embryogenic potential of cultured pollen grains. These compounds accumulate immediately following the embryogenic 'window' in pollen grain development and the onset of their synthesis may commit the pollen grain to following its normal developmental pathway instead of the alternative embryogenic pathway. The release of these compounds into the culture medium may also be toxic to younger, potentially embryogenic pollen grains, hence inhibiting embryogenesis.

The contents of the vacuoles are not retained during preparation for microscopy. Most live pollen grains prestained with neutral red, and other 'model' plant cells, soon lose the contents of their vacuoles/vesicles when exposed to chemical fixatives and freeze substitution procedures. This phenomenon results in the mordanting of the vacuole contents throughout the cytoplasm and onto the nucleus and walls in these cells. In the case of the mature pollen grains thought to contain phenolics within their vesicles, this results in dark non-specific cytoplasmic staining and obscured nuclear staining soon after the first mitosis of the pollen grain. These results are similar to those of Grant *et al.* (1986) and Smith and Scarisbrick (1990) who reported the sudden occurrence of heavy cytoplasmic staining at the same stage in pollen grain development of *B. napus* but had no explanation of this effect. No reliable method for staining nuclei in whole pollen grains of *B. napus* was found presumably due to poor dye access through the pollen grain wall (indeed many dyes just stained the wall), and mordanting of the released vacuole/vesicle contents onto the nuclei hence preventing nuclear staining.

APPENDIX

Retention of vacuole compounds following chemical fixation and freeze substitution

'Model' plant cells with large, easily visible vacuoles, and mature *B. napus* pollen grains were used to investigate the ability of some chemical fixation and freeze substitution methods to retain vacuolar compounds. Table 1 outlines the plant cell systems used.

These cells were treated with chemical fixatives as outlined in Tables 2-5. The freeze substitution results are outlined in Table 6. At least 10 trials of each cell 'type' per treatment were examined in detail.

Table 1: Plant material used

<u>Plant Cells¹</u>	<u>Fixation</u>	<u>Size of Tissue</u> <u>Freeze substⁿ2</u>	<u>Vacuolar 'Pigment'</u>
red onion abaxial epidermis	5 X 10 mm	1 mm ²	anthocyanins
white onion adaxial epidermis	5 X 10 mm	1 mm ²	Neutral red dye. Tissue placed in pH 7.8, 0.025 M potassium phosphate buffer for ca. 5 min. and then stained with 0.1% neutral red (Chroma-Gesellschaft, Schmid & Co.) - made up in buffer - for ca. 15 min.
corn root-cap detached cells	not done	1-2 mm ³	Neutral red dye. Prestained as above.
beet root tissue	0.5 mm X 5 mm diam.	1 mm ³	betacyanins
<i>B. napus</i> mature pollen	see note ³	see note ³	Unstained. Others were prestained for 2-3 min. with 0.1% neutral red in pH 7.8, 0.025 M potassium phosphate buffer with 0.3 M sorbitol and 0.3 M mannitol added to prevent pollen from bursting.

Note for Table 1:

1. Onions and beets were obtained from the supermarket. *B. napus* pollen were obtained from plants grown in growth cabinets as outlined in Chapter 2, section 2.1. The corn root detached cap cells were obtained from primary *Zea mays* roots grown axenically on 0.9% agar in the dark.
2. The freeze substitution protocol was done exactly as described in Canny and McCully (1986) except that the infiltration into Spurr's epoxy resin was altered as follows. After warming to room temperature for 24 hrs, the tissue was slowly infiltrated up to 10% resin over the first hour of infiltration, and then changed every two hours with the following solutions, named in order of use: 1:1 resin:ether; 3:1 resin:ether; 100% resin. The cells and tissues in 100% resin were left overnight. This resin was changed once a day for the next four days and finally polymerized at 70°C. (It is worth mentioning that the Spurr's resin precipitates when petroleum ether is used (by mistake) instead of diethyl ether, and that LRWhite resin precipitates with both petroleum and diethyl ether.) The tissue pieces were freeze-substituted as outlined in Table 1.
3. Anthers taken just before anthesis were ground up with a mortar and pestle in the 0.1% neutral red solution (containing 0.3 M mannitol and 0.3 M sorbitol), and then filtered through a 44 µm nylon mesh to isolate the pollen grains. (1) To test the effects of chemical fixatives, a drop of the neutral red solution containing the stained pollen grains was put on a slide and then a drop of the fixative solution was added. (2) For the freeze substitution the neutral red stained pollen were attached to dried polylysine-coated (0.02% aqueous poly-L-lysine 300,000 mw) solution, Sigma P8920) Millipore 1.2 µm filters (Millipore Corp., Massachusetts, lot no. 30545-2) cut into ca. 1 mm² pieces, before being plunged into the freezing solution. In addition, unstained pollen grains still within the anther (cut into ca. 1 mm³ pieces) were freeze substituted as above and then, along with the embedded (and fresh) red onion epidermis and beet tissue, were examined with UV excitation to test for autofluorescence (presumably indicative of phenolic compounds).

Table 2. Effect of fixatives on the retention of vacuolar 'pigments' in white onion adaxial epidermal cells prestained with neutral red

<u>Treatment</u> ¹⁻⁴	<u>Observations</u>
Control: pH 7.8 buffer, 10 min → neutral red, 15 min. → pH 7.8 or 6.8 buffer.	Neutral red stayed in the vacuoles for about 5 hrs. (Some of the contents had leaked out and formed 'aggregates' in the cytoplasm and stained plasmodesmata). The next observations, done at 24 hrs, showed that all vacuoles had lost the dye and the nuclei, cytoplasm and walls were stained (weaker in the pH 6.8 buffer than in the pH 7.8 buffer).
pH 7.8 buffer, 10 min. → neutral red, 15 min. → pH 7.8 buffer rinse → 3% glutaraldehyde in pH 6.8, 0.025M buffer.	Within 15 min. in the fixative, the dye had started to leak out of the vacuole and stain the nuclei in about 40% of the cells. After 30-45 min. in the fixative, 90-100% of the nuclei were stained with neutral red. After 60-90 min. all of the vacuolar contents had leaked out.
pH 7.8 buffer, 10 min. → neutral red, 15 min. → pH 7.8 buffer rinse → 10% formalin or 4% paraformaldehyde in buffer (see section 6.11.7.3B, O'Brien and McCully, 1981).	Within 15-45 minutes the contents had leaked out of all cells leaving only tiny aggregates of precipitated dye within the vacuoles and strongly staining the nuclei, cytoplasm and walls.
pH 7.8 buffer with 0.3 M sorbitol and 0.3 M mannitol.	Cells plasmolysed within 30 min. The neutral red was retained up to this time. No observations taken after 30 min.

Note for Tables 2-5:

1. 0.025 M potassium phosphate buffer used.
2. Freshly-made fixatives used for all these trials. Generally, the action of all fixative solutions (on the release of vacuole contents) slows down with older fixative solutions (no data collected).
3. All experiments done at room temperature.
4. Fixatives used: (1) Paraformaldehyde, Fisher Scientific Co., T-353, lot 741231; (2) Glutaraldehyde, purified, J.B. EM Services Inc., cat no. JBS-013, lot no. 474; Formalin, Formaldehyde, 37% solution; Baker analyzed reagent, no. 2106-3, lot 519626).

Table 3. Effects of fixative treatment on red onion abaxial epidermal cells

<u>Treatment</u>	<u>Observations</u>
Control: pH 6.8 buffer	Vacuolar pigments retained for at least 48 hrs (not observed longer).
pH 6.8 buffer, 10 min. → 3% glutaraldehyde in pH 6.8, 0.025 M buffer.	Within 15-30 min. the anthocyanin pigments were becoming more dilute within the vacuoles. After 45-60 min. the majority of vacuoles had lost most of their anthocyanins. These pigments did not 'stain' the walls, nuclei or cytoplasm when they leaked out of the vacuoles.
pH 6.8 buffer, 10 min. → 10% aqueous formalin or 4% paraformaldehyde in buffer (section 6.11.7.3B, O'Brien and McCully, 1981).	The anthocyanins remained within the vacuoles for 48 hours (cells were not observed longer) and became darker in colour. Some of the cells had tiny aggregates of anthocyanins in their cytoplasm starting after about 60 min. in the fixative solutions.

Table 4: Effect of fixative treatment on beet root slices

<u>Treatment</u>	<u>Observations</u>
Control: pH 6.8 buffer	Most cells lost their vacuolar betacyanins after 24 hours.
pH 6.8 buffer, 10 min. → (1) 3% glutaraldehyde in pH 6.8, 0.025M buffer, or (2) 4% paraformaldehyde in buffer (see section 6.11.7.3B, O'Brien McCully, 1981).	Since (a) the thickness of the slices varied and (b) it was not possible to obtain a monolayer of cells, it was difficult to assess the fixatives' effect on individual cells. However, in general with both fixatives the majority of cells had lost most of their vacuolar betacyanins by 24 hours.

Table 5: Effect of chemical fixatives on neutral red-stained *B. napus* pollen

<u>Treatmen</u>	<u>Observations</u>
Control: pollen isolated into neutral red solution (see Table 1).	Initially, 95-100% of the pollen 'vesicles' were stained. After about 1 hour, about 40% of the pollen had burst, and after ca. 24 hours, most of the pollen had burst.
A drop of 3% glutaraldehyde (in pH 6.8, 0.025 M potassium phosphate buffer) was added to a drop of the pollen grains previously isolated in the neutral red solution (see Table 1).	Within 5-10 min. most of the 'vesicle' contents were leaking out, staining the cytoplasm of entire pollen grains. After about 1 hour, all of the pollen had lost the vacuolar contents and most had burst.
A drop of 4% paraformaldehyde solution in buffer (see O'Brien and McCully, 1981, section 6.11.7.3B) was added to a drop of the pollen grains previously isolated in the neutral red solution (see Table 1).	Same results as for the control, except that some of the 'vesicular' compounds in individual grains started leaking out within 15 min as judged by the 'muddy' appearance of the neutral red staining within these grains.

Table 6. Retention of vacuolar 'pigments' in freeze-substituted cells

<u>Tissue</u>	<u>Vacuolar 'pigment'</u>	<u>Results</u>
beet root	betacyanins	retained in all uncut cells. UV negative
red onion abaxial epidermal cells	anthocyanins	as for beet
white onion adaxial epidermal cells	neutral red	10-20% retained in uncut cells. In others the wall, cytoplasm, and nuclei were stained.
detached cells from corn root caps	neutral red	0% retained. Many cells have stained nuclei and/or walls.
pollen grains	neutral red	Retained in 20-30% of grains. Sections of these grains show many discrete vesicles of various sizes. Many others have solid red cytoplasm and walls while others have lost their protoplasm.
pollen grains	unstained	UV negative

Table 7. Summary of results (i.e. ability to retain vacuolar/vesicular 'pigments')

<u>Tissue</u>	<u>'Pigment'</u>	<u>Buffer control</u>	<u>Glutaraldehyde</u>	<u>Paraformaldehyde</u>	<u>Freeze substitution</u>
beet root	betacyanin	lost after 24 hrs	lost after 24 hrs	lost after 24 hrs	retained. UV -ve
red onion epidermis	anthocyanin	retained 48(+) hrs. UV -ve	lost within 15-45 min.	retained 48(+) hrs	retained. UV -ve
white onion epidermis	neutral red	retained 5(+) hrs, lost after 24 hrs	lost within 15-45 min. and stained nuclei, cytoplasm and walls.	lost within 15-45 min. and stained nuclei, cytoplasm and walls. Dye precipitates in vacuole.	Retained in 20% of cells. others, either (a) only dilute neutral red staining in vacuole, or (b) lightly stained nuclei and walls.
detached cells from corn root caps	neutral red	not done	not done	not done	0% retained in vacuoles. Cells either unstained or have wall, cytoplasm and nuclei stained.

Table 7 continued

<u>Tissue</u>	<u>'Pigment'</u>	<u>Buffer control</u>	<u>Glutaraldehyde</u>	<u>Paraformaldehyde</u>	<u>Freeze substitution</u>
pollen	neutral red	lost after 24 hours	lost within 10-15 min.	lost after 24 hours	20-30% of grains retained most of their 'vacuolar' contents. In others, the cytoplasm was evenly stained with neutral red or the pollen had burst open.
pollen	unstained	UV negative	not done	not done	UV negative

Figures 1-5

Figures 1-5

Figures 1-12. All micrographs are taken using primary green no. 139 transparent filter (Efstonscience Inc., Edmund Scientific Products, Toronto, Canada).

Figures 1-5 are of white onion adaxial epidermal cells which had been stained with 0.1% neutral red solution for *ca.* 15 min and then treated as outlined below.

Fig. 1. About 30 min in neutral red. Vacuoles are darkly stained while cytoplasm at ends of cells and nuclei are not stained. Bright-field optics. 165X.

Fig. 2. After *ca.* 60 min in neutral red solution. Aggregates of dye have formed in the cytoplasm, presumably after leaking out of the vacuoles. Nomarski optics. 265X.

Fig. 3. After 30 min in 3% glutaraldehyde solution all vacuoles have lost most of the neutral red. The cytoplasm, walls and especially the nuclei (shown here) have been stained with the neutral red which leaked out of the vacuoles. Bright-field optics. 400X.

Fig. 4. After 60 min in 4% paraformaldehyde solution all vacuoles have lost the neutral red staining. Now dark red precipitates in the vacuole (?) and cytoplasm are seen as well as a darkly stained nucleus. Bright-field optics. 200X.

Fig. 5. Freeze substituted tissue. Some cells have retained the neutral red in the vacuoles but most have lost it. Nomarski optics. 100X.

Figures 6-12

Figures 6-9 are of red onion adaxial epidermal cells treated as stated below.

Fig. 6. About 60 min in pH 7.8 0.025 M potassium phosphate buffer. Cells have retained their vacuolar anthocyanins except in those cells at the cut edges of the hand section (asterisks) whose vacuoles have been cut. Nomarski optics. 200X.

Fig. 7. After 30 min in 3% glutaraldehyde the majority of cells have lost most of their anthocyanin pigments. Bright-field optics. 100X.

Fig. 8. After *ca.* 60 min in 4% paraformaldehyde solution none of the cells showed loss of anthocyanin pigments. Micrograph shows the damaged cells (asterisks) next to the cut edge which have lost their vacuolar pigments. Nomarski optics. 200X.

Fig. 9. Freeze-substituted cells. Only those cells with vacuoles damaged at the cut edge (asterisks) have lost the anthocyanins from the vacuoles. Nomarski optics. 200X.

Figures 6-12

3 of/de 3

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Figures 10-12 are of *B. napus* pollen grains from anthers just prior to anthesis. Live grains were prestained with 0.1% neutral red solution (with 0.3M sorbitol and 0.3M mannitol) and then treated as below.

Fig. 10. After about 10 min in the neutral red solution vesicles in the grains have accumulated the dye (magnification is too low to see individual vesicles). Bright field optics. 165X.

Fig. 11. After 10 min in 3% glutaraldehyde solution all pollen have either burst and/or the dye has leaked out of all the vesicles and stained the cytoplasm. Bright field optics. 400X.

Fig. 12. Freeze substituted pollen grains. Only a few of the grains (arrows) have retained the neutral red in vesicles. All other grains have burst and/or the dye has leaked out of the vesicles and stained the cytoplasm. Bright field optics. 270X.

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