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Aq2; a highly water-soluble plant growth
regulator from the pollen of Pinus radiata.

A thesis presented for the degree of
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ABSTRACT

Aq2; a highly water-soluble plant growth regulator
from the pollen of Pinus radiata

Aq2 was detected in crude aqueous extracts from the pollen of Pinus radiata by Sweet and Lewis (1971). These workers noted Aq2 possessed some properties of both gibberellins and cytokinin-like compounds and was probably involved in the regulation of pollen tube growth. A further study was undertaken by Gallagher and Aldersley (1972) and these workers concluded after a preliminary investigation that Aq2 was most probably a cytokinin.

The purpose of this thesis was to further investigate the nature of Aq2. The physiological role and chemical composition of pollen, with special reference to P. radiata was studied, and a review of plant growth regulators carried out with a view to classifying Aq2 into one of the four groups. A survey of possible isolation techniques was also made.

Anion and cation exchange columns were run at various pH's and a portion of the activity attributable to Aq2 was found to bind to an anion column at pH 8.5. The remainder passed straight through the column. An aqueous alcohol treatment has been employed to remove some of the excess carbohydrate material, and freeze drying was shown not to affect the activity of Aq2.

Chemical evidence tends to mitigate against Aq2 being a gibberellin; however, the possibility that Aq2 is a cytokinin has not yet been ruled out. No additional physiological studies have been carried out since those of Sweet and Lewis (1971); however, good responses are still obtained in the radish cotyledon assay for cytokinins.

If Aq2 is indeed a cytokinin it does not appear to resemble any of those known to date.

errata

Page 15. Figure 1, gluobrassicin,
Should read glucobrassicin.

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INTRODUCTION

A study of plant growth regulators occurring in the pollen of Pinus radiata D. Don was recently carried out by Sweet and Lewis (1971). In this investigation, both a qualitative and quantitative comparison between the levels occurring in germinated and ungerminated pollen was carried out, and thirteen growth regulating compounds were detected. These included five auxins, three inhibitors, four gibberellins and one compound with some properties characteristic of both gibberellins and cytokinins.

This latter substance was designated as Aq2 by Sweet and Lewis (1971), as it remained in the aqueous phase after extraction at pH 8.5 and pH 2.7 with diethyl ether and was the second growth regulating substance to be found in this fraction.

Aq2 was first detected in the radish cotyledon expansion assay used primarily for the detection of cytokinins; responses were also obtained in the barley endosperm assay and in the Rumex assay for gibberellins. No response was obtained in the carrot bioassay or the Spirodela bioassays for cytokinins. On the other hand, Aq2 appeared to parallel kinetin in its effect on pollen tube growth. Thus circumstantial evidence seemed to indicate Aq2 was a cytokinin. Sweet and Lewis found evidence to suggest Aq2 was involved in the modulation of pollen tube growth. Further work was undertaken by Gallagher and Aldersley (1972).

Initially Gallagher and Aldersley's work encountered a troublesome toxicity problem. Having solved this, these workers developed a large scale extraction procedure based upon mechanical disintegration of pollen grains in water.

Plant growth regulators are typically present in plants in extremely low concentrations. Thus the isolation and characterisation of such compounds usually involves large quantities of plant extract. For example, the best reported yield obtained for a cytokinin was by Letham (1966a), where 0.7 mg of zeatin was obtained from 60 Kg of maize kernels.

Preliminary results of Gallagher and Aldersley (1972) indicated that Aq2 was a low molecular weight, highly polar, water-soluble compound. Bearing in mind the biological activity

attributed to Aq2, Gallagher and Aldersley decided that it was possible that Aq2 was a cytokinin nucleotide or a "bound" form (e.g. glycoside) of a cytokinin. However, further investigation by these workers mitigated against the possible nucleotide nature of Aq2. Thus, it is known that nucleotides can be absorbed onto charcoal and eluted with aqueous base; attempts to absorb Aq2 onto activated charcoal were unsuccessful, whereas under the same conditions, the nucleotide, AMP absorbed quantitatively onto the charcoal. Again, ion exchange chromatography has been widely used in the purification of cytokinins; nucleotides binding strongly to anion exchange columns under neutral conditions. Attempts to purify Aq2 by anion exchange chromatography (Dowex-1) proved unsuccessful; no activity could be detected in the column eluates, however, a toxic effect in the radish cotyledon assay was obtained from the initial column effluent. From these latter results the possibility could not be excluded that a strong synergism existed in the partly purified extract.

It was the purpose of this thesis to investigate further, aspects of purification, isolation and characterisation of Aq2.

POLLEN

As the hormone Aq2 investigated in this thesis was extracted from pollen, it is clearly pertinent to consider the nature of pollen grains and their general chemical composition. However, before doing this, it was thought worthwhile to briefly consider the biological role of pollen, especially since it has been proposed (Sweet and Lewis, 1971) that the physiological role of the hormone appears to be the modulation of pollen tube growth.

A great deal of research has been carried out on pollens; their morphology and physiology being well studied. In addition to research of a purely botanical nature, pollen has been of interest in the studies of nutrition, and allergies (Nielsen *et al.*, 1955; Kapp, 1969; Barbier, 1970; Heslop-Harrison, 1971; Wodehouse, 1965).

Pollen Function

Pollen is derived from the seed bearing plants which can be divided into two well defined groups: gymnosperms and angiosperms. The gymnosperms are woody perennial plants and while there are relatively few species, these are of great abundance and of economic importance as sources of timber.

The reproductive cycle of Pinus radiata D. Don (a gymnosperm) is illustrated in Figure 1.

Pinus produces both male and female cones. The male cones are produced in autumn, in groups, and fall from the tree at the end of the following spring, having remained fairly small in size. The cone contains many microsporophylls arranged spirally about the long axis. Each microsporophyll contains two pollen sacs on the under side, see Figure 2. Pollen, which is partly germinated microspore, is released by these sacs splitting longitudinally during spring. The pollen from Pinus is wind borne and released in very large quantities.

The female cones grow considerably larger than the male cones and occur singly. Scales are arranged spirally about the long axis of the cone. Each scale has two ovules on the upper side. The ovule has archegonia embedded in the endosperm. The latter is completely surrounded by the nucellus which in turn is

LIFE CYCLE OF PINUS

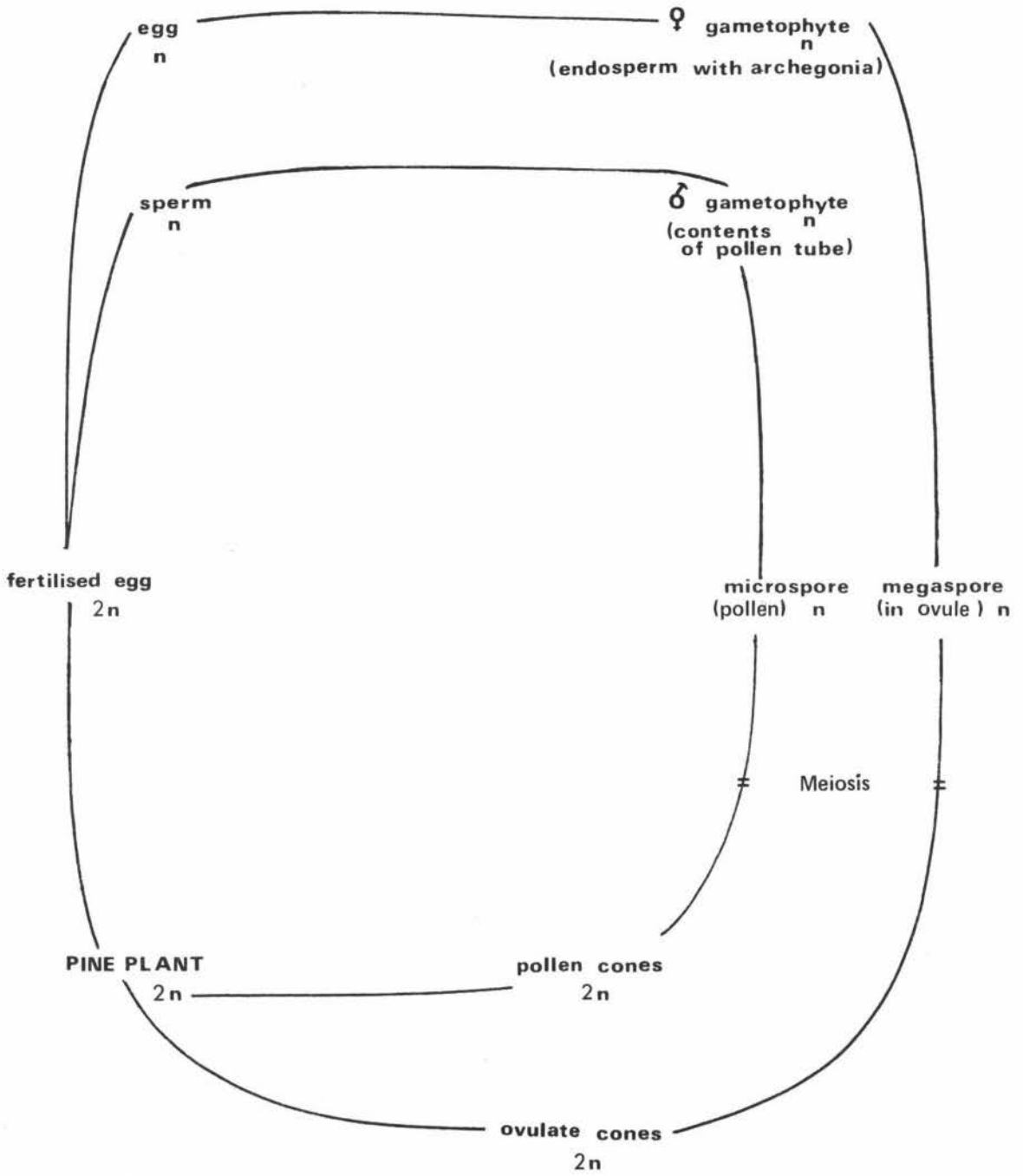
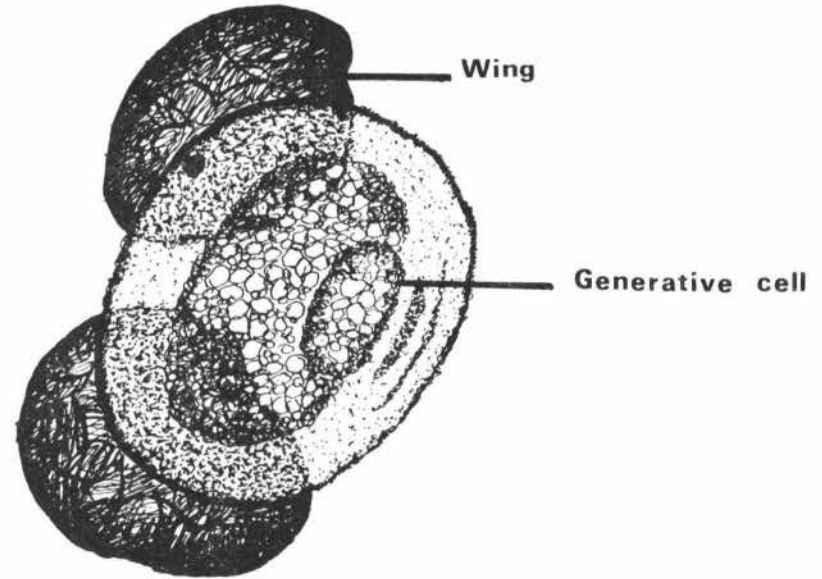
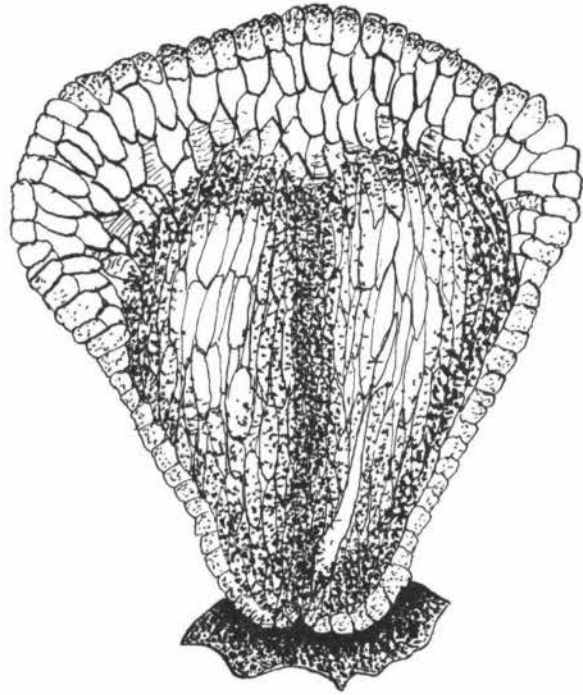


Figure 1



**ENLARGED DRAWINGS OF A MICROSPOROPHYLL SEEN FROM THE LOWER SIDE,
SHOWING THE POLLEN SACS, AND A MATURE POLLEN GRAIN**

Figure 2

surrounded by the integument except at the micropyle (the point of access for the pollen grain), see Figure 3. Each archegonium has one ovum (egg).

At pollination, the scales of the female ovulate cone are slightly separated. Pollen settles between these and comes to rest close to the micropyle of the ovule. It is subsequently drawn in through this opening and comes into contact with the nucellus. The pollen grains now form a short pollen tube which starts to grow down through the nucellus. It remains dormant during the winter and recommences growth in the following spring, at which time the male gametophyte completes its development. Simultaneously, in the ovule the female gametophyte (endosperm and archegonia) completes its development. When the pollen tube reaches and grows into the egg fertilisation occurs by the fusion of a sperm, from the pollen tube, with the egg. Subsequently the fertilised egg develops into an embryo and the whole ovule forms a seed. The scales separate when the seed is mature and permit it to fall from the cone.⁽¹⁾

Pollen Structure

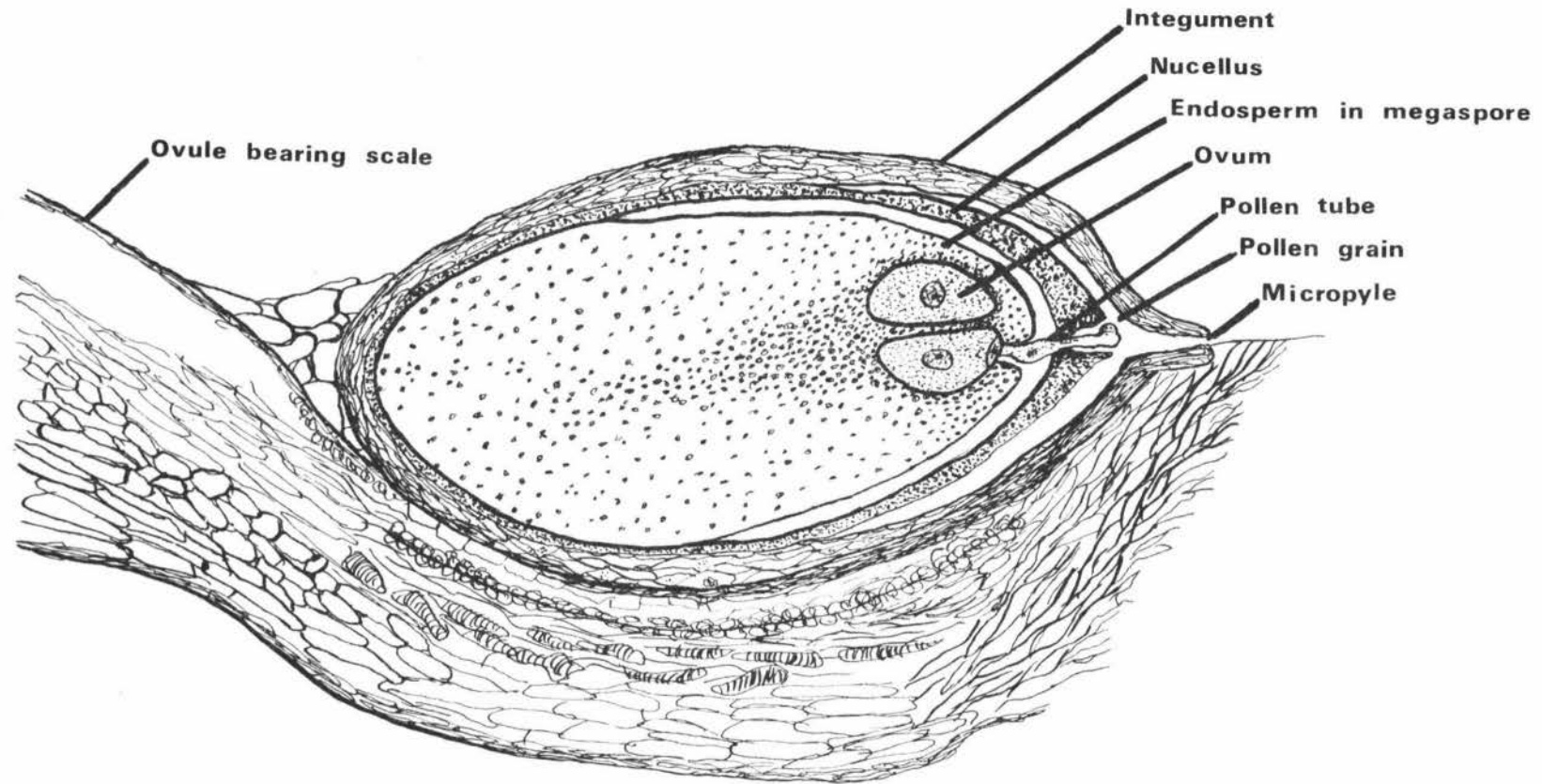
Pollen grains vary in size from about $5\ \mu$ to $250\ \mu$ (Shaw, 1970). Many wind pollinated forms have walls modified to enhance bouyancy, while insect pollinated forms, the presence of surface rods, spines, and other sculptured features, serve to improve dissemination (Kapp, 1969).

The pollen grains of the genus Pinus are characterised by the possession of two large, conspicuous, air-filled bladders.⁽²⁾ The grains are rather large, ranging in the different species from about $45\ \mu$ to $65\ \mu$ in diameter, the bladders measuring about two-thirds the diameter of the body of the grain (Wodehouse, 1965).

The wall of the pollen grain is composed of two basic layers: the intine and the exine. The intine is the cell wall, which immediately surrounds the living protoplasm. It is partly cellulose, but appears to have a different chemical composition from most cell walls, with higher proportions of pectic substances, callose, and other polysaccharides (Kapp, 1969). In some pollens,

(1) Priestley et.al. (1964)

(2) To enhance buoyancy.



AN ENLARGED SECTION OF AN OVULE FROM A SECOND-YEAR *Pinus* CONE

Figure 3

the cellulose layer is embedded with layers of protein (Heslop-Harrison, 1971).

The resistant outer wall layer, the exine,⁽³⁾ of pollen grains, is a chemically resistant layer which has the function of the protection of the organism from injury by external agencies, such as excessive desiccation, destruction by light, and mechanical injury. This wall invariably possesses pores and these appear to allow a certain degree of diffusion of water (Kapp, 1969; Wodehouse, 1965) and a diffusion of enzymes and other soluble material (Stanley and Search, 1971).

The Chemical Composition of Pollen

A number of reviews have been published on the chemical composition of pollen (e.g. Stanley, 1971; Barbier, 1970; Neilsen *et al.*, 1955).

At dehiscence time grass pollen, including Zea mays, has a water content above 50%; other pollens, e.g. Pinus, usually contain about 20% or less water at time of shedding.

Some components, such as carbohydrates, vary more between and within species than other chemical constituents. The carbohydrate content of grass pollen (corn) may be more than twice that of other angiosperm pollens; carbohydrates in pine and most other gymnosperms are considerably lower. Typical results of analyses are shown in Table 1 (reproduced from Heslop-Harrison *loc. cit.*), Table 2 (reproduced from Nielsen *et al.*, *loc. cit.*), and Table 3 (reproduced from Barbier *loc. cit.*)

It can be seen that proteins vary widely with species, usually accounting for 10-30% of the pollen dry weight. Lipid contents average 1.5-4% and may be higher in those with oils on the grain surface. Ash content is usually about 2-4%, but may be as high as 7% in some species.

Variation in chemical constituents of pollen is accounted for by:

- (1) species differences, and

(3) The exine layer of the pollen wall is almost completely composed of sporopollenins - a diverse group of biopolymers with similar empirical formulae (e.g. $C_{90}H_{144}O_{20}$ Pinus sylvestris). A source of suitable monomers has been suggested by Brooks and Shaw (1971).

Table 1
Gross Chemical Analysis of Pollen

Species	% dry weight				Reference
	Ash	Carbohydrates	Protein	Lipid	
Zea mays	2.55	36.59	20.32	3.67	(2)
Zea mays	3.46	34.26	28.30	1.48	(3)
Typha latifolia	3.70	17.78	18.90	1.16	(4)
Pinus sabiniana	2.59	13.15	11.36	2.73	(2)
Pinus radiata	2.35	13.92	13.45	1.80	(2)

Table 2

	Zea mays 1953	Zea mays 1954	Alnus glutinosa	Alnus incana	Pinus montana
N %	4.1	4.2	4.1	4.2	2.2
Protein % (Nx6.25)	25.6	26.3	25.6	26.2	13.8
Sulphate ash %	4.9	4.9	2.4	2.8	3.0
P %	0.58	0.75	0.42	0.28	0.30
S %	0.43	0.30	0.24	0.32	0.18
Reducing sugars (as glucose) %	10.3	7.3	8.4	5.7	2.7
Total carbohydrates (as glucose) %	35.1	34.6	27.4	22.5	29.5
Water-soluble substances %	35.9	49.7	41.2	33.3	31.9
Ether-soluble substances %	5.0	1.8	9.4	13.2	7.1

Table 3

The Percentage Composition of 6 Hand-collected Pollens and 18
Bee-gathered Pollens (Todd and Bretherick)

	Proteins	Ether extract	Sugars	Water	Ashes	Inde- terminate
Hand-collected pollens						
Pinus sabiniana	11.36	2.73	13.15	14.8	2.59	56.09
Pinus radiata	13.45	1.80	13.92	11.25	2.35	57.23
Typha latifolia	18.83	1.28	31.93	6.43	3.82	37.71
Mais	20.32	3.67	36.59	5.53	2.55	31.31
Juglans nigra	23.15	17.55	13.72	3.91	3.07	39.60
Phoenix dactylifera	35.50	3.08	1.20	17.14	6.36	36.73
Bee-gathered pollens						
Pinus contorta	7.02	2.04	48.35	7.01	1.32	34.26
Taraxacum vulgare	11.12	14.44	34.93	10.96	0.91	27.64
Salix sp. I	15.38	5.25	41.92	13.61	2.19	21.65
Salix nigra	22.33	4.15	33.18	12.30	2.61	26.43

(2) environmental differences during maturation and after dehiscence.

During maturation excessively high temperatures tend to reduce the pollen carbohydrate; low light also results in less carbohydrate accumulating in mature pollen, probably as a direct effect of reduced photosynthesis. If the plant nutrient supply, particularly microelements, is below optimum, pollen mineral content may be reduced, modifying the protein-enzyme levels.

Carbohydrates

Simple sugars are the principal metabolic substrates used by germinating pollen. The total sugar composition for P. radiata is given in Table 3 as 13.92%. While other soluble sugars occur in most pollens a high percentage of the free sugar in pine pollen is sucrose. Thus soluble carbohydrates can be seen to significantly contribute to the solids content of aqueous extracts of pollens. The separation of the hormone Aq2 from this carbohydrate material has been a major difficulty in this study.

Proteins and Enzymes

Again from Table 3 it is seen that a significant amount of proteinaceous material would occur in the aqueous extract. While no detailed information on the enzyme content of P. radiata is available, Brewbaker (1971) has published a list of enzymes known to occur in the pollens of higher plants. In all, thirty-nine are listed consisting of dehydrogenases, oxidases, transferases, hydrolases, lyases, and ligases. Knox (1971) reports the localised presence of certain enzymes such as acid phosphatase, ribonuclease, esterase, amylase, and protease in the pollen wall of many species. Stanley and Search (1971) report on protein and enzyme constituents which rapidly diffuse from germinating pollen. Pinus species were noted to undergo a smaller loss of weight when eluted with water than many other species. The rapidity and ease of loss of these proteins and enzymes suggest that some enzymes are surface localised or very near the surface of exine (Stanley and Search, 1971).

While the pollen used in this investigation for this thesis study was ungerminated it is likely that readily diffusible substances such as those above may be leached out by water washing. Indeed, Sweet and Gallagher (1972) proposed that such material was the cause of troublesome toxic effects experienced

in early extraction work.

Organic Acids

All the Krebs cycle acids have been found in pollen, but quantities vary markedly with the stage of development and handling. Phenolic acids such as p-hydroxybenzoic, p-coumaric and vanillic can be extracted and their levels can be measured. Fatty acids are also common in pollen. Large quantities of certain fatty acids, particularly palmitic, linoleic and linolenic acids exist in many pollens. Pine and other gymnosperm pollens are very high in linolenic acid. The other major fatty acids in *Pinus* are oleic, palmitic and stearic acid. The major portion of fatty acids exists in pollen as esters combined with sugars, phosphates or other constituents which have not yet been studied in any great detail (Stanley, 1971). Phospholipids from *P. ponderosa* were examined by McIlwain and Ballou (1966). Phosphatidyl-choline, phosphatidyl-ethanolamine, phosphatidyl-glycerol, phosphatidyl-myoinositol, phosphatidylserine and biphosphatidylglycerol were identified. The major fatty acids of each were palmitic, oleic and linoleic.

Callose and Sporopollenin

Callose is a β -1,3-glucose polymer present in high levels in pollen at maturity. This is a fairly common carbohydrate in plants, however its exact role is not known. The low water solubility of such polymers (compare cellulose) indicates that they are not likely to be present to any significant extent in aqueous pollen extracts.

The chemical composition of sporopollenins and possible precursors for synthesis are now known (Brooks and Shaw, 1971). In addition, the degradation products have been well characterised, giving mainly dicarboxylic acids and aromatic hydroxy acids. As significant degradation is not likely to occur under the mild extraction procedure used in the present investigation, the sporopollenins will not be further considered in this discussion.

Pigments

The chemistry of pollen pigments has been relatively well investigated, but their physiological role is not well understood. Pollens are predominantly yellow in colour and this is due to the presence of carotenoids or flavonoids. However, free carotenes

or their derivatives have never been isolated from pine pollen and thus whilst it is possible that carotenes exist in these pollens in some highly modified form, the visible pigments in the exine are primarily flavonoids. Flavonoids frequently occur as glucosides. Some pigments, in particular flavones, are water soluble and readily diffuse from pollen into water.

Hormones and Plant Growth Regulators

Many pollens have been assayed for their nutritive value, especially pollens which are bee-gathered. Stanley (1971), provides data on the vitamin content of Pinus montana pollen. Four B group vitamins, ascorbic acid, and biotin have been found. In addition, steroids similar in structure to some animal hormones have been found. The function of these is as yet unknown.

Indole acetic acid, auxins, inhibitors, and gibberellins have been detected in pollens. Sweet and Lewis (1971) carried out a comprehensive hormone assay analysis of P. radiata pollen. In a series of papers Mitchell et al. (1970, 1971a, 1971b, 1972)

claim the isolation of a new class of hormones, "Brassins", from pollen of rape (Brassica napus Goertn). These hormones were isolated from ether extracts and were of a lipid nature. However, in a recent paper Milborrow and Pryce (1973) have made major criticisms of the work of Mitchell et al. Milborrow and Pryce maintain that as the brassins were not assayed using any of the "reasonably specific bioassays for auxins (oat coleoptile elongation), gibberellins (growth stimulation of dwarf maize or amylase synthesis in barley aleurone), or cytokinins (tobacco pith callus growth), therefore a contribution of these hormones to the activity of the brassin fraction has not been excluded", These authors also contest much of the other data presented by Mitchell et al.

Mineral content

The predominant elements found in pollens are potassium, phosphorus, calcium and magnesium. Stanley (1971) presents data for the mineral content of Pinus sabiniana and P. radiata. These have an ash content of 2.59 and 2.35 per cent of their dry weight. Aluminium, copper, iron, manganese, nickel, titanium and zinc occur in trace amounts. Estimations of chloride and boron are difficult as these may volatilise during ashing.