AN ABSTRACT OF THE THESIS OF

Roger Craig Muren for the degree of Master of Science
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Title: A STUDY OF IN VITRO METABOLIC CHANGES IN GERMINATING DOUGLAS FIR POLLEN

Abstract approved: Redacted for Privacy

Dr. Te May Citing

The ability of Douglas fir pollen to germinate and develop into a viable gamete in a sterile mineral medium supplemented with mannitol as osmotant is demonstrated. In addition to the high percentage of gamete formation that was accomplished in 4 days, the often encountered bursting due to hypotonic medium and microorganism contamination was minimized. Rapid oxygen uptake was observed at the commencement of germination. After a decrease following 24 hours of germination, oxygen uptake increased to the original level after 48 hours. That the respiratory quotient remained at approximately 1.0 after 24 hours in culture demonstrated the utilization of stored carbohydrate for respiration. Starch constituted about 30% of the dry weight of mature pollen and decreased by 75% over the 4 days of germination. The content of starch was estimated by an enzyme hydrolysis method which was proved to be more complete in hydrolyzing pollen starch compared to conventional acid hydrolysis. The
enzymes used in the hydrolysis were a combination of wheat endosperm extract and commercially pure \( \alpha \)- and \( \beta \)-amylases. The decrease of starch content during germination in culture was further demonstrated by photomicrographs of pollen stained with iodine. Utilization of the products of amylolysis was followed by the incorporation of \( U^{14}C \)-glucose into various chemical fractions of 2-day germinating pollen. In two hours 80\% of the incorporated tracer was metabolized. The largest amount of label appeared as amino acids accounting for 30\% of the incorporated \( ^{14}C \). The tracer was also found in \( CO_2 \) (14\%), organic acids (22\%), lipid (5\%) and an ethanol insoluble fraction (3\%), including cell wall components (1\%). The enzyme responsible for the hydrolysis of starch in Douglas fir was studied quantitatively and electrophoretically. Experimental data suggest that the starch hydrolyzing enzyme in Douglas fir pollen is a phosphorylase which is present in mature pollen and does not increase with germination and gamete formation.
A Study of in vitro Metabolic Changes
in Germinating Douglas Fir Pollen

by

Roger Craig Muren

A THESIS

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degree of

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Redacted for Privacy

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Redacted for Privacy

Dean of Graduate School

Date thesis is presented May 24, 1978

Typed by Opal Grossnicklaus for Roger Craig Muren
ACKNOWLEDGEMENT

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TABLE OF CONTENTS

INTRODUCTION

MATERIALS AND METHODS

Pollen
Culture Conditions
Determination of Adenosine Phosphates
Chemical Analysis and Respiratory
   Measurement of Germinating Pollen
Utilization of Starch Hydrolytic Products
   by 14C-glucose Incorporation
Assay of Starch Hydrolyzing Enzymes

RESULTS AND DISCUSSION

Growth in Selected Medium
Chemical Composition
Respiration
Adenine Nucleotide Pool and Energy Charge
Starch Content
Utilization of Glucose
Amylolytic Enzymes
Bibliography

APPENDIX I  Review of Literature

Development of Pollen Grains
In vivo Pollination and Fertilization
In vitro Mass Culturing
Effects and Metabolism of Exogenous
   Carbohydrates in vitro
Respiration
Starch Content
The Utilization of Carbon Backbones
   During Germination
Metabolic Sequence of Germination
APPENDIX II  Experimental Procedures  51

Fractionation of $^{14}$C-labelled Components  52

BIBLIOGRAPHY (Literature Review)  54
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Morphological changes in Douglas fir pollen during germination time in culture: A and E, 1 hr; B and F, 24 hrs; G, 48 hrs; C and H, 72 hrs; D and I, 96 hrs. A, B, C, and D no stain x 160. E, F, G, H, and I stained in 0.5% I₂ in 1% KI x 420.</td>
</tr>
<tr>
<td>2.</td>
<td>Starch content of Douglas fir pollen germinated in basal medium supplemented with 0.3 M mannitol. Starch determined by acid hydolysis (o---o), and by enzymatic hydrolysis (---).</td>
</tr>
<tr>
<td>4.</td>
<td>Electrophoretic patterns of starch-degrading enzymes in germinating Douglas fir pollen. A. Two different incubation conditions, both extracted in calcium acetate buffer: I. incubated in 1% starch solution containing 10 mM calcium acetate for 2 hours at 25°C prior to Iodine staining. II. Incubated in 1% starch solution containing 50 mM phosphate for 2 hrs at 25°C prior to Iodine staining. B. Pattern after 0, 1, 2, 3, 4 days of germination following incubation in 50 mM phosphate for 2 hrs at 25°C.</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Percent viability and growth of Douglas fir pollen in selected medium. 100 mg of Douglas fir pollen was germinated in 10 ml selected medium at 30°C with shaking. Values represent the mean and standard deviation of four replications.</td>
<td>22</td>
</tr>
<tr>
<td>2.</td>
<td>Chemical changes in germinating Douglas fir pollen over a 48 hour time period in basal medium supplemented with 0.3 M mannitol.</td>
<td>23</td>
</tr>
<tr>
<td>3.</td>
<td>Respiratory rate and respiratory quotient of germinating Douglas fir pollen. 200 mg of pollen was incubated in 0.5 ml medium at 25°C.</td>
<td>24</td>
</tr>
<tr>
<td>4.</td>
<td>Distribution of radioactivity recovered in various fractions of germinating Douglas fir pollen incubated with 14C-glucose for 2 hours at 25°C. The mean of 3 replications plus and minus standard deviation.</td>
<td>25</td>
</tr>
<tr>
<td>5.</td>
<td>Amylolytic activity ($A_{620}$-starch reduction or $A_{570}$-reducing sugar increase) of Douglas fir pollen cell free preparations extracted and incubated under various conditions.</td>
<td>26</td>
</tr>
<tr>
<td>6.</td>
<td>Phosphorylase activity in germinating Douglas fir pollen. The data are the average of 3 experiments.</td>
<td>27</td>
</tr>
</tbody>
</table>
A STUDY OF IN VITRO METABOLIC CHANGES IN
GERMINATING DOUGLAS FIR POLLEN

INTRODUCTION

In vitro culture techniques of pollen have been aimed at sus-
taining pollen growth to a length necessary for fertilization in nature
(10, 12). Thus the carbohydrate requirements provided by the
medium, polysaccharide (wall components) synthesis in the pollen,
and other metabolic processes accompanying germination and
growth have been well documented (2, 10, 12, 16, 23). Starch
comprises a small percent of the total dry weight of most kinds of
pollen! and in most cases plays a minor role in the carbohydrate pool.
For continued growth the pollen generally utilizes exogenously sup-
plied sugars (10, 12).

Pine pollen is the only gymnosperm species which has been
given significant attention concerning the physiological aspects of
its pollen germination (11, 19, 20, 21, 22, 23, 25). Other gymno-
sperm species, most notably Douglas fir (Pseudotsuga menziesii
Franco), have received inadequate attention because of their slow-
ness in growth and lack of a pollen tube at the initial stages of germi-
nation. In nature the pollen tube is observable several days after
the beginning of germination presumably after the penetration into
the nucellus. However, the high starch content of mature Douglas fir
pollen (Figure 1) is of particular interest in carbohydrate metabolism of the haploid gymnosperm tissue which is relatively unknown at the present.

This report examines the metabolism of the starch reserve in Douglas fir pollen germinated in vitro in an osmotically controlled mineral medium. The utilization of starch was traced by the changes in starch content and by the distribution of radioactive glucose into different cellular components in germinating pollen. The identity of the enzyme responsible for starch hydrolysis was explored in electrophoretically separated enzymes. Morphological changes were depicted by photomicrographs and the patterns of respiration and energy (ATP) pool were also followed during germination.
MATERIALS AND METHODS

Pollen

Pollen of Douglas fir (*Pseudotsuga menziesii* Franco) was obtained from a single tree in Corvallis, Oregon in April, 1977. Male strobile were collected over a two day period just as they were beginning to shed pollen. The collected strobile were spread out on a layer of wax paper and allowed to air dry for 48 hours at 23°C with a relative humidity of 20-50%. After passing the shed pollen through a fine sieve (200 mesh), drying was continued for an additional 24 hours. The air dried pollen contained about 6% water and was stored in air tight vials at 5°C where it retained its original viability throughout the subsequent year.

Culture Conditions

Pollen was cultured in medium containing 1.62 mM H$_2$BO$_3$, 1.27 mM Ca(NO$_3$)$_2$ · 4H$_2$O, 0.81 mM Mg SO$_4$ · 7H$_2$O, 2.2 mM KH$_2$PO$_4$ in glass distilled water adjusted to pH 5.2 with 0.01 N NaOH and designated as basal medium (2). The basal medium was supplemented with 0.3 M sucrose or 0.3 M mannitol.

All the results, excluding the original germination and growth conditions were based on pollen hydrated for 12 hours prior to
germination in a moisture chamber at 5°C to a moisture content of approximately 45%, which is comparable to the water content of naturally shed pollen. One hundred milligrams of hydrated pollen was cultured in 10 ml sterilized basal medium supplemented with 0.3 M-mannitol in a 25 ml Erlenmeyer flask at 100 oscillations per minute. To prevent growth of microorganisms, 15 µg ml⁻¹ of chloramphenicol and mycostatin (Calbiochem. Co.) were added to the medium. In experiments lasting longer than 48 hours pollen was collected on Whatman no. 1 filter paper, washed with 10 ml sterile medium and transferred to fresh medium with the addition of 15 µg ml⁻¹ chloramphenicol and mycostatin.

**Determination of Adenosine Phosphates**

Four cultures of 50 mg pollen at the designated time after the beginning of germination were collected by filtration. The pollen was rapidly added to 5 ml boiling glass distilled water for 5 minutes at 100°C. The extract was cooled in an ice bath, and to the extract was added 5 ml buffer containing 0.05 M N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and 0.05 M magnesium acetate pH 7.5. As previously described, the diluted extract was incubated with additives and assayed for the determination of ATP, ADP and Total Adenosine Phosphates (4).
Chemical Analysis and Respiratory Measurement of Germinating Pollen

Three cultures of 100 mg pollen were collected after 24 hours and 48 hours germination on Whatman no. 1 filter paper and washed with 10 mls fresh, sterile medium. The pollen was hand ground in a mortar with pestle in 20 ml 85% ethanol. The slurry was centrifuged at 30,000 g for 10 minutes to separate ethanol soluble and insoluble components. For further fractionation into chemical components the general procedure for Douglas fir seeds was followed (6).

The acid hydrolysis of starch did not completely dissolve the starch granules in pollen as evidenced by the positive iodine stained granules in the residue of hydrolysis. Another method for the determination of pollen starch content was developed using enzymatic hydrolysis. Four replicates of 100 mg pollen were collected after 0, 24, 48, 72 and 96 hours of germination by filtration and washed with fresh medium. The pollen was hand ground in 10 ml 10 mM calcium acetate pH 6.0. The slurry was heated for 10 minutes at 70°C to inactivate most enzymes except α-amylase. This slurry constituted the pollen starch extract used as substrate for further enzyme hydrolysis. Wheat endosperm amylases were prepared by homogenizing ten, three-day-germinated wheat endosperm in 8 ml 100 mM calcium acetate pH 6.0 in a polytron for 30 seconds at 4°C.
The homogenized mixture was centrifuged at 30,000 g for 10 minutes and the supernatant was used as the wheat amylase extract. To determine the amount of starch in the pollen, 0.5 ml pollen starch extract was incubated at 30°C for 2 hours with 0.4 ml wheat amylase extract plus 100 μg purified β-amylase and 20 μg purified α-amylase (sigma). At the end of the incubation period the total reducing sugar in the reaction mixture as well as in the incubated wheat amylase preparation alone (blank 1) and the original pollen starch extract alone (blank 2) was determined spectrophotometrically by the method of Bernfield (1945). The total reducing sugar from the reaction mixture minus blank 1 and blank 2 is presumably the starch of the pollen material. Based on the iodine test of the residue this appears as the only method among many tested that hydrolyzed starch completely in Douglas fir pollen.

Utilization of Starch Hydrolytic Products by $^{14}$C-glucose Incorporation

For the utilization of the product of starch hydrolysis during in vitro germination three 100 mg samples of pollen were collected after 48 hours of germination and transferred to a Warburg flask with 0.5 mls fresh medium containing 10 μC $^{14}$C-glucose (uniformly labeled, sp. act. 12.7 m C/m mole). Incorporation time was 2 hours at 25°C, and the respired $^{14}$CO$_2$ was trapped in 40%
potassium hydroxide. Pollen was collected by filtration, rinsed with 10 ml fresh medium and hand ground in 25 ml 85% ethanol. Fractionation into chemical components was conducted by the method of Ching and Fang (1969).

Assay of Starch Hydrolyzing Enzymes

Starch hydrolyzing enzymes were prepared by hand grinding 200 mg of 0, 24, 48, 72 and 96 hour germinated pollen for 2 minutes in 5 mls of 100 mM calcium acetate pH 6.0 plus 2 mM 2-mercapto-ethanol at 4°C. The slurry was centrifuged for 10 minutes at 30,000 g. After removal of floating fat layer and an additional 10 minutes of 30,000 g centrifugation the supernatant was used as the soluble enzyme preparation. The protein content of the enzyme preparation was determined in 10% TCA precipitate of the preparation by the method of Lowry et al. (1951). Amylase assay followed the method of Mitchell (1972) by incubation of 0.5 ml of the enzyme preparation at room temperature for 3 minutes with 1% soluble potato starch in 10 mM calcium acetate pH 4.8. To determine hydrolytic activity in the presence of inorganic phosphate, 0.5 ml enzyme preparation was incubated with 1% soluble starch in 0.04 M NaH$_2$PO$_4$ pH 4.8.

For isozyme analysis polyacrylamide gel electrophoresis was
performed by the method of Davies (1964) using 7% small-pore gel. Electrophoresis was conducted for 4 hrs at 4°C under a constant current of 5 mamp per gel. The extracts were prepared as described for total activity and 200 µl per gel were charged. After electrophoresis the gels were incubated with 1% solution of soluble starch in 10 mM calcium acetate pH 5.0 or 0.04 M NaH$_2$PO$_4$ pH 5.0 for 2 hours at 26°C. After incubation the gels were rinsed in distilled water, immersed for 30 seconds in an I$_2$-KI solution (0.5% I$_2$ in 1% KI) and rinsed again in distilled water. The enzyme bands were clear in a blue background.
RESULTS AND DISCUSSION

Growth in Selected Medium

Preliminary studies showed that Douglas fir pollen often burst in suspension cultures lacking an osmotic regulator. Unlike pine pollen (25) and lily pollen (14), which grow well with only small amounts of sugar, Douglas fir pollen had only a 35% survival rate after 72 hrs in a similar culture medium (Table 1). By 96 hrs the pollen is totally burst in the basal medium which successfully supported pine pollen growth (25). Addition of 0.3 M sucrose significantly increased pollen survival but did not increase growth, and bursting occurred after 48 hours. Mannitol, however, appeared to be a highly superior osmotic regulator reducing bursting considerably over the 96 hour time course of germination. In addition, mannitol-supplemented medium resulted in an increased growth rate compared to other Douglas fir pollen culture media (Table 1) (6, 20). The growth rate was also increased by hydrating the pollen for 12 hours prior to germination at 4°C. A similar effect on pollen growth by pre-hydration has also been demonstrated in pine pollen (26). Although pollen growth continued for the 4 day period studied, the most rapid elongation occurred in the first 2 days (Table 1).
Chemical Composition

The levels of amino acids, sugars and protein (Table 2) are similar to those reported for pollen of other species (32). Over the first 48 hours, during the greatest rate of pollen growth, sugars decreased 23%, amino acids decreased 30%, and soluble protein increased the first day followed by a reduction the second day while insoluble protein reduced after one day.

Respiration

The respiratory characteristics of Douglas fir pollen during the first 48 hours of germination are summarized in Table 3. Oxygen uptake decreased along with CO₂ uptake after the first 4 hrs of germination then increased to the original level by the end of 48 hours in culture. Carbon dioxide liberation was very high at the beginning of germination, presumably liberating CO₂ which has accumulated as a result of storage. By the end of 48 hours in culture the respiratory quotient stabilized at approximately 1.0, reflecting the utilization of carbohydrate in the form of stored starch for metabolic processes. The level of oxygen consumption is similar to that already reported for viable Douglas fir pollen (2).
Adenine Nucleotide Pool and Energy Charge

Nucleotide levels in germinating pollen over 24 hours of germination are shown in Figure 3. The quantity of ATP in ungerminated pollen was within the range previously reported for Douglas fir pollen. In ungerminated pollen there was an average of 4.5 nmoles ATP, 5.04 nmoles ADP and 4.112 nmoles AMP. In the initial 24 hours of germination there was an 86% increase in ATP and substantial decreases in the levels of ADP and AMP. These results are considerably different from the changes in nucleotide levels of germinating pine seedlings, where there is a large increase in all three species of adenosine phosphates accompanying germination (7). Energy charge increased from .51 to .906 after the first 16 hours of germination and over the following 8 hours increased to a level of .923. This pattern also differs from the pattern of energy charge reported for germinating pine seedlings (7). In quiescent pine seedlings there was a lower energy charge associated with rapid growth and a higher energy charge during the latter stages of germination when there was slower growth. The pattern of increasing energy charge in Douglas fir pollen with increasing growth during the initial stages of germination is closer to the pattern of energy metabolism in the bacterial system (11). This probably reflects the simplicity of the bacterial and pollen systems.
Using a P:O ratio of 3 and 1.656 µl of oxygen consumed per 4 hours per mg pollen (Table 3), an estimated 444 µmoles ATP could be esterified during the first 4 hours of germination. By subtracting the residue pool of 0.21 nmoles ATP increased during the first 4 hours of germination (Figure 3), an estimate of 443.79 nmoles of ATP must be utilized during the first 4 hours for anabolic activities. This represents close to 100% of the ATP produced and is a further evidence that Douglas fir pollen is highly metabolically active under these in vitro conditions.

Starch Content

The level of starch in ungerminated Douglas fir pollen was found to be considerably higher than previously reported for Douglas fir and for pollen of other species (10, 15, 31). The rapid utilization of starch during germination in mannitol medium can clearly be seen by the decreasing size and number of iodine stained starch grains in pollen (Figure 1) and by the lower level of starch as determined by chemical analysis (Figure 2). After complete enzyme hydrolysis starch content amounted to 30% of the total dry weight of ungerminated pollen. Acid hydrolysis method (reflux for 2 hours in 2 N H₂SO₄) of starch resulted in 75% efficiency of the enzyme hydrolysis method in air dried, ungerminated pollen. As germination advanced 45% and 40% efficiency were obtained in 24 and 48 hr
germinated pollen, respectively. This observation probably indicated that starch grains become complexed with enzymes as germination advanced. This particular change has already been discussed in pine pollen (12).

**Utilization of Glucose**

Because of the large concentration of starch in ungerminated Douglas fir pollen and its rapid hydrolysis during germination the metabolism of starch was studied by following the two hour incorporation of $^{14}$C-glucose into chemical constituents of 48 hour germinated pollen. Table 4 summarizes the recovery of $^{14}$C from various fractions. Sugar accounts for only 18.5% of the total incorporated label, demonstrating the rapid utilization of the product of starch hydrolysis. The largest accumulation of tracer (30%) was found in amino acids. A considerable amount of $^{14}$C was found in organic acids (22%) and $\text{CO}_2$ (15%) indicating no need for exogenously supplied carbohydrate for *in vitro* growth of Douglas fir pollen. In addition, $^{14}$C was incorporated into lipid and ethanol insoluble components. The ethanol insoluble material was further separated into pectinase-cellulase soluble and insoluble fractions. Cell wall components composed of cellulose and pectin accounted for 0.88% of the total incorporated $^{14}$C while the insoluble material made up 1.48% of the total incorporated label. This represents a
substantial portion of the $^{14}$C-glucose being incorporated into cellulose and pectin, the primary cell wall materials being synthesized during pollen growth (16, 32). The specific activity for sugars, amino acids and lipids was calculated to be 1.48, 0.65 and 0.07 DPM x $10^6$ per mg, respectively. All these data verify the movement of glucose through the various metabolic pathways for germination and growth.

Amylolytic Enzymes

Table 5 summarizes a number of experiments conducted in an attempt to characterize and quantify the enzyme(s) responsible for the starch hydrolysis in germinating pollen. By extracting in buffer for $\alpha$-amylase (CA$^{++}$-acetate) and following standard assay procedures (23, 1), substrate reduction was not found to be linear with either time or concentration of enzyme used (A and B). Assaying the enzyme preparation following denaturation of heat labile amylolytic enzymes and leaving heat stable $\alpha$-amylase or extracting without CA$^{++}$ but in Tris or buffer for all enzymes did not demonstrate linearity in terms of incubation time and enzyme amount (C and D).

Also shown in Table 5 is the result of experiments designed to follow the enzymatic hydrolysis of starch in the presence of
inorganic phosphate. Although starch degradation is apparent in the presence of inorganic phosphate (E), reducing sugars were not accumulated in the reaction mixture, indicating that the starch hydrolyzing enzyme is tightly coupled to further metabolic pathways making the shunt of the first product difficult and the demonstration of its accumulation impossible.

The difficulties encountered in quantifying the enzymatic hydrolysis of starch with conventional assay procedures led to the utilization of electrophoresis to visualize the enzyme(s). Electrophoresis physically separated the enzymes responsible for starch degradation from other carbohydrate oxidizing enzymes which are mixed in the extract with amylolytic enzyme(s). The gel containing the electrophoretically separated enzymes was then incubated in various pH conditions with different cofactors to discern the characteristics of the starch hydrolyzing enzyme(s). Figure 4 shows the characteristics of electrophoretically separated enzyme(s). The amylolytic enzyme(s) in Douglas fir pollen was not active in calcium acetate buffer but required inorganic phosphate for starch hydrolytic activity. The enzyme(s) was more active in the presence of 2-mercaptoethanol and the pH optimum was 5.0. These observations suggest that the amylolytic enzyme(s) in germinating Douglas fir pollen is not α-amylase which is heat insensitive and requires calcium for
reaction (23). Although the enzyme could be a β-amylase, the dependence of the enzyme upon inorganic phosphate (Figure 4, Table 5) strongly suggests that it is a phosphorylase. At least 3 isozymes are present in the pollen. By using shorter time of incubation, the reduction of starch by phosphorylase can be demonstrated (Table 5-(E)).

Table 6 shows an increase in the phosphorylase activity on the second day of germination followed by a decrease in the total activity to a level that is 5% higher than the initial rate after 4 days of germination. The same pattern of an increased activity after the first day of germination followed by a decreased activity has been shown for glycolytic enzymes in germinating pine pollen in vitro (29). The total and specific activity of amylolytic enzymes was considerably lower in Douglas fir pollen than has been reported in germinating seeds (5) and in Douglas fir pollen when only 1 minute reaction time was used (7).

The amylolytic enzymes were separated by electrophoresis to 3 bands (Figure 4). Upon longer electrophoretic separation (5 hours), 5 bands were observed, but activity was reduced because of the dilution and possible degradation of the enzymes with lengthened time of electrophoresis. An additional minor band appeared consistently after 2 days of germination at the top or anodic end
of the gel. The phosphorylase isozymes did not coincide with major protein bands appearing on gels stained with amido black. Therefore the quantity of amylolytic enzymes was very small in the whole complement of soluble proteins.

The enzymatic activity responsible for starch degradation in germinating Douglas fir pollen, therefore, is considerably different from the amylolytic enzymes in seeds. The phosphorylase appeared to be pre-existing in ungerminated pollen (Table 5, Figure 4) similar to the phosphorylase in pea and cereal seeds (5, 19). Unlike the seed phosphorylase, however, the pollen phosphorylase does not decrease drastically after the initial stages of germination. In Douglas fir pollen there appears to be little de novo synthesis of amylolytic enzymes other than phosphorylase during germination. Although a new band appeared after 2 days germination, it was inactive in the absence of inorganic phosphate. Furthermore, the pollen phosphorylase is unlike the pea and cereal seed systems where phosphorylase hydrolyzes starch only at the onset of germination to supply phosphorylated substrate for glycolysis and respiration without further depletion of ATP for substrate oxidation. In germinating seeds α-amylase is produced 2-3 days later and is responsible for the majority of starch hydrolysis. The phosphorylase observed here is apparently responsible for the 75% reduction in starch content during 4 days of
germination (Figure 2). Further characterization of the enzyme will be of interest.


**TABLE 1.** In vitro growth of Douglas fir pollen in selected medium.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µm</td>
<td>% Viable</td>
<td>µm</td>
<td>% Viable</td>
</tr>
<tr>
<td>Basal medium</td>
<td>83 ± 11</td>
<td>85±23</td>
<td>157±8</td>
<td>78±15</td>
</tr>
<tr>
<td>Basal medium + 0.3 M sucrose</td>
<td>83 ± 11</td>
<td>84±14</td>
<td>170±4</td>
<td>67±10</td>
</tr>
<tr>
<td>Basal medium + 0.3 M mannitol</td>
<td>83 ± 11</td>
<td>93±5</td>
<td>163±7</td>
<td>92±7</td>
</tr>
<tr>
<td>Basal medium + 0.3 M mannitol hydrated pollen</td>
<td>100±5</td>
<td>96±1</td>
<td>159±5</td>
<td>90±8</td>
</tr>
</tbody>
</table>
Table 2. Chemical changes in germinating Douglas fir pollen over a 48 hour time period in basal medium supplemented with 0.3 M mannitol.

<table>
<thead>
<tr>
<th>Mg of component per 100 mg pollen</th>
<th>Hours in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Soluble sugars</td>
<td>20.95±3.7</td>
</tr>
<tr>
<td>ETOH soluble amino acids</td>
<td>8.15±0.3</td>
</tr>
<tr>
<td>Acid hydrolyzed amino acids</td>
<td>11.35±0.7</td>
</tr>
<tr>
<td>(insoluble protein)</td>
<td></td>
</tr>
<tr>
<td>Soluble protein</td>
<td>2.13±0.1</td>
</tr>
</tbody>
</table>
Table 3. Respiratory rate and respiratory quotient of germinating Douglas fir pollen.

<table>
<thead>
<tr>
<th>Hours of Germination</th>
<th>$QO_2^{**}$</th>
<th>$QCO_2^{**}$</th>
<th>RQ $^{***}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>0.575±0.002</td>
<td>1.489±0.028</td>
<td>2.560±0.10</td>
</tr>
<tr>
<td>4-6</td>
<td>0.253±0.002</td>
<td>0.200±0.016</td>
<td>0.810±0.025</td>
</tr>
<tr>
<td>24-26</td>
<td>0.3977±0.003</td>
<td>0.418±0.002</td>
<td>1.040±0.018</td>
</tr>
<tr>
<td>48-50</td>
<td>0.593±0.004</td>
<td>0.541±0.012</td>
<td>0.914±0.016</td>
</tr>
</tbody>
</table>

$^{*}$μlO$_2$ uptake/mg pollen/hour

$^{**}$μl CO$_2$ production/mg pollen/hour

$^{***}$RQ = $\frac{QCO_2}{QO_2}$
Table 4. Distribution of radioactivity recovered in various fractions of germinating Douglas fir pollen incubated with $^{14}$C-glucose for 2 hours at 25°C. The mean of 3 replications plus or minus standard deviations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\text{dpm} \times 10^6$</th>
<th>Incorporation$^\dagger$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{CO}_2$</td>
<td>15.39 ± 2.90</td>
<td>14.30 ± 2.74</td>
</tr>
<tr>
<td>Organic acids</td>
<td>22.50 ± 5.15</td>
<td>21.50 ± 6.01</td>
</tr>
<tr>
<td>Amino Acids</td>
<td>32.43 ± 1.14</td>
<td>30.15 ± 1.06</td>
</tr>
<tr>
<td>Sugars</td>
<td>19.90 ± 0.98</td>
<td>18.50 ± 0.98</td>
</tr>
<tr>
<td>Lipids</td>
<td>5.18 ± 1.70</td>
<td>4.80 ± 1.55</td>
</tr>
<tr>
<td>Ethanol insoluble material</td>
<td>3.70 ± 0.16</td>
<td>3.40 ± 0.18</td>
</tr>
<tr>
<td>Total uptake</td>
<td>107.40 ± 0.16</td>
<td>100%</td>
</tr>
</tbody>
</table>

$^\dagger$disintegrations per minute

$^\ddagger$Incorporation, percent of total recovery
Table 5. Amylolytic activity (A\textsubscript{620} or A\textsubscript{570}) of Douglas fir pollen cell free preparation extracted and incubated under various conditions.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Extraction Buffer</th>
<th>Reaction Buffer</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CaAc</td>
<td>CaAc</td>
<td>.283 ± .02</td>
<td>.246 ± .02</td>
<td>.230 ± .03</td>
<td>.218 ± .01</td>
</tr>
<tr>
<td>B</td>
<td>CaAc</td>
<td>CaAc [2x vol. emz]</td>
<td>.271 ± .02</td>
<td>.257 ± .03</td>
<td>.251 ± .02</td>
<td>.248 ± .02</td>
</tr>
<tr>
<td>C</td>
<td>CaAc</td>
<td>CoAc [following 70°C]</td>
<td>.266 ± .04</td>
<td>.247 ± .04</td>
<td>.244 ± .05</td>
<td>.255 ± .04</td>
</tr>
<tr>
<td>D</td>
<td>TRIS</td>
<td>CaAc</td>
<td>.262 ± .01</td>
<td>.243 ± .03</td>
<td>.232 ± .02</td>
<td>.235 ± .01</td>
</tr>
<tr>
<td>E</td>
<td>CaAc</td>
<td>Na\textsubscript{2}PO\textsubscript{4}</td>
<td>.264 ± .02</td>
<td>.233 ± .05</td>
<td>.192 ± .05</td>
<td>.160 ± .03</td>
</tr>
<tr>
<td>F</td>
<td>CaAc</td>
<td>Na\textsubscript{2}PO\textsubscript{4}</td>
<td>.233 ± .05</td>
<td>.257 ± .03</td>
<td>.251 ± .02</td>
<td>.248 ± .02</td>
</tr>
</tbody>
</table>

A, B, C, D and E values for starch assayed at A\textsubscript{620}.

Sample B is assayed using double concentration of enzyme.

F values for reducing sugar at A\textsubscript{570}.

26
Table 6. Phosphorylase activity in germinating Douglas fir pollen. Pollen was germinated in Basal medium supplemented with 0.3 M mannitol. The data are the average of 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>Days in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Total Activity</td>
<td>133.5±5.6</td>
</tr>
<tr>
<td>Specific Activity</td>
<td>0.0624</td>
</tr>
</tbody>
</table>

Total Activity = μg starch hydrolyzed per 100 mg per minute at 25°C.

Specific Activity = mg starch hydrolyzed per mg protein per minute at 25°C.
Figure 1. Morphological changes in Douglas fir pollen during germination.
Figure 1 (Continued)
Figure 2. Starch content of germinating Douglas fir pollen.
Figure 3. Changes in nucleotide pools in germinating Douglas fir pollen.
Figure 4. Electrophoretic patterns of starch-degrading enzymes in germinating Douglas fir pollen.
A considerable amount of work has been done in recent years in the area of pollen growth and development for a better understanding of controlled hybridization, male sterility, incompatibility, and pollen storage. With extensive efforts in tissue culture and attempts to perfect anther culture and the production of haploid plants, the implications of pollen studies are being realized in many areas of the basic and applied plant sciences. Although the bulk of the research on pollen physiology and development has been with angiosperm species, some attention is now being given to gymnosperm species with the increased activity in the past decade in forest genetics and forest improvement programs. To date, the majority of the research concerned with forest species has been centered around viability estimation and attempts to increase the longevity of pollen in storage (3, 9, 10). The study of physiological events of pollen germination has been primarily concentrated on the short time period between initiation of germination and fertilization which is characteristic of angiosperm species. Less work has been done on the more slowly growing gymnosperm pollens which may take up to one year (pines) and, in most cases, at least two months to result in fertilization once germination has begun. Physiological information
pertaining to Douglas fir pollen germination is particularly lacking not only because of its slow growing nature but also because of the lack of an evident pollen tube which generally appears long after the initiation of germination.

Several reviews summarize the state of our knowledge concerning pollen physiology and development (50, 34, 46).

**Development of Pollen Grains**

The developmental processes of the male gamete in Douglas fir is of special interest here because they are distinctive to Douglas fir and because of their possible effects on pollen growth and metabolism during germination in vitro. An excellent review of the development of Douglas fir pollen appears in Allen (1970) and is summarized here.

Pollen development begins in Douglas fir with meiotic pairing of the 26 chromosomes within the thin-walled pollen mother cell. By late October pachytene has occurred, resulting in thick, fuzzy chromosomes filling only a portion of the nucleus. Diplotene, the next step of meiosis in which the chromosomes usually continue to shorten, is modified in Douglas fir pollen. The chromosomes become diffuse and appear as they did in diplotene. The pollen mother cell remains in this state of diffused diplotene from early November through mid-February during which time starch is accumulated. In mid-February
meiosis resumes with a more typical diplotene activity followed by
diakinesis and continued chromosomal condensation. Meiosis is
completed by the end of February and in March each microspore
develops into a pollen grain by a thickening of the cell wall, slight
cell enlargement, and further accumulation of starch.

By the end of March the microspores have reached the stage
where the mother cell wall has ruptured. At this point the micro-
spore nucleus divides. Two cells are formed by mitotic division of
the microspore nucleus and uneven division of the cytoplasm. The
second mitotic division results in two small prothallial cells, a gen-
erative cell, and a larger tube cell containing the tube nucleus which
remains in the center of the developing pollen grain. Maturation is
completed when the generative cell divides equally to produce a stalk
cell adjacent to the second prothallial cell and the body cell adjacent
to the tube cell. The mature pollen grain at the time of pollination
consists of five cells: two lens shaped prothallial cells, a stalk cell,
a body cell, and a large tube cell. Owens and Molder (1971) have re-
ported that 50% of the pollen has reached the five-celled stage at the
time of pollination. Other reports, however, maintain that the major-
ity of the pollen observed has only reached the four-celled stage at the
time of pollination (7).

Unlike the short time interval between pollination and fertilization
characteristic of the angiosperms, fertilization in the gymnosperms
occurs after a more extended period of time following pollination. Several conifers, including *Pinus*, have a period of 14 months between pollination and fertilization. McWilliam (1954) has demonstrated that pine pollen germination is divided into two periods of growth over the year following pollination. Douglas fir is typical of conifers in that pollination normally occurs in April and fertilization occurs 10 weeks later.

**In vivo Pollination and Fertilization**

About three weeks after it is shed, pollen of Douglas fir is engulfed by the tip of the integument. The exine splits as germination and elongation begin, leaving the highly flexible intine intact. Douglas fir does not respond in germination as do other gymnosperms and angiosperms because no ordinary pollen tube is produced. The fertilization mechanism is similar to that of Gingko, and the structure of the pollen grain is much more complicated than that of pine (12). The cytoplasm of the tube cell becomes vacuolated and enlarges as does the body cell. At this time the body cell moves away from the stalk cell toward the advancing tip of the pollen grain preceded by the cell nucleus. During this stage the body cell nucleus divides unequally to form two gametes. Together with the tube nucleus and the stalk cell, the body cell forms a sac-like structure referred to as the body cell complex. A pollen tube is formed which
penetrates 300 μ of nucellar tissue carrying the tube nucleus. The body cell containing the two gametes and the stalk cell move along the surface of the female gametophyte to the archegonium which it penetrates to reach the egg. The larger of the two male gametes moves towards the female egg and nuclear fusion occurs. The fate of the other male gamete has not been ascertained.

In vitro Mass Culturing

In order to study metabolic and morphologic changes in germinating pollen in vitro, a considerable effort has been made to successfully culture pollen in mass. Typical culture conditions include a carbohydrate supply of ten to twenty percent sucrose which acts as an energy source as well as an osmoregulator. The carbohydrate requirements for angiosperm pollen have been studied extensively (43, 4, 21, 14). Nygaard (1969, 1977) has studied the effect of various carbohydrates on in vitro germination and growth of pine pollen and has reported that in the continued presence of metabolizable sugar pollen produces the same growth rate for the first ten days as in culture devoid of carbohydrate. At the end of ten days the pollen supplied with an external carbohydrate source remains metabolically active and growing. However, pollen deprived of sugars has ceased growth by the tenth day in culture. Using a modified
Brewbaker (1963) medium substituted with 50 mM fructose or 10 mM sucrose Nygaard successfully grew pine pollen to a length of 1.5 mm in three weeks, exceeding the length required to induce fertilization in nature. No significant effect was found on varying the pH between 4.5 and 6.5.

Using another variation in carbohydrate, Dickinson (1968), in his study of endogenous carbohydrate metabolism in lily pollen worked out a culture medium which permitted normal pollen tube growth in the absence of metabolizable carbohydrates. Using a number of osmotic regulators (pentaerythritol, mannitol, sorbitol) he observed pollen tube growth in these media similar to growth in natural conditions.

For angiosperm pollen it is well established that boric acid and calcium, as well as sugar, are usually required for growth (4). Nygaard (1969) observed similar requirements for calcium and boric acid on germination and $^{32}P$ uptake in pine pollen.

Calcium is widespread in plant tissues, averaging 1.5% in dry weight of leaves, 1.2% in shoots and 0.2% in seeds. Pollen grains are low in calcium averaging 0.03% (49). Nevertheless, calcium was found to be the most significant constituent in pine pollen culture medium with respect to tube growth. Nygaard (1969) showed that the necessity of calcium for maximal $^{32}P$ uptake became apparent within 6 hrs and that within 48 hrs tube growth was also affected. The
structural and functional integrity of the cell membrane was shown to be deleteriously affected in the absence of calcium. Upon stimulation of tube growth with sucrose the calcium requirement becomes more pronounced and metabolic activity as well as tube growth are inhibited by EDTA.

In contrast to the significant role of calcium in gymnosperm pollen culture, boron has the most dramatic effect on pollen germination and tube growth in angiosperms (25). There is evidence that boron acts in a number of ways in the germinating pollen tube including: water uptake, translocation of sugars, and by affecting directly or indirectly enzymatic steps in carbohydrate biosynthesis (50). The ability of borate ions to react with hydroxyl rich compounds and to form ionizable sugar borate complexes may facilitate entrance of sugars into the cell (50).

Several other factors have been studied and shown to significantly affect pollen tube growth in vitro. Besides the requirements for carbohydrate and calcium for continued tube growth, Nygaard (1969) has shown that tube growth is greatly dependent upon a short period of equilibration at room temperature and high relative humidity before the beginning of germination. Hormones have been found to have varied effects on pollen tube growth and germination in vitro. Kinetin, indole acetic acid, 2,4-dichlorophenoxyacetic acid, abscisic acid and myoinositol had no effect on tube growth in
pine pollen (43, 38). However, giberellic acid was shown to significantly affect growth and cytological development in Douglas fir pollen (7).

Irradiation produces a more striking stimulation of tube growth in Douglas fir pollen (30). Elongation of Douglas fir pollen was increased with exposure of up to 256 kR while having little effect on germination percentage. This is in contrast to the effect of ionizing radiation on other pollen including Pinus and Picea (18). Livingston (1973) also demonstrated that irradiation increased metabolic activity. Enhancement of growth with ionizing radiation brings up questions concerning the nature of pre-existing enzymes necessary for the germination process and modification of activation of these enzymes with irradiation.

Effects and Metabolism of Exogenous Carbohydrates in vitro

As has been noted exogenous sugars have a significant effect on pollen germination in vitro. The process of tube wall formation, pollen tube growth, and other energy requiring processes involved in the metabolism of germination are all dependent on carbon sources that are stored in the pollen grain or provided by the external environment. Early studies of pollen germination and the growth established the necessity for sugar in the in vitro germination
of many pollen types (43).

To demonstrate the utilization of exogenous carbohydrates and the necessity for environmentally supplied sugars Nygaard (1977) showed rapid tube growth in pine pollen starting at 12 hours of germination and stopping at 60 hours unless sucrose was added to the medium. This showed that pine pollen could initiate germination without exogenous sugar but stops growth if no sugars are made available once endogenous levels drop to a critical level. Hellmers et al. (1956) have shown that the substrate for respiration in pine pollen is almost exclusively sucrose. A drop in sucrose concentration from 16.4% of the dry weight to 1.4% in the first 24 hours of germination occurred. The expected cleavage of sucrose into monomeric components was demonstrated by an increase in reducing sugars from 2% in non-germinated lily pollen to 16% after three hours incubation (16).

The importance of endogenous sugars as a readily metabolizable substrate is reflected in the concentration of sugars in pollen prior to germination which ranges from 10% to 20% of the total dry weight (11, 19, 48). In addition, levels of sugars and organic acids have been directly correlated with pollen viability (48). In all the pollens studied by Hrabatova and Tupy (1964) sucrose was the primary sugar present and provided a stimulatory effect on growth when added to the germination medium.
Contradictory reports exist in the literature concerning the uptake of sucrose from the culture medium by germinating pollen. The ability of pine pollen to take up mono-, di- and tri-saccharides, incorporate them into polysaccharides and oxidize them has been shown by Hellmers (1956). In contrast Nygaard (1977) has recently reported that sucrose is hydrolyzed in the medium to fructose and glucose before being taken up by pollen. This result indicates that uptake mechanisms exist only for fructose, glucose, galactose, and xylose. The cleavage of oligosaccharides is believed by Nygaard to occur in the outside of the pollen tube because monomeric units were present in the culture medium of pollen germinating in an oligosaccharide medium and the only major internal free sugar was sucrose. Once within the pollen, the sucrose pool is thought to be regulated by an equilibrium between synthesis and degradation rather than by availability from the surroundings. However, no tracer experiments were conducted and the origin of the monosaccharide in the medium is unknown.

Respiration

Exogenously supplied sugars are also expected to have a significant effect on the respiration of metabolically active pollen. Sugars active in polysaccharide synthesis increased oxygen uptake from 9% to 61%, arabinose and ribose having no effect (48). Aerobically in
phosphate buffer pollen has a respiratory quotient of 0.85 which increases to 1.0 with the addition of glucose, sucrose and other metabolizable sugars to the medium. However, at the beginning of germination Stanley (1961) found that CO$_2$ is used in a priming step in the first few hours of germination. Presumably this utilization of CO$_2$ is through carboxylation which is circumvented by the addition of sugars to the system.

In both gymnosperms and angiosperms there is a strict correlation between respiration, and metabolic and morphological changes occurring during specific periods of germination. Nygaard (1970) reported an increase in oxygen uptake after 14 hrs of germination and oxygen uptake was correlated with rapid tube growth in pine pollen. In lily pollen a three phase respiratory rate pattern exists (14). An initial high rate of oxygen consumption occurs for about the first 30 minutes after the commencement of germination. This was followed by a 38% lower rate for the second 30 minutes during which time tubes were initiated. The third phase of high respiratory rate began after 60 minutes and continued up to 180 minutes which was accompanied by rapid tube elongation (15, 32).

**Starch Content**

The endogenous levels of starch in dry, ungerminated pollen as
well as its synthesis and degradation during germination vary considerably, reflecting the growth characteristics of the particular species under study. In 34 species of pollen that were studied by Todd and Bretherick (1960) 70% contained less than 3% starch and only 3 species contained over 10%. *Zea mays* contains 22.4%, the highest content of an angiosperm studied and pine contained an average of 2.3% starch. More recently Douglas fir pollen has been shown to contain 13.4% starch (11).

Typically, in angiosperm pollen, because of the short time period between the onset of pollen germination and fertilization and the high concentration of sugars within the micropyle, germinating pollen contains the enzymatic machinery necessary for the storage of available carbohydrate in the form of starch. Dickinson (1968) has shown that the initial high rate of respiration in lily pollen prior to tube elongation coincides with starch synthesis. Lily pollen, prior to germination consists of only 2.5% starch, reaching a maximum level of 7.5% after four hours of germination in sucrose medium. Dickinson has also shown that endogenous sucrose decreases rapidly during this initial period. Further, the rate of starch synthesis is reduced markedly in the presence of abundant endogenous sugars, demonstrating that starch synthesis is under some type of regulational control in lily pollen. Adequate evidence
has not been obtained to identify the site or sites of this regulation.

At the onset of germination pine pollen has reportedly contained only 1.29% starch decreasing to 1.05% after 48 hours of germination (48). Although the level of starch in pine pollen was reported as small, Christiensen (1973) had difficulty in observing the interior of the pollen grain because of the large number of starch granules.

Although starch represents an important constituent both as a primer in pollen types that synthesize starch in the presence of exogenous sucrose as well as a carbohydrate reserve in those types with high starch content prior to germination, the processes by which starch is enzymatically hydrolyzed in germinating pollen has not been studied. However, starch content has been used for some time as an indicator of pollen maturity and a predictive tool for viability estimates (50).

Enzymatic processes involved in starch utilization have however received considerable attention in seed development. Peas and cereal seeds contain between 45% and 55%, and 70% and 80% starch respectively. The hydrolysis of starch provides energy and synthetic requirements for seedlings. In both cereal seeds and peas phosphorylase activity is high in early stages of germination. In contrast, $\alpha$-amylase activity is low at early stages increasing over the first 4 days of germination to 80 times the level of phosphorylase.
activity (8). The activation of pre-existing phosphorylase (22) provides the substrate for glycolysis and respiration without using ATP. The phosphoryolytic pathway for the degradation of starch reserves in germinating lentils seems not to be very important since during the maximum activity the starch content remains almost intact (61). At later stages of germination α-amylase is synthesized and accounts for the major degradation of starch (22).

The Utilization of Carbon Backbones During Germination

The fate of carbon from metabolized sugar in germinating gymnosperm pollen has to date not been clarified. The metabolism of sucrose-$^{14}$C in apple pollen has been studied by Hrabutova and Tupy (1964) and after one hour the main part of the carbon from metabolized sugar appears in free amino acids. Less appears in protein, polysaccharide, CO$_2$, ribonucleic acid, 'deoxyribonucleic acid and the least amount appears in lipid.

A number of interesting occurrences in germinating pollen make the incorporation into amino acids worth noting. Total free amino acids are usually higher in pollen than in any other plant part (50). Linskens (1969) has shown that in petunia 25% of the pollen dry weight is composed of amino acid; proline is one of the most abundant free amino acids representing 2.2% of the total dry
weight (50). More work is necessary to elucidate the role of proline and the other amino acids during pollen germination.

**Metabolic Sequence of Germination**

The metabolic processes occurring at the onset of pollen germination are dependent upon genetic information which has been expressed in the form of enzymes or stored in mRNA either during microsporogenesis or during germination itself. In seeds certain of the hydrolytic enzymes are present prior to seed germination, others are activated during the germination process and still others are synthesized de novo upon hormonal stimulation and according to developmental patterns (6).

The synthetic machinery of protein synthesis as well as enzymes subject to post translational control have been given some consideration in germinating pollen. The pollen of lily and petunia have been shown to be equipped with pre-formed polysomes which become active immediately after hydration resulting in protein synthesis along with the initiation of pollen germination (26, 27, 32). Ungerminated pollen has been shown by Linskens (1970) to contain only monosomes as their ribosomal preparations have no amino acid incorporation in a cell free protein synthesizing system. Polysome formation begins as soon as the pollen has started taking up moisture. Linskens also demonstrated that there is a decrease in the monosomal
peak during the first minute of germination indicating that the newly formed polysomes were made up from ribosomes already present in the dry pollen grain.

Mescahrenas (1969) has shown that the same system of pre-existing ribosomes ready to function upon hydration exists in Tradescantia pollen. About 38% of the ribosomes in ungerminated pollen of this species are in the form of polysomes prior to germination. He demonstrated that these aggregates of ribosomes in dehydrated, ungerminated pollen are sensitive to ribonuclease, indicating that a fraction of the ribosomes in metabolically inactive pollen are pre-packaged with mRNA. During germination there is a very rapid formation of additional polysomes beginning with as short a period as two minutes. Mescahrenus also showed that no additional ribosomes are formed during pollen tube growth in Tradescantia.

The availability of RNA upon the onset of germination and the dependence of the germination process upon de novo RNA synthesis have been studied. Inhibition of RNA synthesis during the first 20 minutes of germination in petunia pollen had no effect on the increase in polysomes, indicating that at the initial phase of germination de novo synthesis of mRNA is not required for the observed increase in polypeptide formation (28). The rapid formation of polysomes upon imbibition of water along with the inability of actinomycin-D to
prevent polysome formation (28, 34) suggests that mRNA may be present in a masked form in resting pollen. Linskens concluded that imbibition resulted in the freeing of protein bound mRNA directly or by activation of enzyme systems which detach the RNA from protein carriers. Therefore, at the translational level germination is controlled by activation and inactivation of long lived mRNA.

In petunia after the initial 15-20 minutes of rapid polysome formation, RNA synthesis begins and is continuous up to 60 minutes (34). At the end of this period, which would be sufficient time for the tube in vivo to be formed and enter the stylar tissue, RNA content decreases and only minor amounts of RNA synthesis occurs (26, 27). Mescarhenus (1969) has also shown that once pollen tube formation has started there is a breakdown of polysomes. In a similar fashion protein content remains constant during the first two hours of germination, then decreases continuously throughout the germination period in lily and petunia pollen (26, 14).

The presence of needed mRNA and proteins in dry pollen for the metabolic pathways of germination has been further demonstrated by inhibitory studies. Although cyclohexamide and actinomycin-D inhibited protein and RNA synthesis, respectively, in germinating pollen, the antibiotics did not prevent continued tube growth (34). In fact the inhibitor of RNA synthesis, 2 thiouracil, has been shown to stimulate tube growth (28).
Enzymes which convert simple sugars into the sugar nucleotide precursors of cell wall polysaccharides have been shown to be present in ungerminated pollen and do not increase in activity during germination (14, 47). Nygaard (1977) reported in pine pollen that carbohydrate metabolizing enzymes were already present in ungerminated pollen. However, unlike angiosperm pollen where there appears to be little de novo synthesis of enzymes, pine pollen in his analysis shows a marked effect on the activity of key enzymes involved in a carbohydrate metabolism by cyclohexamide and chloramphenicol, suggesting that protein synthesis is an important event during germination of gymnosperm pollen.
APPENDIX II

Detailed Protocol of Chemical Analysis of Germinating Pollen

Three cultures of 100 mg pollen were collected after 0, 24, and 48 hrs germination on Whatman no. 1 filter paper and washed with 10 ml fresh sterile medium. The pollen was hand ground in a mortar and pestle in 20 ml 85% ethanol followed by centrifugation at 10,000 g for 10 min to separate ethanol soluble and insoluble components. The ethanol soluble fraction was concentrated by vacuum evaporation and the concentrated extract dissolved in H$_2$O. This extract was filtered and the volume of the filtrate adjusted to 10 ml. Sugars and ethanol soluble amino acids were determined by Anthrone (8) and Ninhydrin (7) methods respectively. The residue from the initial ethanol extraction was hydrolyzed in 20 ml 0.2 N H$_2$SO$_4$ for 1 hour by refluxing. The hydrolyzed residue was filtered and the hydrolyzed starch determined by the Anthrone method with a correction factor of 0.9. Amino acids from proteins in the residue freed by hydrolysis were determined by ninhydrin after neutralization of the acid hydrolysate. The residue from the 0.2 N H$_2$SO$_4$ treatment was hydrolyzed further in 6 N HCl for 1 hour by refluxing, filtering, and then amino acid content of the hydrolysate determined by Ninhydrin method.
Fractionation of $^{14}$C-labelleled Components

After incubation of the pollen with $^{14}$C-glucose as explained in Materials and Methods the pollen was collected by filtration, hand ground in 25 mls 85% ethanol and an aliquot of this slurry removed and counted for the total $^{14}$C-glucose uptake. The ethanol insoluble material was precipitated by centrifugation at 10,000 g for 10 minutes. The ethanol soluble fraction was concentrated by vacuum evaporation and made to a total volume of 10 mls in water. The lipids and other ether soluble components were separated by extracting the ethanol soluble fraction in 15 mls absolute ether. An aliquot of the ether extract was dried and the weight of the ether soluble material determined. Another sample of the ether extract was counted to determine incorporation of $^{14}$C.

The ethanol soluble fraction was separated into amino acids, sugars and organic acids by first passing the extract through 10 g of washed Dowex 50-W-x4 cation exchanger. The effluent was passed through a column of Dowex 1-x4 anion exchanger. An aliquot of the effluent from the second column was counted and sugars determined in this fraction by the Anthrone method. Organic acids were eluted from the second column with 10 mls 1 N acetic acid and an aliquot was counted. Amino acids were eluted from the first column with 20 mls 2 N ammonium hydroxide and the radioactivity
in the fraction determined. Quantitative estimate of amino acids was assayed by the method of Moore and Stein (1954).

The original ethanol insoluble fraction composed primarily of exine, intine, cytoplasmic membranes, proteins, starch, nucleic acids, etc. was suspended in 10 mls water and split into 2 equal fractions. One of these was made to 1 N in NaOH and solubilized by heating at 100°C for 10 min. A sample of the resulting suspension was taken to determine the total counts in the ethanol insoluble fraction. The remaining sample of ethanol insoluble residue was made to pH 4.5 in 0.04 M phosphate buffer and incubated for 2 hours at 30°C with a mixture of 0.1% cellulase and pectinase (Calbiochem.). At the end of the incubation period the insoluble material was precipitated with 20% trichloroacetic acid at 4°C for 10 min. followed by centrifugation for 10 min. The precipitate was washed with 10% trichloroacetic acid and suspended in 10 mls water, and both the insoluble and soluble fractions were counted. All samples were counted directly in 9 ml Handifluor in a Packard Tricarb liquid scintillation spectrometer. The counting efficiency was calculated by adding $^{14}$C-toluene with known DPM to all samples as internal standard. Generally a counting efficiency of 65% was obtained.
BIBLIOGRAPHY (Literature Review)


