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The Nutritional Role of Pistil Exudate in Pollen Tube Wall Formation in *Lilium longiflorum*

I. UTILIZATION OF INJECTED STIGMATIC EXUDATE[1]

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C. LABARCA[2] AND F. LOEWUS
Department of Biology, State University of New York at Buffalo, Buffalo, New York 14214

ABSTRACT

A quantity of labeled stigmatic exudate, collected from detached *Lilium longiflorum* (cv. Ace) pistils labeled with D-glucose-1-^{14}C, was fractionated on Sephadex G-100 and the polysaccharide component, G-100-I, was injected into the hollow styles of unlabeled detached pistils (cv. Ace) which had been removed on the day after anthesis from the plant. Injected pistils were immediately cross-pollinated with *L. longiflorum* (cv. No. 44) pollen. Eighty-four hours later, pistils were dissected to recover the pollen tubes, expended exudate, and labeled tissues of the stigma and style. Distribution of label revealed that at least 25% of the carbohydrate substance in excised pollen tubes was derived from G-100-I. The composition of expended exudate adhering to pollen tubes, of pollen tube cytoplasm, and of pollen tube walls suggests that utilization of exudate by growing pollen tubes involves uptake and incorporation into pollen tube cytoplasm of exudate polysaccharide fragments followed by extensive metabolism of at least a portion of the incorporated carbohydrate prior to its utilization for pollen tube wall biosynthesis. Results suggest the presence of at least two polysaccharide components in G-100-I, one which resists major degradation following injection into the style and another which undergoes measurable degradation both before and after entry into the pollen tube.

In pollination, success depends on a process of pollen tube formation leading to fertilization (21). For study of this process, *Lilium longiflorum* has advantages which enable the experimentalist to proceed with assurance that an answer to the question of the nutritional dependence of the growing tube on the pistil will be found. Lily pistils secrete a stigmatic exudate in such quantities that droplets accumulate on the surface of the stigma and are shed at irregular intervals, once or twice a day (35). Detached flowers will survive for days and continue to produce stigmatic exudate as long as the cut end is immersed in water. The plant produces pollen in abundance, about 5 mg per anther. The pollen is viable over long periods of time and under a variety of storage conditions. Due to the large stigma, as much as 10 mg of pollen can be applied to a single pistil. Pollination leads to formation of a mass of pollen tubes. In cross-compatible pollinations (20), 3 days of tube growth leads to formation of long strands of pollen tubes that extend from stigma to ovary. These rope-like strands occupy a superficial position in relation to cells which line the stylar canal and are readily recovered by dissection. By making practical use of these qualities, it is possible to examine the nutritional dependence of pollen tube formation on pistil secretion and obtain evidence that this dependence is a process that accounts for a significant part of wall substance during tube development.

The stigmatic exudate produced by *L. longiflorum* is an aqueous solution in which about 95% of the solute is polysaccharide with a monosaccharide composition similar to the gum exudates (3, 18). This exudate is secreted from surface tissues of the stigma and from cells which line the hollow duct that forms the stylar canal (5, 30, 34, 35). If labeled glucose or myoinositol is introduced into the vascular system of a detached pistil through the cut surface of the pedicel, a portion of the label ultimately appears in stigmatic exudate as labeled polysaccharide. If the exudate is collected over a period of 4 to 5 days, about 5% of the label accumulates in the exudate (14, 18). If the labeled pistil is pollinated, upwards of 1% of the administered label is incorporated into tube wall polysaccharide, primarily as pectic substance (15). Clearly, exudate is utilized for pollen tube wall formation in a selective way that involves pectin biosynthesis. Additional support for this is found in experiments in which labeled exudate or just its polysaccharide component is incorporated into pectic substance of pollen tubes grown *in vitro* or *in vivo* (12).

This paper describes an experiment in which labeled polysaccharide from exudate that was collected from detached pistils fed D-glucose-1-^{14}C is injected into stylar canals of unlabeled pistils and followed by cross-compatible pollination. The composition and specific radioactivity of injected polysaccharide are compared with that portion of the label recovered in pollen tubes. A quantitative estimate of exudate utilization for pollen tube wall biosynthesis is obtained.

MATERIALS AND METHODS

Preparation of Labeled Exudate. The method used in this paper was similar to one described earlier (18). Ten detached pistils of *L. longiflorum*, cv. Ace, were given a total of 337 μc of D-glucose-1-^{14}C (35.9 c/mole). The labeled sugar was contained in 0.2 ml of water per pistil to allow rapid uptake

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[2] Present address: Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile.

through the cut surface of the pedicel. When all of the labeled solution was taken up, water was added to keep the pedicel immersed throughout the period in which exudate was collected (5.5 days). The total yield of exudate was 1.5 ml containing 15.5 μc of ^{14}C, about 4.6% of the label supplied.

The pooled exudate was separated by gel filtration (Sephadex G-100, column size, 2 × 46 cm) into a polysaccharide fraction, G-100-I, and a low molecular weight fraction, G-100-II (18). G-100-I was freeze-dried and redissolved in 1.5 ml of water. It contained 14 μc of ^{14}C in 30 mg of dry solids. Previous results indicated that 80 to 90% of this was carbohydrate and that nearly all of the ^{14}C was present as carbohydrate. On the basis of these considerations, the G-100-I used here had a specific radioactivity of 0.55 ± 0.03 μc/mg.

Injection of Labeled G-100-I into Detached Pistils. Flower buds of *L. longiflorum*, cv. Ace, were removed from plants 1 day before anthesis and stored at 27 C in a chamber held at 70% humidity. The light regimen consisted of 8 hr of light (500 ft-c, cool white fluorescent plus incandescent) followed by 16 hr of darkness. Anthers were removed at anthesis, tepals and stamens 24 hr later. Prior to injection with labeled G-100-I, each pistil was recut at the pedicel and placed in a separate vial of water. Vials were tilted to bring the stigma and upper portion of the style into a horizontal plane slightly lower than the ovary. This position resembles the natural orientation of the flower and prevents drainage of injected G-100-I into the ovary due to gravity. The labeled polysaccharide was injected with a small glass syringe fitted with a 4-cm No. 23 hypodermic needle. The needle was inserted into the stylar canal through the center of the stigma at the point where the carpels joined. Injection of G-100-I continued until a slight overflow was detected on the stigma surface. A total of 40 pistils were injected with 13.2 μc of G-100-I, about 35 μl per pistil.

After injection, each pistil was cross-pollinated with fresh pollen (10 mg, cv. no. 44, a compatible pollen). Pollination of all pistils was completed within 1 hr and the pistils were returned to the growth chamber for 83 hr. At the end of this time, all pistils contained pollen tubes that extended as a tapering mass within the canal from the stigma to the base of the style.

Dissection of Pistils for the Recovery of Exudate and Pollen Tubes. Before dissection, the ovary and pedicel were removed and discarded. The remaining style-stigma portion was trimmed to remove the secreting upper surface of the stigma with its load of pollen grains. The process of trimming severed pollen tubes from the grains and allowed the tubes to be removed with ease from the canal after dissection. After trimming, the style-stigma section was immersed in 0.3 M pentaerythritol (20 ml) and split open to expose the canal. The pollen tube mass was teased from the canal with a dissecting needle and transferred to fresh pentaerythritol solution. Fresh dissecting medium was used for each group of 10 pistils. Eventually, these separate batches of media were pooled and analyzed for ^{14}C.

Trimmings from the stigmas were combined, rinsed with water to recover adhering exudate, and ground in a glass homogenizer. Adhering exudate, the water-soluble portion of the homogenate, and the insoluble residue were assayed for ^{14}C. Similarly, the style-stigma sections from which the pollen tubes had been removed were rinsed free of adhering exudate with water, ground with pestle and mortar, separated into water-soluble and -insoluble fractions and analyzed for radioactivity.

Pollen tubes were pooled in 0.3 M pentaerythritol (8 ml), centrifuged at 1,000*g* for 5 min, and resuspended in fresh pentaerythritol (1.5 ml). This washing procedure was repeated 9 times to remove all traces of adhering exudate. Washes were combined and set aside for further study of the composition of that portion of exudate (referred to as expended exudate) found in close association with pollen tubes during tube growth.

Fractionation of Excised Pollen Tubes. The washed tubes were resuspended in water and ground for 10 min in a motor-driven glass homogenizer. No visible particles remained in the turbid suspension following grinding. Centrifugation at 1000*g* separated the suspension into a turbid supernatant and a residue. The latter was resuspended 4 times in fresh portions of distilled water and centrifuged between each resuspension. All washes were added to the original supernatant. The washed residue was resuspended in 95% ethanol, centrifuged, and dried (yield 32 mg).

The turbid supernatant from the low speed centrifugation was spun at 20,000*g* for 1 hr at 4 C to give a clear colorless supernatant, referred to as the "high speed supernatant," and a pellet, referred to as the "high speed pellet." The former was reduced to a small volume and placed on Sephadex G-100 (column, 1.2 × 95 cm). Elution was done with 0.1 M acetic acid. Fractions containing significant amounts of radioactivity were pooled on the basis of observed peaks of radioactivity, hydrolyzed with acid, and analyzed for carbohydrate by paper chromatography and GLC.[3]

The high speed pellet was hydrolyzed, first with 2 M TFA at 120 C for 0.5 hr, then with 1% *Aspergillus niger* cellulase (Worthington CSE I) at 25 C and pH 4.5 for 21 hr. After each hydrolysis, the supernatant was collected and analyzed for carbohydrates by paper chromatography and GLC.

A portion of the low speed precipitate which contained the washed pollen tube walls was hydrolyzed in 2 M TFA at 120 C for 0.5 hr. Soluble products were removed by centrifugation and the process repeated once. The residue was treated with cellulase as described for the high speed pellet.

Analysis of Expended Exudate in Washes from Excised Pollen Tubes. Washes containing this fraction were combined, reduced to a small volume, placed on Sephadex G-100 (column, 2 × 46 cm), and eluted with 0.1 M acetic acid. Fractions were analyzed for ^{14}C and selected fractions were pooled and further examined to determine their carbohydrate composition after acid hydrolysis as described above.

Analytical Procedures. Polysaccharides and fractions containing high molecular weight carbohydrate were hydrolyzed with TFA (1). Products were separated by paper chromatography on Whatman no. 1 paper in solvent A (ethyl acetate-pyridine-water, 8:2:1, v/v) to recover neutral sugar residues. Products which remained at the origin of the paper after chromatography in solvent A were eluted and chromatographed in solvent B (ethyl acetate-water-acetic acid-formic acid, 18:4:3:1, v/v). Radioactive regions were located by scanning and the individual sugars by comparison with standards which were run simultaneously on the same papers. After development, control strips were sprayed with the alkaline silver nitrate system.

Water-soluble samples were analyzed for ^{14}C at an efficiency of 70 to 80% in a liquid scintillation spectrometer (Packard Model 3324) with toluene-Triton X-100 scintillation fluid as described by Turner (32). Insoluble residues were suspended in dioxane-naphthalene (22) containing Cab-O-Sil (Cabot Corp., Inc., Boston, Mass.). A windowless radiochromatogram scanner (Packard Model 7201) was used to detect radioactive regions on paper chromatograms.

[3] Abbreviations: GLC: gas-liquid chromatography; TFA: trifluoroacetic acid.

Neutral sugars were also identified by elution from paper after chromatography and conversion to either trimethylsilyl ethers or alditol acetates which were analyzed by GLC. These separations were run in a glass column (0.63 × 180 cm) filled with 3% silicone XE-60 on 80/100 mesh Supelcoport. The instrument was a Packard system equipped with a flame ionization detector and a Model 802 oven. Nitrogen (60 cc/min) was used as carrier gas. Separations were run isothermally, 132 or 150 C for trimethylsilyl ethers and 200 C for alditol acetates. Areas under the detector peaks were measured with a Disc integrator and compared with standards prepared from authentic sugars. To determine specific radioactivities, aliquots of the samples used for GLC were counted by liquid scintillation.

RESULTS

Exudate. Table I records the distribution of ^{14}C in different fractions of dissected pistils. Over one-half of the label was recovered in the dissecting medium and various washes. Stigma, style, and pollen tube tissues accounted for 21%, equally divided after grinding, between water-soluble and -insoluble components. Unrecovered ^{14}C includes label lost as respired CO_2 and any injected exudate that leaked into the locular cavity of the ovary during injection or subsequent pollen tube growth. Significantly, 6.1% of the label was found in washed pollen tubes, over one-half of this as water-insoluble tube wall.

Total carbohydrate content and specific radioactivity of injected G-100-I, expended exudate, and pollen tube wall are given in Table II. In contrast to the 25 mg of G-100-I injected, 119 mg of carbohydrate was recovered in expended exudate from the washes. This is very nearly a 5-fold dilution of injected exudate. Nor does this figure include the ^{14}C-labeled carbohydrate incorporated into pollen tubes and surrounding stylar or stigma tissues or that portion of the label lost as CO_2. When specific radioactivities of these exudates are compared with that of injected G-100-I, dilution values ranging from 7-fold in exudate recovered from pollen tube washes to 9-fold in washes of stigma and style or the dissecting medium are obtained. The specific radioactivity of pollen tube wall was about one-fourth of that representing the bulk of the expended exudate.

Gel filtration profiles of the radioactivity in G-100-I and in the expended exudate associated with excised pollen tubes are given in Figure 1, A and B, respectively. It should be emphasized that G-100-I is the polysaccharide fraction of stigmatic exudate. The low molecular weight fraction, G-100-II, was set aside and will be described separately (19). The profile of expended exudate reveals considerable fragmentation of high molecular weight substance but an examination of the lowest molecular weight fractions uncovered no free monosaccharides. Fractions from points 1 through 6 (Fig. 1B) were measured for total carbohydrate and the specific radioactivities of these fractions are given in Table III. Polysaccharides excluded from the gel (sample 1) contained high specific radioactivity, but subsequent samples from expended exudate partially retarded on the column exhibited a sharp drop due to dilution by unlabeled secretion product produced in the style during pollen tube development. Labarca and Loewus (unpublished observations) have noted that exudate produced by the stylar canal differs in molecular weight distribution from exudate recovered from the stigma in that it is richer in intermediate size polysaccharides which are partially retarded by Sephadex G-100 and is much poorer in very large macromolecules. As a result, samples 2, 3, and 4 have specific radioactivities far lower than one might expect if G-100-I were fragmented and uniformly diluted by unlabeled secretion product of the pistil.

Fractions in Figure 1B corresponding to a (void volume to point 1), b (point 2 to point 3), and c (point 4 to point 5) were pooled, hydrolyzed with acid, and analyzed for monosaccharides by paper chromatography. Results are given in Table IV. Similar patterns of ^{14}C distribution were found in hydrolysis products of b and c. The pattern in a is different, not only from those of b and c, but also from that of G-100-I. In G-100-I, most of the label remaining on the origin after chromatography in solvent A is glucuronic acid and galacturonic acid in the ratio 4:1 (18). Expended exudate from a appears to have been enriched in these acidic residues, possibly as the result of fragmentation of some G-100-I molecules which were rich in galactose and arabinose residues. The latter now appear in regions of the profile from which b and c were taken. Mannose and xylose (or fucose, the latter two sugars are not resolved by solvent A) remain in polysaccharide recovered in a. Rhamnose is distributed throughout the profile. Compounds labeled with ^{14}C that eluted from the paper during development with solvent A were not identified. Galactose, arabinose and rhamnose regions of paper chromatograms were eluted separately, converted to their trimethylsilyl ethers and analyzed by GLC. In each sample, a, b, and c, the ratio of specific radioactivities for galactose-arabinose-rhamnose was 4:2:1.

Further fractionation of G-100-I on Sephadex G-200 separates this polysaccharide mixture into a fraction that is partially excluded from the gel and a much larger fraction that is retained (18). Samples from these two fractions, which we have identified as G-200-I and G-200-II, respectively, were hydro-

Table I. *Distribution of Label from Injected Exudate in Pistil and Pollen*

	Dissecting Medium	Pollen Tubes	Style	Stigma and Pollen Grains	Total
	%				
Expended exudate (washes)	15.9	6.1	6.0	29.7	57.9
Tissues[1]					
Supernatant		2.4	2.7	5.3	10.4
Residue		3.7	1.5	5.7	10.9
Label not recovered					20.8

[1] Tissues were ground to a fine suspension in water and centrifuged at 1000*g*.

Table II. *Total Carbohydrate Content and Specific Radioactivities of Injected Exudate, Expended Exudate, and Pollen Tube Wall*

	Total Carbohydrate[1]	Specific Radioactivity
	mg	*cpm/mg*
Injected exudate	25	920,000
Expended exudate (washes)		
Dissecting medium	34	99,200
Stigma	61	109,600
Style	14	100,300
Pollen tubes	10	130,600
Pollen tube walls	32[2]	25,600

[1] Total carbohydrate as determined by phenol-sulfuric acid assay (7) with exception as noted.

[2] Dry weight of pollen tube walls, recovered as described in the text.

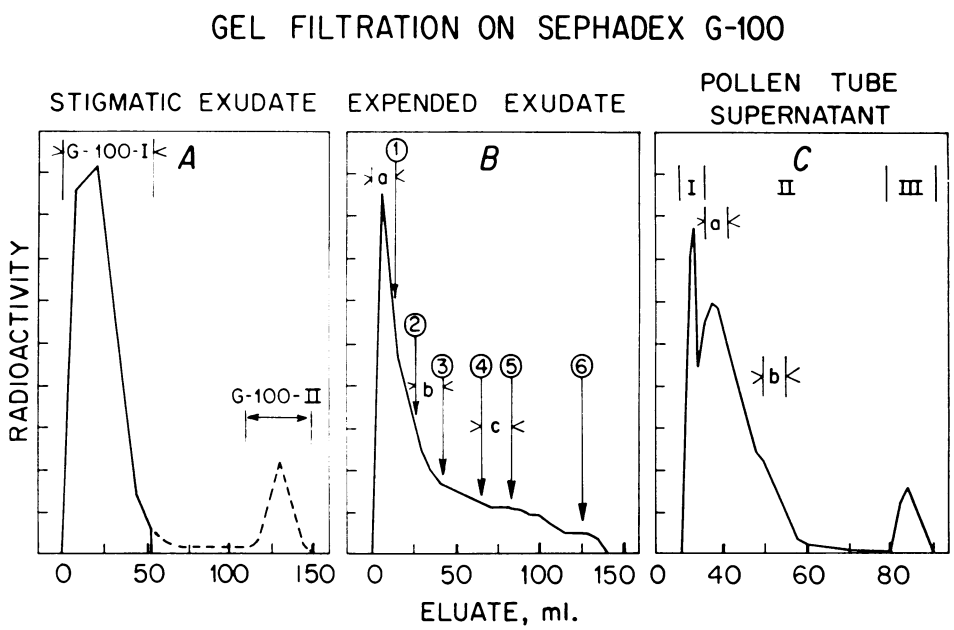

FIG. 1. Gel filtration profiles. Experimental details are provided in the text. Profile A: stigmatic exudate from pistils labeled with D-glucose-1-^{14}C. Only the G-100-I portion corresponding to the first 52 ml of eluate was pooled and used for injection into detached, unlabeled pistils. Profile B: expended exudate recovered from the 0.3 M pentaerythritol rinses of excised pollen tubes. Specific radioactivities were determined at points identified by numbered arrows. Carbohydrate analyses were run on pooled samples from fractions in region a, b and c. Profile C: high speed supernatant fraction of the pollen tube cytoplasm. Carbohydrate analyses were run on samples from fractions I, II, a and b, and III.

Table III. *Total Carbohydrate and Specific Radioactivity of Selected Fractions from Gel Filtration of Expended Exudate (Pollen Tube Washes)*

Sample[1]	Total Carbohydrate	Specific Radioactivity
	μg/ml	*cpm/mg*
1	43	174,900
2	32	93,800
3	26	73,800
4	16	67,500
5	11	85,500
6	10	54,000

[1] See Figure 1B for fractions sampled.

lyzed with 2 M TFA and then separated into acidic and neutral components by ion exchange chromatography (18). The neutral fractions of both G-200-I and G-200-II were converted to their alditol acetates (1) and separated by GLC. Their chromatograms together with that of a mixture of authentic alditol acetates are shown in Figure 2. Myoinositol hexaacetate is the reference marker. The retention times of components in the standard mixture, relative to myoinositol hexaacetate are: rhamnitol, 0.23; arabinitol, 0.30; xylitol, 0.38; mannitol, 0.68; galactitol, 0.73; and glucitol, 0.833, all as their fully acetylated esters. Fucitol pentaacetate, which appears as a shoulder on the trailing edge of rhamnitol pentaacetate, is not included in this standard mixture. Under the same chromatographic conditions, hydrolysis of G-200-I released neutral sugars whose alditol acetates gave a chromatogram with 6 peaks corresponding to rhamnose (peak 1), arabinose (peak 2), xylose (peak 3), mannose (peak 4), galactose (peak 5), and glucose (peak 6). An unknown peak, U, was also found in the trailing edge of the solvent peak in both fractions, G-200-I and G-200-II. G-200-II lacked peaks 3 and 4 corresponding to xylose and mannose. Fucose was present as a shoulder on the rhamnose peak in both fractions.

These results suggest that G-100-I consists of at least two groups of polysaccharides, one a high molecular weight component that is partially excluded from Sephadex G-200 and contains small amounts of xylose and mannose, while the second is a lower molecular weight component that is retarded by Sephadex G-200 and is devoid of xylose and mannose. It is the G-200-I fraction that appears to be most resistant to degradation when G-100-I is utilized by growing pollen tubes. The G-200-II fraction, which approaches the arabinogalactans in structure, appears to be more susceptible to breakdown in the pollinated pistil.

Pollen Tubes. The distribution of ^{14}C in pollen tube fractions is given in Table V. The low speed residue is predominantly tube wall carbohydrate. The low speed supernatant, referred

Table IV. *Distribution of Label in Fractions from Expended Exudate (Pollen Tube Washes) after Acid Hydrolysis*

The fractions reported in this table were selected from regions of the gel filtration profile as seen in Figure 1B. After hydrolysis in 2 M trifluoroacetic acid, each sample was separated into constituent sugars by paper chromatography in solvent A. Sugars are listed in this table in order of increasing R_F.

	Fraction			G-100-I
	a	b	c	
	%			
Origin	30.9	6.1	5.8	11.8
Galactose	35.2	62.8	65.2	58.4
Mannose	8.5	0.7		1.2
Arabinose	11.3	21.3	20.8	15.1
Xylose (Fucose)	6.0	0.3		0.9
Rhamnose	1.1	3.3	2.9	4.9
Eluate[1]	7.1	5.4	5.4	7.8

[1] Eluted from paper in the course of separation of other constituents.

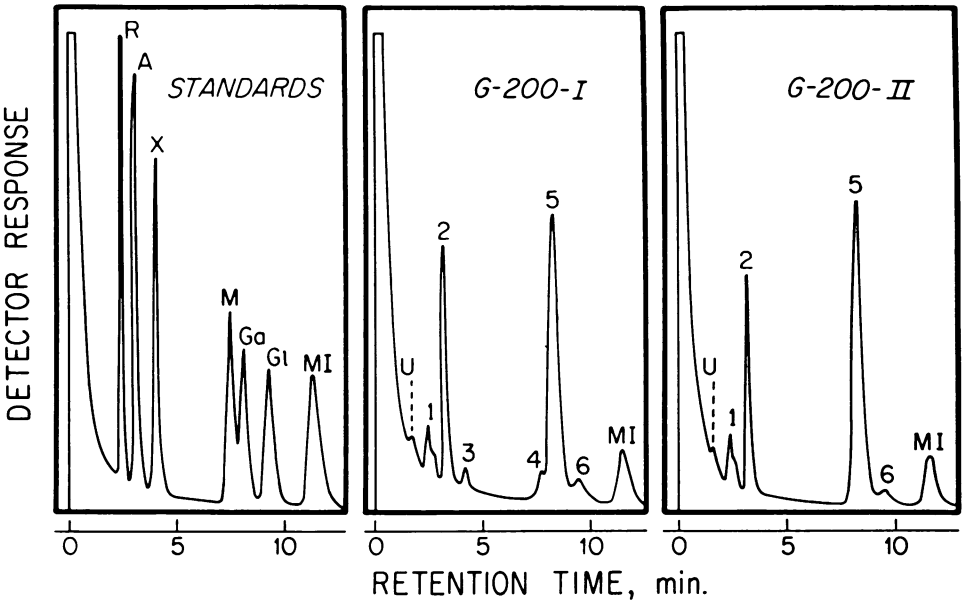

FIG. 2. Gas-liquid chromatography of the alditol acetates prepared from neutral sugars in the hydrolyzates of the two polysaccharide components, G-200-I and G-200-II, of the high molecular weight portion of stigmatic exudate, G-100-I. Comparisons may be made with the chromatogram of mixed standards on the left. The lettered peaks are rhamnitol, R; arabinitol, A; xylitol, X; mannitol, M; galactitol, Ga; glucitol, Gl and myoinositol, MI. See text for retention times, identity of numbered peaks in G-200-I and G-200-II and conditions of separation.

to hereafter as pollen tube cytoplasm, was fractionated at a higher speed of centrifugation into a clear supernatant and a pellet. Gel filtration on Sephadex G-100 of the high speed supernatant gave the radioactivity profile shown in Figure 1C. Fractions were pooled into three samples, I (polysaccharide excluded from the gel), II (polysaccharides partially retained during elution with 0.1 M acetic acid), and III (low molecular weight substances). Portions of fraction II corresponding to fractions in fractions II a and b (Fig. 1C) were withheld and examined separately to determine the degree of homogeneity of labeled residues throughout this broad radioactive band. The distribution of label was determined in each sample as well as the cytoplasmic pellet. With the exception of cytoplasmic fraction III, all were hydrolyzed with acid prior to paper chromatography in solvent A. Results are given in Table VI.

Distribution of ^{14}C among sugar residues of cytoplasmic fraction I is strikingly similar to that found in fraction a of expended exudate from excised pollen tube washes. The presence of significant amounts of mannose and xylose, far more than was found in other cytoplasmic fractions, strengthens the view that this fraction resembles the G-200-I component of G-100-I and that this component has resisted fragmentation during its passage into the pollen tube.

Distribution of ^{14}C within cytoplasmic fraction II at points a and b was almost identical. The ^{14}C pattern in cytoplasmic fraction II resembles fractions b and c of expended exudate in that it is rich in galactose and arabinose, low in uronic acids, and almost devoid of mannose and xylose. Results indicate that fraction II, which contained over 50% of the ^{14}C recovered in the pollen tube cytoplasm, moved from its location in the stylar canal as partially fragmented G-100-I into the pollen tube cytoplasm with little breakdown beyond that already noted for expended exudate.

Analysis of fraction III and the cytoplasmic pellet showed that extensive metabolism of exudate polysaccharide does take place within the pollen tube. Direct chromatography of cytoplasmic fraction III in solvent A revealed that 60% of the label was present in free monosaccharides. Undoubtedly, a portion of the ^{14}C that remained at the origin was also free uronic acids although further identification was not made due to the small amount of material available. The free sugars were identified as galactose, glucose, mannose, arabinose, xylose, and rhamnose. Free glucose accounted for one-half of the ^{14}C found in these sugars. Its identity was confirmed by the appearance of two peaks corresponding to trimethylsilyl ethers of α and β glucose when the glucose spot was eluted from the paper chromatogram, trimethylsilylated and assayed by GLC. The specific radioactivities of glucose, galactose, and arabinose as their trimethylsilyl ethers were measured after separation of these sugars by paper chromatography. Values fell within the range of 110,000 to 140,000 cpm/mg of sugar, similar to values obtained from the bulk of expended exudate (Table II).

Acid hydrolysis of the cytoplasmic pellet released 78% of its ^{14}C as soluble carbohydrate. Another 8% was solubilized with cellulase, and exhibited the same chromatographic properties as hydrolysis products of cellulose. The acid hydrolyzate was chromatographed on paper in solvent A. The distribution of ^{14}C in neutral sugars is given in Table VI. Glucose contained 16% of the label present in this hydrolyzate. The labeled glucose content of the pellet was probably greater as suggested by the ^{14}C released by cellulase. Labeled components that remained on the origin in solvent A were eluted and chromatographed in solvent B. Both glucuronic acid and galacturonic

Table V. *Label in Pollen Tube Fractions*

Fractions	Distribution
	%
Low speed residue (1,000*g*)	60
High speed supernatant[1]	
Fraction I	6
Fraction II	22
Fraction III	2
High speed pellet (20,000*g*)	10

[1] Fractionated by gel filtration on Sephadex G-100. See Figure 1C.

Table VI. *Distribution of Label in Components of Pollen Tube Fractions*

	Cytoplasm					Tube Wall[1]
	I	IIa	IIb	III	Pellet[1]	
	%					
Origin	35	8	7	40	24	27
Galactose	30	63	64	12	30	10
Glucose	1	1		25	16	19
Mannose	8	1		2	4	2
Arabinose	10	18	22	6	12	30
Xylose (Fucose)	7	1		2	2	3
Rhamnose	1	3	3	4	6	5
Eluate	7	6	4	9	6	3

[1] Only radioactivity released into solution by acid hydrolysis is presented here.

acid were detected as well as two unknown components that moved more slowly than either of the free uronic acids.

Pollen tube walls were hydrolyzed with TFA in two steps. The first treatment released 71.5% of the bound ^{14}C and the second, another 7.5%. The results presented in the last column of Table VI represent the sum of chromatographic separations involving these two acid hydrolyses. Treatment with cellulase solubilized the remaining 21% of the label, which had the same chromatographic pattern in solvent A as hydrolysis products of cellulose.

A significant portion of the ^{14}C in tube wall polysaccharides was present as glucose; 19% of the free sugar released by acid hydrolysis and most, if not all, of the ^{14}C released by cellulase. Since 60% of the ^{14}C in excised pollen tubes was tube wall bound, it can be estimated that one-fourth of all the ^{14}C recovered in these tubes (cytoplasm + wall) had been converted to glucose. This observation provides a minimal figure for the portion of G-100-I that underwent degradation to the level of hexose phosphate followed by interconversion to glucose-containing polysaccharides. G-100-I contains only a trace of glucose (Fig. 2).

Labeled components of the tube wall hydrolysis mixture that remained on the origin after chromatography in solvent A were separated with solvent B. At least one-half of the ^{14}C was present as galacturonic acid and a much smaller amount as glucuronic acid. The remaining label was located in a slow moving spot close to the origin. The galacturonic acid accounts for about 7% of the ^{14}C incorporated into the wall. Although the labeled G-100-I used in this experiment contained about 12% uronic acid, most of it was glucuronic acid (9–10%) (18). In the conversion of G-100-I into pollen tube wall polysaccharide, either galacturonic acid residues were utilized selectively or a portion of the glucuronic acid was interconverted to galacturonic acid during incorporation.

All of the arabinose in the ^{14}C distribution pattern given in Table VI for tube wall polysaccharide was released by the first treatment with acid. The proportion of labeled arabinose to other sugars in tube wall was about twice that found in G-100-I.

DISCUSSION

Background of the Present Study. Myoinositol is a specific precursor of uronic acids and pentose units in pectic substance of plant cell walls (23), including pollen tube walls (13, 31, 36). Pollen grains which have germinated in artificial medium containing pentaerythritol, a metabolically inert substitute for glucose or sucrose (6), must rely upon carbon reserves stored in the grain for pectin biosynthesis. If glucose is added, a portion of it will be converted to myoinositol and then to pectic substance through the myoinositol pathway (24). The enzyme that converts glucose 6-P to myoinositol is present in *L. longiflorum* pollen and there is a 3-fold increase in the activity of this enzyme during the first hour of germination (28). If myoinositol is added to the medium, it is utilized for pectin biosynthesis directly (13). Medium containing 0.1 mM myoinositol and 5 mg of pollen/ml is depleted of 80% of its myoinositol within 6 hr and most of this is converted to pectic substance.

Detached pistils of *L. longiflorum* utilize glucose or myoinositol for the biosynthesis of cell wall polysaccharides and pistil secretion products (12, 14, 15, 18, 23). When one of these compounds is supplied in labeled form to the cut surface of the pedicel, it is transported through vascular strands into the distal part of the style and into the stigma, especially those tissues involved in active secretion of exudate. The exudate appears to be a glycoprotein (17, 18, 23), although specific information on this point must still be sought. Its carbohydrate components resemble the class of plant polysaccharides known as gum exudates (3). The exudate from *L. longiflorum* pistils also contains small quantities of low molecular weight compounds but the precise nature of these compounds still awaits investigation.

If stigmatic exudate from *L. longiflorum* is added to pentaerythritol medium in which pollen grains are suspended, the number of grains that will germinate increases and a noticeable stimulation of tube elongation is detected (27). If labeled stigmatic exudate or its polysaccharide component, G-100-I, is added to a system such as that just described, a portion of the label is recovered as pectic substance of pollen tube walls (12). Similarly, if the labeled exudate or G-100-I is injected into the stylar canal of unlabeled detached pistils and the pistils are pollinated, pollen tubes recovered from such pistils are labeled and the label is primarily in pectic substance. Ascher and Drewlow (2) have injected stigmatic exudate into the stylar canal of *L. longiflorum* pistils and found no adverse effects on pollen growth.

The present study examines the nutritional relationship between developing pollen tubes and pistil secretion product in greater detail and makes use of experience gained in the preliminary study. The nutritional dependence of growing pollen tubes on the pistil has been examined by others (8, 9, 16, 35) but except for one, these studies focus on pollen tube penetration in solid styles where pistil secretion is obscured by the morphology of the tissue. In none of these studies has the specific nature of the carbon source supplied by the pistil been characterized.

The decision to use labeled glucose rather than labeled myoinositol in this study was based on economic considerations, but hindsight suggests that this has been a rather fortunate choice. The polysaccharide component of stigmatic exudate is essentially devoid of glucose residues. When detached pistils are labeled with D-glucose-1-^{14}C, all carbohydrate residues in the exudate become labeled, the greatest part appearing in galactose. Thus, the fate of all components of G-100-I can be followed during pollen tube wall formation. Had labeled myoinositol been used instead of labeled glucose, more label would have appeared in uronic acid and pentose units of G-100-I (18) but label would appear in hexose residues only as a result of recycling of pentose through pentose phosphate back into the hexose pool (26) or as the result of label from decarboxylation being fixed photosynthetically. The tissues involved in exudate production in the pistil are unusual in that polysaccharide production is maintained at a very high level and most of the carbon in the metabolic pool is directed

toward this process (30, 34). By contrast, when pollen is germinated in artificial medium containing labeled myoinositol, there is no detectable recycling of label back into the hexose pool during the first 8 hr of tube growth (13). Galacturonic acid, arabinose and xylose are the only pectic units labeled. Use of labeled myoinositol may be appropiate for studies of pectin biosynthesis in pollen tubes grown *in vitro* but extension of this approach to *in vivo* studies will require more information regarding the biochemistry of exudate secretion.

Dilution of G-100-I. Comparison of specific radioactivities of injected G-100-I and washed pollen tube walls provides a minimal value of the amount of carbohydrate removed from the canal and incorporated into tube wall during pollen tube growth. In the present experiment not all injected label remained in the canal. About 15% appeared in stylar and stigma tissues. Another 21% was not recovered, presumably that portion of injected G-100-I that reached the ovary plus the portion lost metabolically as CO_2. The remaining 64% appeared in washes of dissected tissues (58%) and excised pollen tubes (6.1%). On the basis of carbohydrate analyses of exudate in washes of dissected tissues, G-100-I was diluted 5-fold by endogenous secretion product from the unlabeled pistils. If it is assumed that only 60 to 70% of the labeled G-100-I was available during pollen tube growth, then the seven- to ninefold dilution of specific radioactivity observed in expended exudate (Table II) is understandable.

Expended exudate adhering to dissected stylar tissues or present in dissecting medium averaged 1.0×10^5 cpm/mg of carbohydrate. A significantly higher value, 1.3×10^5 cpm/mg, was found in the small quantity of exudate clinging to excised pollen tubes. This higher value is not surprising since the tubes penetrated injected exudate while the bulk of exudate eventually recovered came from actively secreting unlabeled tissues.

Pollen tube wall, after exhaustive washing to remove all water-soluble material, had a specific radioactivity of 25,600 cpm/mg of dry tube wall. Compared to that in the bulk of expended exudate, this is a 4-fold dilution. In other words, at least one-fourth of the carbohydrate incorporated into tube wall substance during pollen tube growth was derived from diluted G-100-I. This is a minimal value. Recent experiments which will be reported shortly (C. Labarca and F. Loewus) indicate that nutritional dependence may account for up to 70% of the pollen tube.

Changes in the Polysaccharide Composition of G-100-I following Injection. When it became evident that transfer of labeled carbohydrate from injected G-100-I accounted for a significant part of newly formed tube wall, our interest shifted to the nature of the carbohydrate involved in this transfer. G-100-I consists of at least two high molecular weight components which are partially separated on Sephadex G-200 gel (18). The highest molecular weight fraction, G-200-I, accounts for 15% of G-100-I and contains 30% of the protein in G-100-I. Most of the mannose and xylose in G-100-I is also found in G-200-I (Fig. 2). When G-100-I is injected into the stylar canal of detached pistils followed by pollination, there is fragmentation of part of the G-100-I but this breakdown of polysaccharide appears to be limited largely to G-200-II, the portion of G-100-I that is retarded during gel filtration, while the G-200-I component resists fragmentation. Polysaccharide degrading enzymes have been found in diffusates of pollen grains and pistil tissue (10, 11, 21) but the functional role of such enzymes in modifying pistil secretion products must still be examined.

Utilization of G-100-I for Pollen Tube Formation. To prevent tube breakage and loss of tube contents, pollen tubes were recovered from the pollinated pistils in the presence of 0.3 M pentaerythritol. Successive rinsing with fresh pentaerythritol effectively removed all traces of expended exudate adhering to the outer surfaces of the tubes. Tube contents were separated into a high speed soluble cytoplasmic fraction and a high speed cytoplasmic pellet. The soluble fraction contained a high molecular weight component, I, similar to the G-200-I fraction of G-100-I as well as fraction a of expended exudate. The soluble cytoplasm also contained a second polysaccharide component, II, which resembled G-200-II and fractions b and c of expended exudate. About 28% of the ^{14}C recovered in the washed pollen tubes was present in these two polysaccharide fractions. The similarities noted here between injected G-100-I polysaccharides and those in expended exudate or tube cytoplasm suggest that a significant part of the labeled polysaccharides has entered the tubes without undergoing complete degradation to monosaccharide constituents. An engulfing process such that macromolecular components of pistil secretion product are swept into tube cytoplasm by growing pollen tube tips has been proposed by Rosen (29) on the basis of electron microscope studies.

Evidence that a portion of the labeled material incorporated into pollen tube cytoplasm had undergone extensive metabolism is seen in the pattern of ^{14}C distribution in fraction III and the high speed pellet. Both are rich in labeled glucose, which is not a significant constituent of G-100-I. It is most probable that galactose residues, released by metabolic breakdown of labeled polysaccharide within the cytoplasm, are interconverted to glucose residues. It was demonstrated earlier (12) that pollen tubes grown in pentaerythritol medium containing labeled stigmatic exudate or grown in detached pistils injected with labeled stigmatic exudate will incorporate label into tube wall polysaccharides. Moreover, the process involves interconversion of labeled galactose residues of stigmatic exudate to labeled glucose residues in tube wall polysaccharides. The discovery of labeled glucose in the high speed pellet from the tube cytoplasm, a membrane-rich fraction, is in accord with the view that tube wall formation is preceded by polysaccharide biosynthesis in secretory vesicles within the cytoplasm (33).

About one-fourth of the ^{14}C in the pollen tubes had been converted to glucose residues, evidence that G-100-I is utilized by growing pollen for glucan biosynthesis as well as pectin biosynthesis. Part of the tube wall glucose was released by acid hydrolysis; possibly this corresponds to callose. There was a further release of glucose-rich fragments after cellulase treatment. Since the high speed cytoplasmic fraction II accounted for the bulk of polysaccharide label present in tube cytoplasm, the suggestion is offered that this fraction provides most of the galactose required by the hexose pool for interconversion to glucose polymer.

At least 50%, possibly more, of the labeled uronic acid in pollen tube wall polysaccharides was galacturonic acid. The ratio of glucuronic acid to galacturonic acid in G-100-I was 4:1. Unfortunately, the corresponding ratios for G-200-I and fraction I of pollen tube cytoplasm were not determined, so it is not possible to determine if selective enrichment of galacturonic acid occurred as polysaccharide moved into pollen tubes or if interconversion of glucuronic acid to galacturonic acid accompanied the process of tube wall formation. The presence of significant amounts of mannose and xylose in the tube wall hydrolysate suggests that part of the G-200-I component of G-100-I was utilized for tube wall biosynthesis, possibly without fragmentation.

Both polysaccharide components of G-100-I contained similar ratios of galactose to arabinose (Fig. 2) but the bulk of the carbohydrate in G-100-I was in the lower molecular weight material and this portion of G-100-I appears to be the major source of arabinose units for pollen tube wall polysaccharides, via cytoplasmic fraction II. The enzymes needed for direct

incorporation of L-arabinose into cell wall pectin are present (4, 25). The unusually low level of labeled arabinose noted in the low molecular weight fraction of the cytoplasm, III, indicates that arabinose residues are used for pectin biosynthesis almost as rapidly as such residues are released during the metabolic breakdown of cytoplasmic fraction II.

In conclusion, it has been demonstrated that injected G-100-I fraction of stigmatic exudate provides a significant portion of the carbohydrate requirement for pollen tube wall biosynthesis. The process involves actual transfer of polysaccharide fragments from the stylar canal into pollen tube cytoplasm followed by breakdown and resynthesis within the cytoplasm and eventual deposition of new polysaccharides which contain monosaccharide residues and, possibly, larger fragments of G-100-I into the growing tube wall. Some fragmentation of G-100-I within the canal may lead to release of small oligosaccharides or monosaccharides which can be used by the pollen tube, but results suggest that this source of carbohydrate makes a smaller contribution to the nutritional needs of the developing tube than G-100-I fragments metabolized within the tube.

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LITERATURE CITED

1. Albersheim, P., D. J. Nevins, P. D. English, and A. Karr. 1967. A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohyd. Res. 5: 340–345.
2. Ascher, P. D. and L. W. Drewlow. 1970. Stigmatic exudate: a potential carrier of exogenous substances into the style of *Lilium longiflorum* Thunb. J. Amer. Soc. Hort. Sci. 95: 706–708.
3. Aspinall, G. O. 1969. Gums and mucilages. Advan. Carbohyd. Chem. Biochem. 24: 333–379.
4. Dashek, W. V., H. I. Harwood, and W. G. Rosen. 1971. The significance of a wall-bound, hydroxyproline-containing glycopeptide in lily pollen tube elongation. *In:* J. Heslop-Harrison, ed., Pollen: Development and Physiology. Butterworths, London, pp. 194–200.
5. Dashek, W. V., H. R. Thomas, and W. G. Rosen. 1971. Secretory cells of lily pistils. II. Electron microscope cytochemistry of canal cells. Amer. J. Bot. 50: 909–920.
6. Dickinson, D. B. 1968. Rapid starch synthesis associated with increased respiration in germinating lily pollen. Plant Physiol. 43: 1–8.
7. Dubois, M., K. A. Gillis, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–356.
8. Esau, K. 1965. Plant Anatomy, 2nd ed. John Wiley and Sons, Inc. New York.
9. Kanno, T. and K. Hinato. 1969. An electron microscopic study of the barrier against pollen-tube growth in self-incompatible Cruciferae. Plant Cell Physiol. 10: 213–216.
10. Konar, R. N. and R. G. Stanley. 1969. Wall-softening enzymes in the gynoecium and pollen of *Hemerocallis fulva*. Planta 84: 304–310.
11. Knox, R. B. and J. Heslop-Harrison. 1969. Cytochemical localization of enzymes in the wall of the pollen grain. Nature 223: 92–94.
12. Kroh, M., C. Labarca, and F. Loewus. 1971. Use of pistil exudate for pollen tube wall biosynthesis in *Lilium longiflorum*. *In:* J. Heslop-Harrison, ed., Pollen: Development and Physiology. Butterworths, London. pp. 273–278.
13. Kroh, M. and F. Loewus. 1968. Biosynthesis of pectic substance in germinating pollen: labeling with myoinositol-2-^{14}C. Science 160: 1352–1354.
14. Kroh, M., H. Miki-Hirosige, W. Rosen, and F. Loewus. 1970. Inositol metabolism in plants. VII. Distribution and utilization of label from myoinositol-U-^{14}C and -2-^{3}H by detached flowers and pistils of *Lilium longiflorum*. Plant Physiol. 45: 86–91.
15. Kroh, M., H. Miki-Hirosige, W. Rosen, and F. Loewus. 1970. Incorporation of label into pollen tube walls from myoinositol-labeled *Lilium longiflorum* pistils. Plant Physiol. 45: 92–94.
16. Kroh, M. and A. J. Munting. 1967. Pollen germination and pollen tube growth in *Diplotaxis tenuifolia* after cross-pollination. Acta Bot. Neer. 16: 182–187.
17. Labarca, C., M. Kroh, M-S. Chen, and F. Loewus. 1969. Studies on stigmatic exudate from *Lilium longiflorum*. Plant Physiol. 44: S2.
18. Labarca, C., M. Kroh, and F. Loewus. 1970. The composition of stigmatic exudate from *Lilium longiflorum*. Labeling studies with myo-inositol, D-glucose and L-proline. Plant Physiol. 46: 150–156.
19. Labarca, C. and F. Loewus. 1970. Bound forms of myo-inositol in stigmatic exudate of *Lilium longiflorum*. Plant Physiol. 46: S27.
20. Linskens, H. and M. Kroh. 1967. Incompatibility of seed plants. *In:* W. Ruhland, ed., Vol. 18, Encyclopedia of Plant Physiology, Springer, New York. pp. 506–530.
21. Linskens, H. and M. Kroh. 1970. Regulation of pollen tube growth. *In:* A. A. Moscona, ed., Current Topics in Developmental Biology, Vol. 5. Academic Press, New York. pp. 89–113.
22. Loewus, F. 1961. The use of bis-(2-alkoxyethyl)ethers as antifreeze in naphthalene-1,4-dioxane scintillation mixtures. Intern. J. Appl. Rad. Isot. 12: 6–9.
23. Loewus, F. 1969. Metabolism of inositol in higher plants. Ann. N.Y. Acad. Sci. 165: 577–598.
24. Loewus, F. 1971. Carbohydrate interconversions. Annu. Rev. Plant Physiol. 22: 337–364.
25. Loewus, F. and R. Jang. 1958. The conversion of C^{14}-labeled sugars to L-ascorbic acid in ripening strawberries. III. Labeling patterns from berries administered pentose-1-C^{14}. J. Biol. Chem. 232: 521–532.
26. Loewus, F. and S. Kelly. 1963. Inositol metabolism in plants. I. Labeling patterns in cell wall polysaccharides from detached plants given myo-inositol-2-t or -2-^{14}C. Arch. Biochem. Biophys. 102: 96–105.
27. Loewus, F. and C. Labarca. 1970. Substances in stigmatic exudate promoting germination and growth of *Lilium* pollen. Fed. Proc. 29: 901.
28. Loewus, M. W. and F. Loewus. 1971. The isolation and characterization of D-glucose 6-phosphate cycloaldolase (NAD-dependent) from *Acer pseudoplatanus* L. cell cultures. Plant Physiol. 48: 255–260.
29. Rosen, W. G. 1971. Pollen tube growth and fine structure. *In:* J. Heslop-Harrison, ed., Pollen: Development and Physiology. Butterworths, London. pp. 177–185.
30. Rosen, W. G. and H. R. Thoams. 1970. Secreting cells of lily pistils. I. Fine structure and function. Amer. J. Bot. 57: 1108–1114.
31. Stanley, R. G. and F. Loewus. 1964. Boron and myo-inositol in pollen pectin biosynthesis. *In:* H. Linskens, ed., Pollen Physiology and Fertilization. North Holland, Amsterdam. pp. 128–136.
32. Turner, J. C. 1968. Triton X-100 scintillant for carbon-14 labelled materials. Intern. J. Appl. Rad. Isot. 19: 557–563.
33. VanDerWoude, W. J., D. J. Morré, and C. E. Bracker. 1971. Isolation and characterization of secretory vesicles in germinated pollen of *Lilium longiflorum*. J. Cell Sci. 8: 331–351.
34. Vasiliev, A. E. 1970. The ultrastructure of stigmatoid cells in *Lilium*. Soviet Plant Physiol. 17: 1240–1247.
35. Yamada, Y. 1965. Studies on the histological and cytological changes in the tissues of pistil after pollination. Jap. J. Bot. 19: 69–82.
36. Young, L. C. T., R. G. Stanley, and F. Loewus. 1966. Myo-inositol-2-t incorporation by germinating pollen. Nature 209: 530–531.