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Rapid Starch Synthesis Associated with Increased Respiration in Germinating Lily Pollen[1]

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Abstract. In vitro studies of germinating *Lilium longiflorum* pollen showed that starch increased more rapidly during the first 30 minutes of incubation than during the next several hours. The period of rapid starch formation coincided with the first period of high respiration. An estimate was made of the extra ATP utilized to form extra starch during the first 30 minutes, and this estimate indicates that starch synthesis accounts for a significant portion of the initial high rate of respiration. This pattern of respiration and starch synthesis was not altered when pollen germinated in a pentaerythritol medium that could not be metabolized instead of the standard sucrose medium.

Sucrose was the predominant sugar in mature lily pollen. This sugar decreased 50 % during several hours incubation in pentaerythritol culture medium. Reducing sugars remained low during incubation which may indicate that sucrose breakdown is regulated by the rate of utilization of hexose units.

The work on pollen germination reported here is part of a continuing study to learn the metabolic steps required for pollen tube initiation and to learn how tube growth is controlled through regulation of the rates of the underlying metabolic processes.

Germinating lily pollen exhibits an initial high rate of respiration which is followed by a period of decreased respiration; the transition occurs after 30 minutes of incubation (7). A second period of high respiration occurs after about 2 hours of incubation, coinciding with extensive tube growth. Experiments with oligomycin and 2,4-dinitrophenol showed that respiration of lily pollen was limited by the rate of oxidative phosphorylation, and it was suggested that the distinctive respiratory pattern resulted from variations in rates of ATP synthesis (8). ATP utilization associated with cell wall synthesis could account for the second period of high respiration, but the cause of the initial high rate of respiration was not clear. It was hypothesized that a brief period of rapid synthesis of starch or membranes was responsible for the initial high rate of respiration. Experiments to test the hypotheses are reported here.

Other studies were conducted 1) to find a culture medium which permitted normal pollen tube growth but which lacked organic substances that could be metabolized by lily pollen, and 2) to learn how endogenous sugars and starch change during germination in a metabolically inert medium. To prevent bursting of the pollen grains, a sugar such as sucrose (10 %, w/v) is routinely added to the culture medium, so it is difficult to study interconversions of endogenous carbohydrates during germination.

Materials and Methods

Pollen from *Lilium longiflorum*, variety Ace, was used unless otherwise noted. Methods of germinating the pollen and of measuring tube length were reported earlier (7). Respiration was measured manometrically at 30°, and each 15 ml Warburg flask contained 10.0 mg pollen and 1.7 ml of culture medium. The standard culture medium (pH 5.2) contained 0.29 M sucrose, 1.27 mM $Ca(NO_3)_2$, 0.16 mM H_3BO_3, 0.99 mM KNO_3, and 10 μg/ml tetracycline. In some culture mediums, sucrose (Mallinkrodt) was replaced by 0.29 M

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mannitol (Eastman-Kodak), 0.29 M pentaerythritol (Matheson), or other organic substances.

In the radioisotope studies a 7 mg sample of pollen was spread on a 2.1 cm diameter fiber glass filter disc (Reeve Angel grade 934AH) which was placed in a 25 ml Erlenmeyer flask. Culture medium (0.15 ml) containing the isotope was added, and the flask was closed with a serum stopper from which hung a glass cup (5) containing 0.2 ml of 2.5 N NaOH. These assemblies were incubated in a water bath shaker set at 30°. Experiments were terminated by placing the flasks in ice. Each cup with its NaOH was immediately put into a scintillation bottle containing Cab-O-Sil and the toluene-ethanol scintillation fluid of Harlan (12). Pollen and disc were rinsed on a suction filter with 7 ml of ice cold non-radioactive culture medium and placed in 80% (v/v) ethanol at 70° for 30 minutes. Pollen and glass fibers were dispersed with a spatula and sedimented by centrifugation (20,000 × *g*, 15 min). The residue was rinsed repeatedly in ethanol until a negligible amount of radioactivity appeared in the ethanol wash, and then the residue was oxidized with Van Slyke dichromate reagent (1). $^{14}CO_2$ was absorbed in 2.5 N NaOH and counted according to Harlan (12). The same scintillation fluid with Cab-O-Sil omitted was used for aqueous or ethanolic solutions; counting efficiency was determined by the channels ratio method.

Plastic foils coated with cellulose (Brinkmann Instruments) were used for thin layer chromatography of ethanol extracts from pollen. Radioactive compounds were located with a Packard radiochromatogram scanner, and sugars were visualized with analine-diphenylamine reagent (11). Solvents 1 (formic acid:methyl ethyl ketone:*tert.*-butyl alcohol:water, 15:30:40:15, v/v) and 8 (*iso*-propyl alcohol:pyridine:acetic acid:water, 8:8:1:4, v/v) of Vomhof and Tucker (24) were used to study pollen sugars. Three developments (1 dimension) in solvent 1 separated sucrose, glucose, and fructose from one another. Pentaerythritol was chromatographed in solvent C (ethanol:water, 9:1, v/v) of DiCarlo, *et al.* (6) and solvent 1 of Vomhof and Tucker (24). The R_F in solvent C was 0.73. A single development in solvent 1 gave good separation of pentaerythritol (R_F about 0.62) from sucrose, glucose, and fructose (R_F values of 0.10–0.23). In several experiments, pollen extracts were incubated with invertase (2 mg/ml) for 15 minutes at 30° before the extracts were spotted on chromatograms. The invertase was grade II from Sigma (122 units/mg). Isotopes used were sucrose-U-^{14}C (10.0 mc/mmole) from Nuclear-Chicago and pentaerythritol-1,2-^{14}C (283 μc/mmole) from Baird-Atomic. Pentaerythritol-^{14}C used in preliminary experiments was obtained from Warner-Lambert Research Institute through the courtesy of Edward J. Merrill.

For studies of starch and sugar content, samples of pollen (10 mg) were incubated in a water bath shaker (30°) in 25 ml Erlenmeyer flasks containing 1.3 ml of culture medium. Incubation was terminated by adding hot ethanol (final concentration 80% ethanol) and holding the flasks at about 75° for 15 minutes. Groups of 5 replicate flasks were pooled, and a starch fraction was determined according to Hassid and Neufeld (13). The ethanol supernatant was analyzed for reducing sugars (20), and total ethanol-soluble sugars were measured by the anthrone method (15). Glucose standards were used, so starch and sugar content was calculated as glucose. Pentaerythritol did not react with anthrone reagent. Similar samples of pollen were extracted with methanol-chloroform according to Bligh and Dyer (3), and lipid phosphate was determined on the extract (18).

Results and Interpretation

Germination in Various Culture Media. Lily pollen was incubated in culture media which contained the salts listed under methods plus a range of concentrations of a number of substances which acted as osmotic agents but which might not be metabolized. These substances included Ficoll, pentaerythritol, mannitol, sorbitol, glycerol, ethylene glycol, and propylene glycol. Several polyethylene glycols (molecular weights 380–7500) and polypropylene glycols (molecular weights 400 and 1200) were also tried. Rapidly growing pollen tubes which resembled those in sucrose were observed only in pentaerythritol, mannitol, and sorbitol.

Pentaerythritol and mannitol were selected for more detailed studies of tube growth and respiration. Figure 1 shows O_2 uptake of lily pollen germinating in media that contained sucrose, mannitol, pentaerythritol, or 1:1 mixtures of sucrose with 1 of the alcohols. Average percent germination and tube length were determined at 3 hours when the respiration experiment was terminated. The pattern of respiration was not affected by substituting an alcohol for one-half of the sucrose, but the second period of high respiration was markedly reduced when pollen germinated in either alcohol alone. Percent germination was about 70% in all media, and the pollen tubes looked similar in them all. Average tube length at 3 hours was about 1.0 to 1.2 mm for all except the mannitol medium in which the length was about 0.7 mm. Pentaerythritol was selected for further study because 1) mannitol may cause a slight inhibition of tube growth, and 2) mannitol is metabolized by some species of higher plants (23).

Radioisotope Studies. It was decided to study absorption and possible metabolism of pentaerythritol-^{14}C under conditions where at least 1% of administered sucrose-^{14}C appeared as $^{14}CO_2$. More

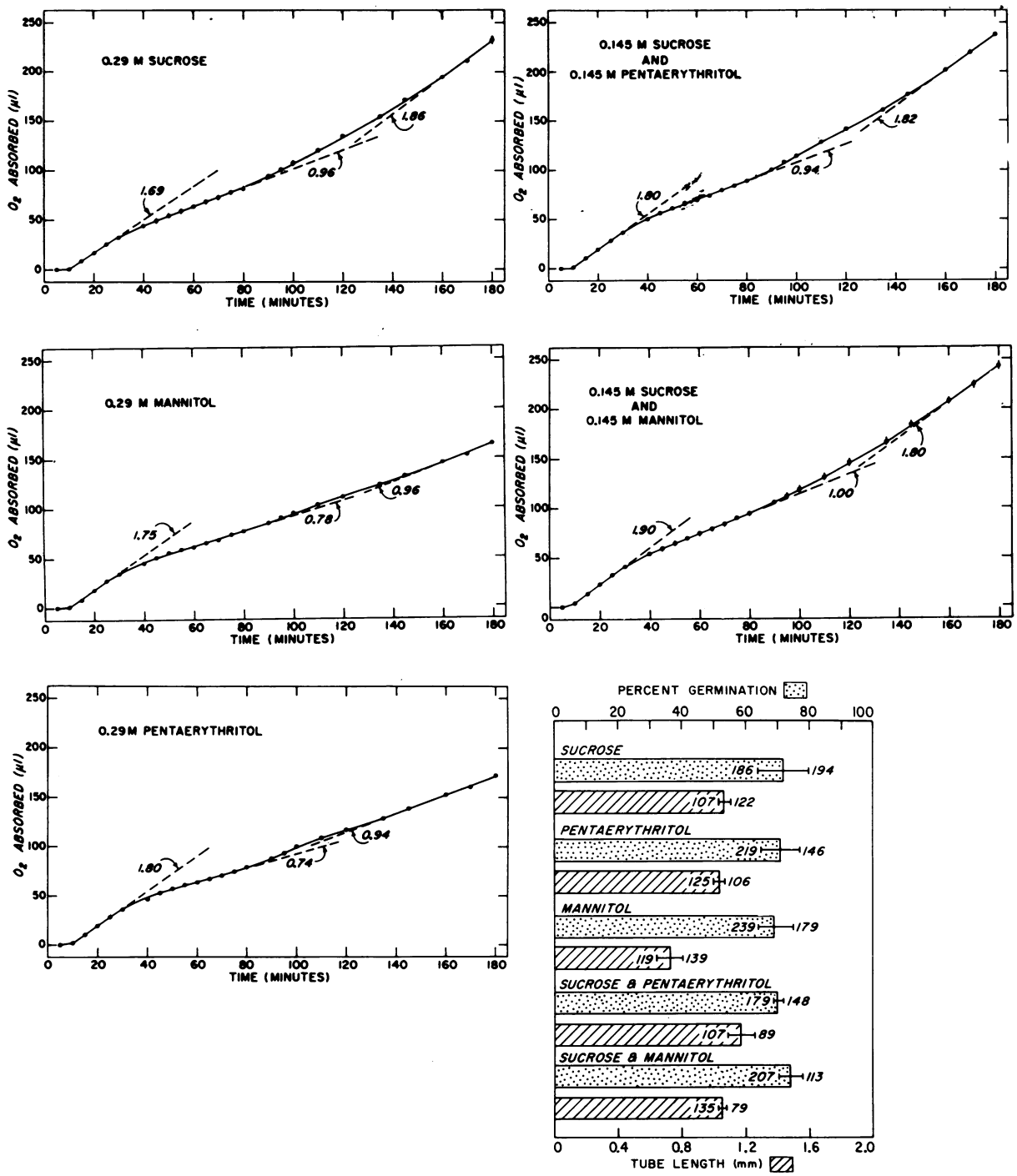

FIG. 1. O_2 uptake, percent germination, and tube lengths of lily pollen germinating in various culture media. The culture medium contained the indicated sugar or alcohol, 3.0 mM KH_2PO_4, and the salts described in the text. Each point on the curves is an average from duplicate Warburg flasks; vertical bars show dispersion between duplicates. Numbers near the curves are rates of O_2 uptake in µl/min. Growth data (lower right) is from the same samples after 180 minutes of incubation, and horizontal bars indicate dispersion between duplicates. Numbers near the bars indicate how many pollen grains were counted or tubes measured in each duplicate.

Table I. *Radioactivity of Various Fractions From Lily Pollen Incubated with Sucrose-U-^{14}C or Pentaerythritol-1,2-^{14}C*

Samples of pollen (7.0 mg) were incubated 6 hours at 30° with 0.15 ml sucrose or pentaerythritol culture medium as described under Methods.

Fraction	Treatment 1 Sucrose-^{14}C		Treatment 2 Pentaerythritol-^{14}C	
	dpm	%	*dpm*	%
Radioactivity administered to each replicate	2,075,000	100	1,999,000	100
$^{14}CO_2$ evolved				
Repl. A	40,600	2.0	72	0.004
Repl. B.	40,400	2.0	55	0.003
Repl. C	31,500	1.5	62	0.003
Activity absorbed (total of the pooled replicates)				
Soluble in 80 % ethanol	439,700	7.1	271,900	4.5
Not soluble in 80 % ethanol	216,600	3.5	275	0.005

than 1 % of administered sucrose-^{14}C did appear after 6 hours of incubation when 7.0 mg pollen was placed with 0.15 ml culture medium on a fiber glass disc. This procedure was adopted in subsequent studies. Lily pollen was incubated for 6 hours with pentaerythritol-^{14}C on 3 separate occasions, and no $^{14}CO_2$ was detected. The third experiment is summarized in table I. Data for evolved $^{14}CO_2$ is given for each replicate, and the replicates were pooled during extraction with ethanol. Both pentaerythritol and sucrose were absorbed by the pollen; some absorbed sucrose was metabolized to $^{14}CO_2$ and to an alcohol insoluble fraction. In contrast, the absorbed pentaerythritol did not appear as $^{14}CO_2$ nor was it converted to products insoluble in ethanol. Radioactivity absorbed from the pentaerythritol culture medium migrated as a single compound when chromatographed in 2 different solvents. R_F values were similar to those of authentic pentaerythritol. Sucrose-^{14}C was the predominant ethanol soluble compound when pollen germinated in sucrose-^{14}C culture medium. This was demonstrated by similar R_F values of the unknown and authentic sucrose and by complete hydrolysis of the unknown to glucose and fructose after incubation with invertase.

Measurement of Endogenous Constituents During Germination. Similar patterns of starch accumulation during germination were observed in preliminary experiments, and in 2 experiments shown in figure 2. Nongerminated lily pollen contained little or no starch. Starch accumulated rapidly after pollen was placed in the culture medium, but a sharp decrease in rate of accumulation occurred at about 30 minutes. Data of Rosen *et al.* (22) and Iwanami (14) also indicated that starch was absent from nongerminated lily pollen but that starch grains appeared during germination. Starch content and respiration were determined on replicate samples in experiment B (fig 2). Decreases in rates of respiration and starch accumulation occurred about the same time. Pollen tube growth began at 45 to 60 minutes, and rapid tube elongation ensued (fig 2). Thus, the changes in

Table II. *Balance Sheet for Estimated Extra ATP Formation and Utilization During the First 30 Minutes of Germination*

These calculations are based on data in figure 3.

Description	Expt A	Expt B
μl O_2 absorbed/10 mg pollen		
0 to 30 mins incubation		39.6
30 to 60 mins incubation		26.9
Extra O_2 absorbed during the first 30 mins		12.7
Estimated ATP formation resulting from the extra O_2 absorbed (mμmoles ATP/pollen grain)[1]		0.082
Increase in starch (calculated as μmoles glucose/10 mg pollen)		
0 to 30 mins incubation	1.58	1.84
30 to 60 mins incubation	0.23	0.59
Extra starch formed during the first 30 mins	1.35	1.25
Estimated ATP utilized in synthesis of extra starch (mμmoles ATP/pollen grain)[2]	0.058	0.063

[1] Assuming an average P/O of 3 in the intact cells.
[2] Assuming 2 ATP utilized/1 hexose incorporated into starch.

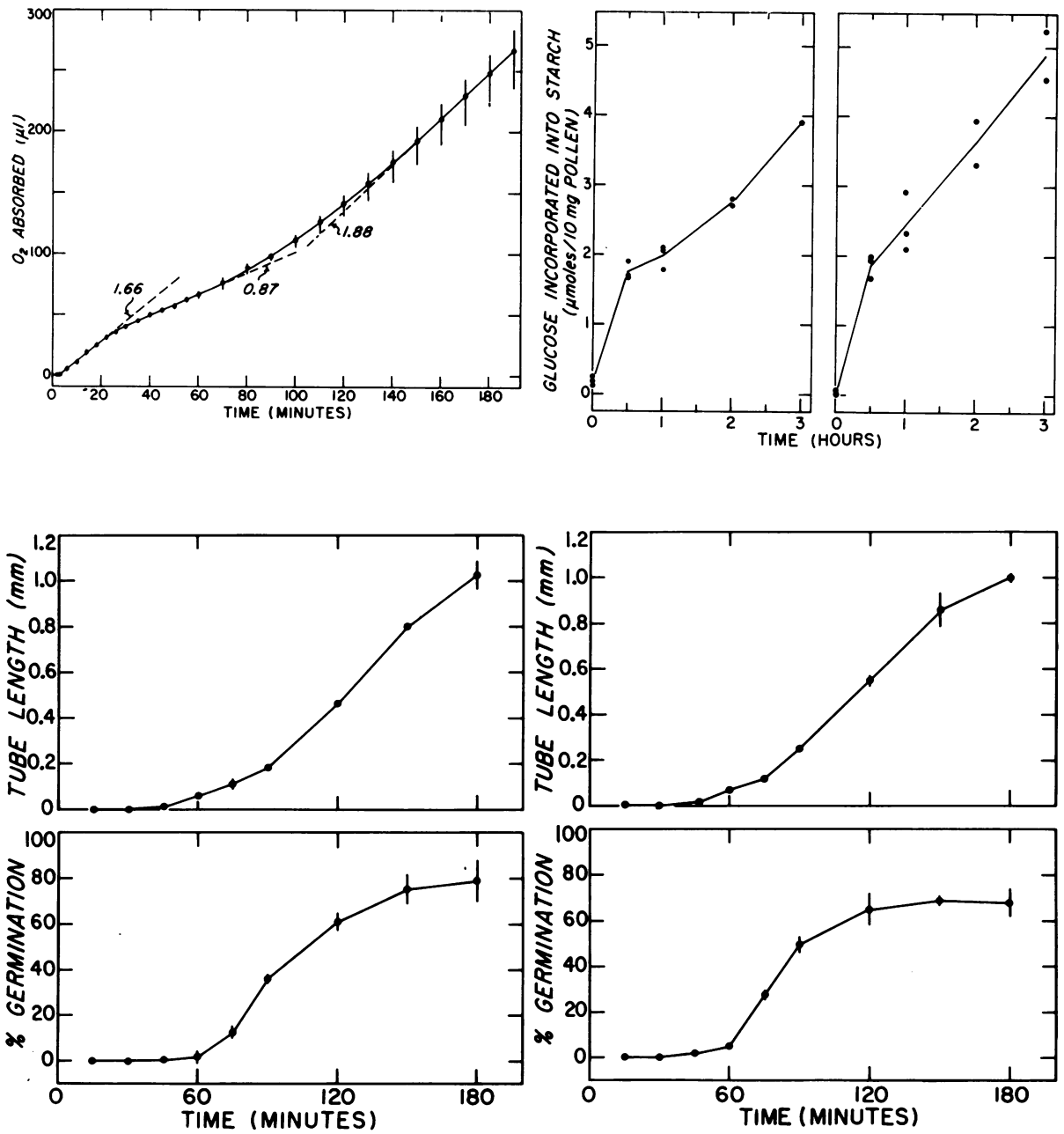

FIG. 2. Starch content and respiration of germinating pollen. All samples were incubated in the standard culture medium. Starch (upper right) was calculated as glucose; experiment A is on the left, and B is on the right. Each point represents a starch determination. There were 4600 ± 244 (mean ± S.E.) pollen grains/mg in Expt. A and 4150 ± 155 grains/mg in Expt. B. O_2 uptake (upper left). Each point is the average of 4 Warburg flasks, and vertical bars show extent of variation among the flasks. Pollen samples were replicates of those used in starch experiment B. Rates of O_2 uptake are given in µl/min. Average tube length and percent germination (Expt. A, lower left; B, lower right). Vertical lines indicate variation between duplicate samples.

rates of respiration and starch accumulation occurred at least 15 minutes prior to tube initiation.

Assuming an average P/O of 3 in the intact cells and formation of starch from free hexoses, an estimate may be made whether rapid starch formation contributes significantly to the initial high rate of respiration. Extra O_2 uptake during the first 30 minutes of incubation may be equated to ATP formation; extra starch formed during the same time may be equated to ATP utilization. Phosphorylations during the reactions catalyzed by hexokinase and ADP-glucose pyrophosphorylase would utilize 2 moles of ATP for each mole of hexose incorporated into starch. The assumption that the average P/O equals 3 seems justified by earlier data (8) which indicated that mitochondrial O_2 uptake accounted for the bulk of O_2 uptake by intact pollen grains.

Table II shows estimates of ATP formation and utilization which are based on the data in figure 2. The estimate of extra ATP formed is 0.082 mμmole/pollen grain. Estimates of ATP utilization for extra starch formation are 0.058 and 0.063 mμmole/pollen grain. Hence, starch synthesis accounted for about three-fourths of the extra ATP utilization, a minimum estimate since starch turnover is not taken into account. Only one-half as much ATP would be utilized if starch arises from sucrose by the action of sucrose-UDP glucosyltransferase. This enzyme has not been studied in lily pollen, but it is widely distributed among higher plants (4). The enzyme has an equilibrium constant near unity (2), and it may be involved in starch synthesis in maturing seeds of corn (9) and rice (19). *Lilium* pollen contains sucrose as well as glucose and fructose (14) so starch may form either from hexoses or it may be synthesized via the step involving sucrose transglucosylase.

In a subsequent experiment, the pattern of starch accumulation was about the same whether pollen germinated in sucrose or pentaerythritol (fig 3). Although some starch was present initially, this pattern resembled earlier data in that starch accumulated more rapidly during the first 30 minutes of incubation than during the next several hours. In sucrose culture medium, extra starch formed during the first 30 minutes compared to the second 30 minutes of incubation was equivalent to 0.11 μmole glucose/10 mg pollen. The figure for pentaerythritol medium was 0.07 μmole glucose/10 mg pollen.

Total sugars decreased continually during germination in pentaerythritol medium, and the greatest decline occurred during the first 30 minutes of incubation when starch increased most rapidly (fig 3). Reducing sugars were about 2 % of total sugar in nongerminated pollen and 16 % after 3 hours incubation. Growth data for this pollen are also given in figure 3. In a second experiment total and reducing sugars were determined on Ace and Georgia pollen germinating in pentaerythritol medium, and the results were similar to those given above. In both varieties the reducing sugar content was about 1 % of total sugar in nongerminated pollen and 6 % after 4 hours of incubation. In a third experiment with Georgia pollen, reducing sugars were unusually high, 140 μg/mg pollen, and declined to about 40 μg/mg pollen after 3 hours of incubation. Total sugar declined from about 270 to 180 μg/mg pollen during the same period. Thus, nonreducing sugars always were predominant during germination.

Thin layer chromatography showed that sucrose was the nonreducing sugar in Ace and Georgia pollen. Chromatograms of ethanol extracts from germinated and nongerminated pollen showed a single spot after development with solvents 1 or 8 of Vomhof and Tucker (24). Identity was confirmed when incubating the extract with invertase before development in solvent 1 caused complete disappearance of the supposed sucrose and appearance of 2 new compounds which cochromatographed with glucose and fructose.

In 2 separate experiments, lipid phosphate was determined on nongerminated and on germinating pollen. In both experiments the pollen contained about 1.75 μg lipid P/mg pollen (0.01 mμmole lipid P/pollen grain), and this amount remained constant during several hours of incubation. In both experiments germination was about 50 %, and average tube length exceeded 1 mm.

Discussion

A significant portion of the initial high rate of respiration appears to be caused by starch synthesis within the pollen grains. Endogenous sucrose decreases rapidly during incubation, and this compound is the probable source of carbon skeletons for starch synthesis and respiratory processes. The respiratory quotient was 1 during 2 hours incubation in pentaerythritol medium which also indicates that an endogenous carbohydrate was the major respiratory substrate.

The first steps of sucrose metabolism may be hydrolysis by β-fructofuranosidase to yield free hexoses which are transformed into hexose phosphates by hexokinase. Alternatively, sucrose glucosyltransferase (4, 19) may convert sucrose to UDP- or ADP-glucose plus fructose which is subsequently phosphorylated. In either case, control of sucrose breakdown is indicated by the fact that reducing sugars (glucose and fructose account for this fraction) remain low while sucrose decreases about 50 % in 4 hours. Furthermore, absorbed sucrose-^{14}C was present primarily as sucrose even after 6 hours of incubation. Some regulatory mechanism may link the rate of sucrose metabolism to synthetic and respiratory processes. Such a mechanism may involve regulation *in vivo* of the activity of sucrose glucosyltransferase or β-fructo-

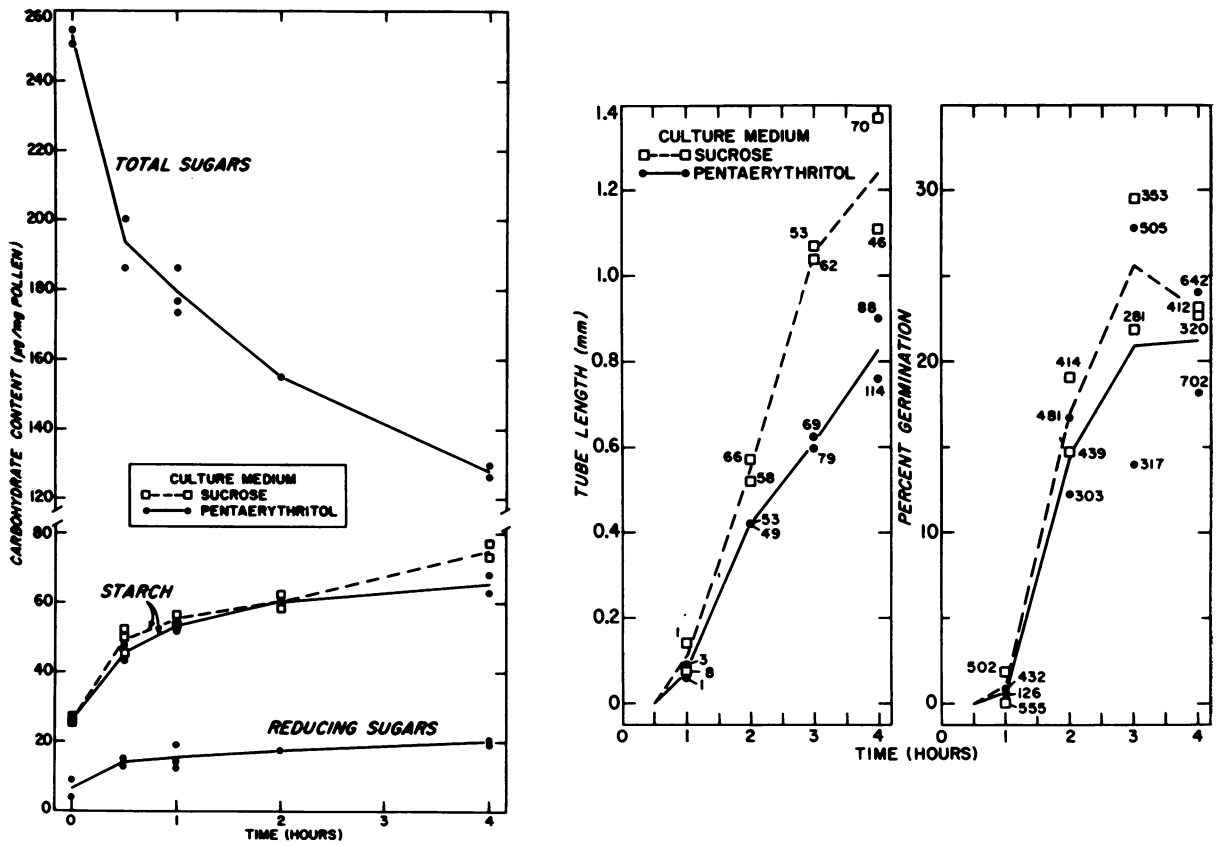

FIG. 3. Starch and sugar content of lily pollen germinating in standard sucrose and pentaerythritol mediums. Each point for starch and sugar (left) is a determination made on a 50 mg sample of pollen. Results are expressed as μg glucose/mg pollen. There were 4190 ± 167 (mean ± S.E.) pollen grains/mg pollen. Average tube length and percent germination of replicate samples are on the right. The number of tubes measured or grains counted is placed next to the point which is an average value.

furanosidase. Kidby found that the latter enzyme is activated by P_i and several organic and amino acids (17). Perhaps this enzyme is also reversibly inhibited by some other metabolite which increases at 30 minutes when starch synthesis and respiration decrease. The same may be true of sucrose glucosyltransferase; Pridham *et al.* (21) report that this enzyme is strongly inhibited by D-glucose and D-fructose.

Regulation of starch synthesis is indicated by 1) the marked reduction in rate of starch accumulation in the presence of relatively abundant endogenous sugars and 2) finding that the pattern of starch synthesis is the same for at least 2 hours whether lily pollen is incubated in 10% sucrose or in a metabolically inert culture medium. Identifying the site(s) of regulation is difficult without knowing whether lily starch is formed from free hexoses or whether sucrose-UDP glucosyltransferase plays an important role. In either case ADP-glucose pyrophosphorylase would act on at least part of the hexose being transformed to starch, and regulation might occur at this step. This enzyme is thought to regulate starch synthesis in spinach chloroplasts (10). Its catalytic activity is greatly enhanced by several glycolytic intermediates, and the most effective compound (3-phosphoglycerate) causes as much as 58-fold activation. Whether the lily pollen enzyme has regulatory properties remains to be seen.

Formation of lipid phosphate does not contribute significantly to either the first or second period of high respiration during germination, barring the unlikely possibility of extensive breakdown and resynthesis of pollen membranes. Dictyosomes which are secreting vesicles are found in lily pollen tubes, and these vesicles seem to be the source of new cell membrane at the tips of the growing tubes (22). Such new membranes may represent a very small percentage of the total lipid P in the cells, and this probably accounts for the apparent constancy of lipid P reported here.

Lily pollen lacks an alcohol dehydrogenase capable of oxidizing pentaerythritol since the compound remained unchanged within the cells. Such an enzyme does occur in some living cells, however. Pentaerythritol is oxidized by a particulate fraction from *Gluconobacter oxydans* (16).

Acknowledgment

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