Plant Physiol. (1970) 45, 86–91

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*[1]

Received for publication August 21, 1969

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ABSTRACT

When detached flowers or isolated pistils of *Lilium longiflorum* are given myoinositol-U-^{14}C or -2-^{3}H as dilute solution through the severed pedicel, label is quickly distributed by the vascular system. In the case of pistils, a pattern of labeling in ovary, style, and stigma is obtained which indicates that products of myoinositol metabolism are utilized in the biosynthesis of exudate (secretion product) of the stigma and style as well as for components of pistil cell walls. Pollination had no discernible effect on labeling pattern.

Polysaccharides account for a major part of pollen tube walls. Since the reserves of the pollen grain are limited and a relatively large amount of tube wall is synthesized during the growth of the tube, it appears reasonable to assume that a part of this material derives from carbohydrates provided by the pistil. Pollen tubes growing in pistils that contain a solid transmitting tissue in the styles are in contact with the intercellular pectin-containing material. Electronmicroscopical studies have shown that pollen tubes during their growth through the transmitting tissue hydrolyze part of this wall material, and it is assumed that the hydrolysis products are metabolized by the growing tubes (4, 5, 8, 14). Pollen tubes growing in pistils containing a style canal, as in the case of *Lilium*, are in contact with secretory cells which line the surfaces of both stigma and canal and with the secretion product of these cells (18). The wall material as well as the secretion product could serve as a source of carbohydrate for growing pollen tubes (7). Rosen and Gawlik (17) proposed a heterotrophic phase of tube growth in *Lilium*. They suggested that a portion of the tube wall substance is derived from secretory products of the style.

A suitable method of labeling polysaccharide material of pistils is a prerequisite for testing the ability of pollen tubes to take up labeled substances from the pistil and to utilize such substances for cell wall synthesis. Myoinositol, which was reported by Loewus (11, 12) to be a specific precursor of pectic substances in several plant materials, was used as the source of label. In the experiments described in this report, MI[4] was applied to (*a*) detached *Lilium longiflorum* flowers via the cut pedicel, (*b*) detached pistils via the cut pedicel, and (*c*) styles with stigma via the cut style. The effects of different labeling procedures and of pollination on the uptake and distribution of label into selected flower parts were determined.

MATERIAL AND METHODS

Flowers of Easter lily, *L. longiflorum* (cv. Ace, Croft and Georgia), from plants grown under greenhouse conditions were used in this study. Pollen was collected on the day of anther dehiscence and stored in covered Petri dishes at 4° until used. Flower buds to be used for labeling experiments were excised 1 day before anthesis, and the cut stem was immediately immersed in water and then recut under water. Buds were kept 24 hr at 25° under constant light in a covered glass container prior to labeling.

Labeled Compounds. In all experiments involving chemical determination of labeled compounds, the plant material was labeled with MI-U-^{14}C. In autoradiographic studies MI-2-^{3}H was used. It was known from previous studies (10, 13) that tritium attached to carbon 2 of MI is stereospecifically conserved in pentose and cell wall pectic substance of plant tissues thus labeled. MI-U-^{14}C (324 c/mole) was purchased from Amersham/Searle. Vial contents (50 μc) were taken up in 50% ethanol (1 ml) and stored at −15°. MI-2-^{3}H (approx. 25 μc/mg) was prepared by reduction of myoinososose-2 with sodium borohydride-^{3}H (15).

Labeling Procedure. The concentration of MI used in all experiments except those involving MI-2-^{3}H was 10^{-3} M. This concentration is optimal for maximum uptake and incorporation of label into pectic substance in pollen tubes grown *in vitro* (7). Flower parts were labeled by placing the cut surface of the pedicel of a flower or pistil, or the cut surface of a style with stigma in a small glass vial containing 10^{-3} M MI-U-^{14}C (50 μl, 0.5 μc). Severed flowers or flower parts, together with the "feeder" vial, were placed in covered glass containers. In experiments in which labeled exudate was collected, pistils were given 5 μc of MI-U-^{14}C. After the labeled solution had been taken up, distilled water was added to keep the cut surface submerged.

Pollination. The pistils were pollinated by applying 5 mg of pollen to the stigma, distributing it as uniformly as possible over the stigmatic surface.

Fresh Weight. Labeled flowers were sectioned into pedicel, ovary, style, stigma, stamens, and tepals and then weighed. The

[1] This investigation was supported, in part, by grants from the National Institute of General Medical Sciences, United States Public Health Service (GM-12422), and the National Science Foundation (GB-5402).

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[4] Abbreviation: MI: myoinositol.

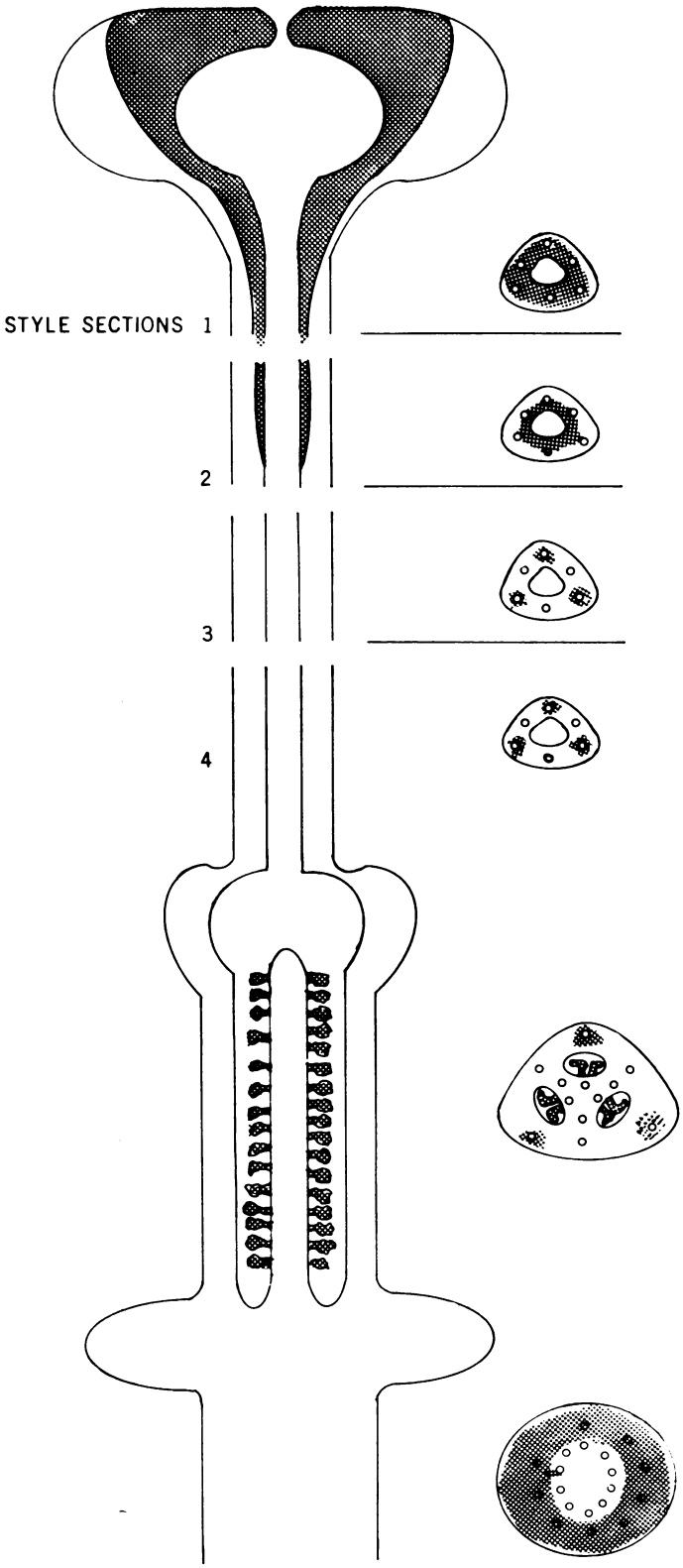

FIG. 1. Diagram of a detached pistil labeled with myoinositol-2-^{3}H showing the distribution pattern of silver grains (stippled areas) obtained from autoradiographs of selected sections.

style was subdivided into four or five 2-cm sections as measured from the stigma (Fig. 1).

Determination of Radioactivity. Labeled tissues were homogenized in a glass homogenizer (Kontes K-88545B) either in 80% ethanol or in water. An aliquot of the finely divided suspension was transferred to a centrifugal filter holder (Gelman No. 4305) fitted with a GA-4 membrane and spun for 10 min at 1000*g*. Each residue was washed twice (2.5 ml per wash) with 80% ethanol or water and then transferred together with its supporting membrane to a counting vial containing Cab-O-Sil plus naphthalene-dioxane liquid scintillation counting fluid. After membranes had dissolved in the counting fluid, vials were counted in a Packard liquid scintillation spectrometer (model 3324) at an efficiency of approximately 80% for ^{14}C.

Chemical Analysis of Radioactive Compounds. Washed residues were hydrolyzed with 0.5% pectinase (Rohm and Haas Pectinol R-10) plus 0.25% disodium EDTA (25°, 22 hr) or with 2 N trifluoracetic acid (121°, 1 hr) (1).

Separation of soluble compounds in the hydrolysate was accomplished by ion exchange chromatography, descending paper chromatography (ethyl acetate–pyridine–water, 8:2:1, v/v) for neutral sugars, and paper electrophoresis (16) for sugar acids. Arabinose was characterized by carrier dilution with authentic arabinose, oxidation to potassium arabinonate, and recrystallization to constant specific radioactivity. Galacturonic acid was also characterized by carrier dilution as its sodium calcium salt (13).

Autoradiography of MI-2-^{3}H-labeled Pistils. Pistils were fed MI-2-H^3 approx. 25 μc per pistil) via the cut pedicel. Forty-eight hours after labeling, pistils were cross-pollinated, and 72 hr after pollination they were fixed in ethanol-acetic acid (3:1, v/v, 24 hr), dehydrated in an ethanol-xylol series, and embedded in paraffin. Small punctures were made at intervals along the style and ovary to facilitate entrance of fixative into the style canal. Sections (5μ) were cut with a rotary microtome and stained with periodic acid-Schiffs reagent (3). Tritium was detected with Kodak NTB-2 emulsion.

RESULTS

Distribution of ^{14}C in Unpollinated and Pollinated Flower, Pistil, and Style-Stigma. Table I presents the amount of label recovered in the different parts of unpollinated and pollinated flowers, pistils, and style-stigmas 72 hr after label was introduced to the flower or flower part. A comparison of the ^{14}C content in stigma and style under different labeling conditions shows that the uptake of label into the stigma is independent of the labeling procedure. It varied between 6 and 10%. The amount of ^{14}C present in the styles, however, depended on whether label is introduced through the pedicel or directly into the style. Styles from flowers and pistils labeled through the pedicel contained, respectively, 13.3 and 19.4% of the label. Styles brought directly in contact with labeled solution contained an average of 91.2% of the label applied. Pollination did not affect the uptake and

Table I. *Distribution of Carbon-14 in Unpollinated (Experiment 1) and Pollinated (Experiment 2) Pistils, Flowers, and Style-stigmas*

Experiment[1]	Tissues labeled	Percentage of Total ^{14}C Recovered at End of Labeling Period					
		Stigma	Style	Ovary	Pedicel	Perianth	Stamen
1a	Pistil	8.4	19.4	7.5	64.7	...	...
1b	Flower	6.1	11.3	3.6	42.6	21.6	14.8
1c	Style-stigma	10.5	89.5	...	...	...	...
2a	Pistil	6.4	19.6	4.4	69.6	...	...
2b	Flower	9.8	15.3	4.0	34.9	32.3	12.7
2c	Style-stigma	7.0	93.0	...	...	...	...

[1] Pollination interval from 24 to 72 hr, ♀ cv. Ace, ♂ cv. Croft. The labeling period was 72 hr.

distribution of label in the different flower parts (compare experiments 1a, 1b, 1c with experiments 2a, 2b, 2c). During the labeling procedure it was observed that uptake of labeled MI through the pedicel of detached flowers and pistils was completed in about 3 hr, but uptake by excised style-stigmas took about 22 hr.

Distribution of ^{14}C in Style Sections of Unpollinated and Pollinated Flower, Pistil, and Style-Stigma. The style of *L. longiflorum* flowers is 8 to 11 cm in length. It was of interest to know if the label taken up by pistils is evenly distributed over the whole style length. To check this, styles were divided into 2-cm sections, section 1 being the one next to the stigma (Fig. 1).

Styles of labeled pistils or flowers generally accumulated ^{14}C label to a larger extent in the upper than in the lower style sections. In contrast to this, in labeled style-stigmas the style section in contact with the radioactive solution (section 4 or 5) was the most radioactive, and the amount of label decreased progressively with increasing distance up the style (away from the source of label). These points are illustrated in Table II, where the data are given in counts per minute per milligram fresh weight to minimize differences caused by variations in styles taken from different flowers.

Radioactivity not extracted by 80% ethanol may be regarded as a measure of that portion of MI which was converted to polysaccharide. In styles of unpollinated flowers and pistils an average of 17.5 and 29.8% of the counts per minute per unit fresh weight was found in insoluble residue. There was no correlation between the amount of label taken up by the style section and the amount that was incorporated. However, in detached unpollinated style-stigmas that contained an average of 44% of the label in ethanol-insoluble form, the progressive decrease in uptake of label from section 4 to 1 was accompanied by a decrease in ^{14}C bound to insoluble residue. Section 4, which contained the highest amount of label, showed also the highest percentage of incorporation (75%); section 1 with the lowest percentage of uptake showed the lowest incorporation (27%). Pollinated pistils had a distribution and incorporation of radioactivity in style sections (after removal of the pollen tubes) similar to that obtained in experiments with unpollinated material (compare experiments 1 and 2, Table II).

In the course of this study it was observed that style tissue, extracted and washed with 80% ethanol, was contaminated with labeled secretion product which is, in part, of carbohydrate nature (9). Because of this, radioactivity in the style residue included labeled polysaccharide from the secretion product, and therefore the amount of ^{14}C actually incorporated into style tissue was overestimated. Grinding and washing the style tissue in water revealed that the fraction of ^{14}C remaining in water-washed residues was only about one-half of that remaining after extraction with 80% ethanol (36% compared to 18%, Table III). Water-soluble polysaccharide and related carbohydrate substances normally associated with cell walls also may have been removed, a possibility that must be considered when comparing the extraction procedures.

Incorporation of ^{14}C in Stigmatic Exudate. To examine incorporation of label into secretion product (exudate) of detached pistils, two unpollinated Ace pistils were each given MI-U-^{14}C (5μc) through the pedicel. Stigmatic exudate was collected during the production period (5–6 days), pooled, and assayed for ^{14}C. As shown in Table IV, about 5% of the ^{14}C supplied to each pistil was recovered in secretion product. This represents a minimal figure since collection involved recovery of only clear droplets which appeared about to fall from the stigma. Much milky exudate on the stigma surface was not collected for fear of contaminating the exudate with adhering stigma cells.

Identification of Labeled Compounds. Gel filtration on Sephadex G-25 of ethanol-soluble label from the stigma of pistils fed MI-U-^{14}C revealed that 82% of the ^{14}C was present in low molecular weight compounds. Dowex 50-H^+ resin retained very little of the low molecular weight fraction, but Dowex 1-formate resin retained about 50%. The latter could be eluted with 1 N formic acid. In the neutral effluent, only MI was positively identified as a labeled constituent. It accounted for less than one-

Table II. *Distribution of Radioactivity in Style Sections of Unpollinated (Experiment 1) and Pollinated (Experiment 2) Pistils, Flowers and Style-stigmas*

Experiment[1]	Tissues Labeled	Style Section 1	2	3	4	5
		cpm/mg, fresh wt[2]				
1a	Pistil	600	440	510	490	320
		(20)	(16)	(13)	(18)	(12)
1a′	Pistil	430	500	320	220	140
		(21)	(16)	(22)	(9)	(28)
1b	Flower	310	230	150	110	100
		(16)	(21)	(20)	(27)	(30)
1b′	Flower	130	190	130	100	80
		(31)	(31)	(38)	(40)	(44)
1c	Style-stigma	1130	1540	2300	3200	...
		(27)	(34)	(39)	(75)	
2a	Pistil	240	450	420	380	250
		(33)	(20)	(9)	(16)	(33)
2a′	Pistil	120	80	70	60	50
		(33)	(25)	(33)	(33)	(40)
2b	Flower	620	420	270	180	120
		(13)	(14)	(11)	(17)	(17)
2b′	Flower	130	120	130	100	50
		(23)	(18)	(46)	...	(50)
2c	Style-stigma	460	950	1090	4250	4620
		(26)	(26)	(30)	(38)	(85)

[1] Pollination interval from 24 to 72 hr, ♀ cv. Ace, ♂ cv. Croft. The labeling period was 72 hr.

[2] Sections (2 cm) are numbered from stigma toward ovary. Numbers in parentheses record the per cent of ^{14}C not extracted by 80% ethanol.

Table III. *Radioactivity Present in Styles of Pollinated Pistils after Extraction with Ethanol or Water*

Pollination[1]	Extracting Medium	Total Radioactivity	Specific Radioactivity	Radioactivity Remaining in Insoluble Residue
		cpm	*cpm/mg, fresh wt*	%
Cross	Ethanol	82,200	275	32
Self	Ethanol	103,700	315	40
Cross	Water	130,100	450	17
Self	Water	128,500	350	19

[1] The labeling period was 72 hr.

Table IV. *Incorporation of ^{14}C in Stigmatic Exudate*

Experiment	Exudate Collected	^{14}C in Exudate	^{14}C Applied
	mg, fresh wt	*cpm*	%
A	90	504,000	5.6
B	132	468,000	5.2

fourth of the ^{14}C in this fraction. Attempts to characterize other components of the neutral and acidic fractions were not successful.

Pectinase solubilized 89% of the ^{14}C present in the ethanol-insoluble residue from a MI-U-^{14}C-labeled stigma. Further separation of the hydrolyzed mixture by ion exchange resins, paper chromatography, and paper electrophoresis yielded arabinose (20%), xylose (7%), galacturonic acid (32%) plus a series of unidentified, partially hydrolyzed oligosaccharides. Arabinose was characterized by carrier dilution and crystallization to constant specific radioactivity. Galacturonic acid was distinguished from glucuronic acid by paper electrophoresis.

In order to obtain a more complete hydrolysis, trifluoroacetic acid was used for the hydrolysis of water-insoluble residue from a MI-U-^{14}C-labeled style. It released about 95% of the ^{14}C present in the residue. The distribution of radioactivity in different components of the trifluoroacetic acid hydrolysate is shown in Table V. Separation of components of the acid hydrolysate by paper chromatography showed radioactivity to be present in compounds with R_F values corresponding to galactose, arabinose, and xylose. Labeled substances remaining at the origin of the paper chromatogram were eluted and further separated by paper electrophoresis. Of the latter radioactivity, 89% was recovered as galacturonic acid. Arabinose and galacturonic acid were characterized by dilution analysis.

Table V. *Distribution of Radioactivity in Components of a Trifluoroacetic Acid Hydrolysate from Residues of an Unpollinated Style*

Eluted Region	Radioactivity Recovered
	%
Origin (galacturonic acid)	69
Galactose	12
Arabinose	9
Xylose	10

Autoradiography of Cross-pollinated Pistils. The diagram in Figure 1 summarized information taken from autoradiographs of a number of sections of a cross-pollinated pistil. Stippled portions indicate regions in the pistil where cell wall bound tritium derived from MI-2-^{3}H had produced abundant silver grains. Parenchymal cell walls of the pedicel were heavily labeled, as one might expect since the pedicel was in direct contact with radioactive solution (Fig. 2). Distribution of silver grains above the pedicel suggested that movement of label into other parts of the pistil was controlled by a process involving vascular transport. Within vascular bundles, labeling was heavy in sieve tube walls and especially heavy in walls of companion cells. Xylem elements contained very little label (Fig. 2). In the ovary, heavy labeling was evident in ovules and in cells bordering vascular bundles. As vascular strands approached the stigma, regions of heavy labeling around each bundle became more extensive until, in the region of style section 1, they fused into a collar of uniformly labeled cell walls surrounding the style canal. Although the region between vascular bundles and the canal become labeled, little label was found in tissues between the bundles and the external surface of the style. In the stigma, silver grains were distributed fairly uniformly over all parenchymal cells facing the upper stigma surface and extending downward to the region represented by the collar of labeled cells around the style canal.

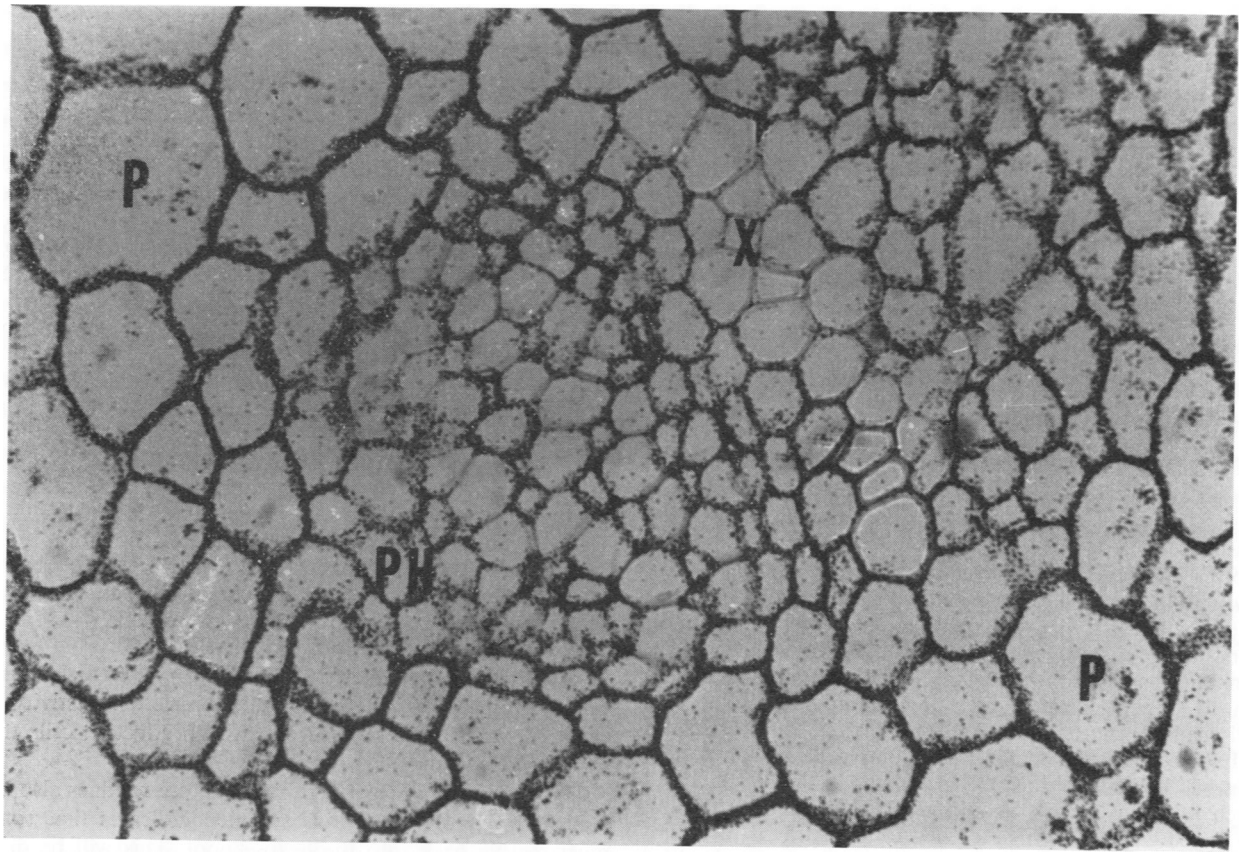

FIG. 2. Autoradiograph of a section through the pedicel of a myoinositol-2-^{3}H-labeled pistil. X: Xylem; PH: phloem; P: parenchymal cells. × 450.

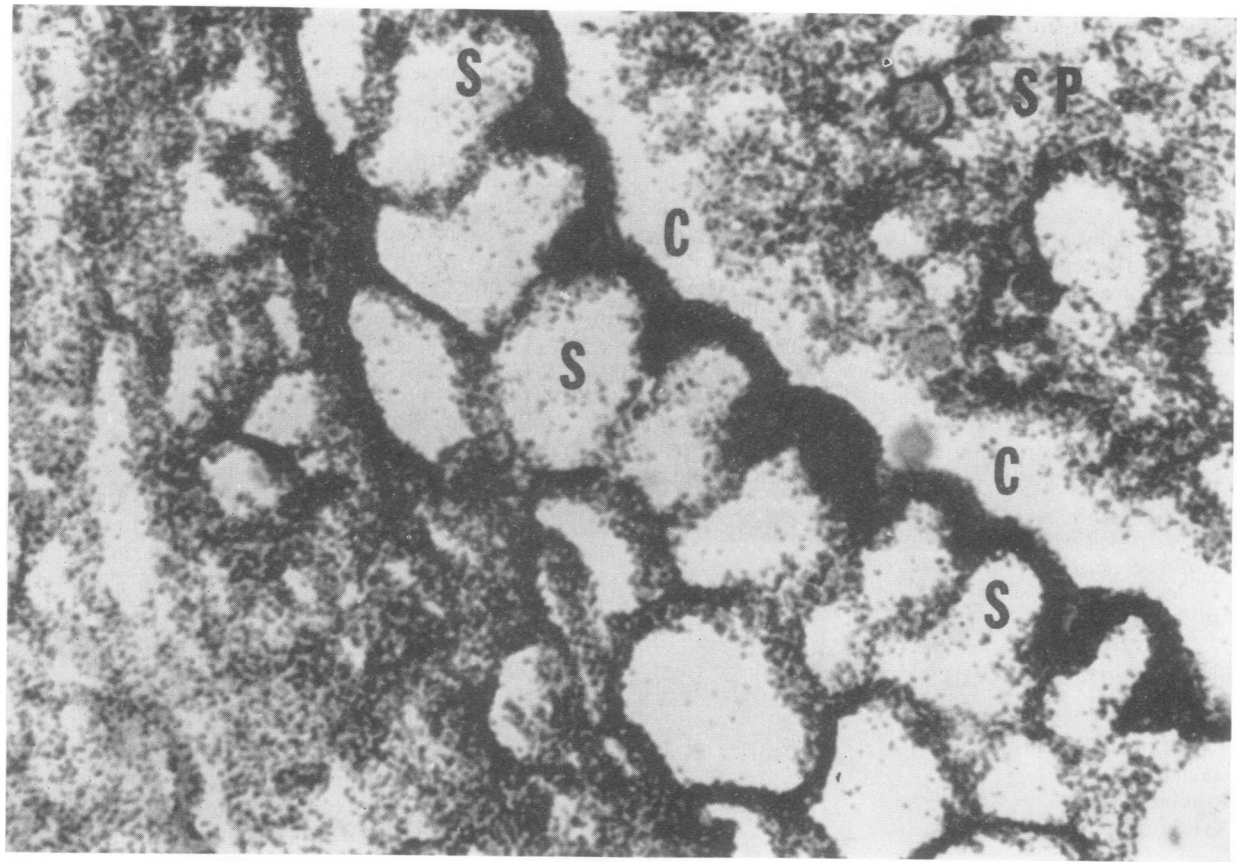

FIG. 3. Autoradiograph of a section through the style of a myoinositol-2-^{3}H-labeled detached pistil. S: Secretory cells; C: style canal which is filled with secretion product (SP). × 920.

Papillae and trichomes covering the stigmatic surface had labeled cell walls.

Secretory cells lining the style canal and the inner surface of the stigma in the region where it joins the canal bore an extraordinarily large amount of label in cell walls (Fig. 3). A dense zone of radioactivity marked the middle portion of the wall that faced the canal. Other regions of the wall were less heavily labeled. Labeled secretion product, presumably derived from these cells, can be seen in Figure 3 as an irregular mass of silver grains filling the canal.

DISCUSSION

When a detached *L. longiflorum* flower or pistil is placed in a solution of radioactive MI, label is taken up by the vascular system and transported to all parts of the pistil. The cells of the pistil tissue use the cyclitol for the synthesis of pectin and related polysaccharides. Developmental studies of *L. longiflorum*, pistils *in vivo* and *in vitro* (2) have shown that growth is uniform throughout styles which are larger than 40 mm, a condition that persists to postanthesis. Some cell divisions accompany cell elongation. Pectin biosynthesis, therefore, will include, in addition to possible turnover of pre-existing pectin deposits, new cell wall formation and deposition of newly synthesized pectin. Labeled MI utilized for pectin biosynthesis during such processes will be deposited as polysaccharide in these cells. Dead cells, such as xylem vessels, should not be labeled. Distribution of label in cell walls of pistils recorded in this study is in accord with this projection.

In detached pistils which were fed radioactive MI through the pedicel, vascular processes controlled the distribution of label into tissues above the pedicel. Sections of style closest to the stigma, and the stigma itself, were most heavily labeled with ^{14}C. Autoradiographic studies with tritiated MI produced a similar picture. The latter revealed that diffusion from vascular bundles appeared to be directed toward cells in the vicinity of the style canal and implied the presence of a gradient. Autoradiograms suggested that in the stigma, too, label accumulated preferentially in the direction of secreting surfaces.

Detached styles exhibited a gradual diffusion of label from the site at which labeled MI was introduced. Cells close to the radioactive solution incorporated most of the ^{14}C that was taken up into wall polysaccharide. Processes involving vascular transport appeared to play little, if any, role in movement of label to upper parts of the style or stigma.

Secretion product of the pistil is present on the surface of the stigma, and, after pollination, in the style. When MI-U-^{14}C or MI-2-^{3}H was fed to detached pistils, label could be found in secretion product as it accumulated on the stigma and in the style. Autoradiograms of cross sections through secretory cells which line the style canal revealed a dense zone of radioactivity deposited in the central portion of those cell walls facing the canal. The way in which MI-2-^{3}H was given to the pistils (a single feeding followed by 120 hr of metabolic activity before analysis) might have created a pulsed product in cells that actively secrete in one direction.

The observation (Table V) that galactose residues also became labeled when MI-U-^{14}C was supplied indicates that some ^{14}C must recycle back into the hexose pool. This will be discussed more thoroughly in a subsequent report (C. Labarca, M. Kroh, and F. Loewus, manuscript in preparation).

In summary, the uptake, distribution, and incorporation studies have shown that rate of uptake in style tissue depends on the mode of application of label. Feeding the label to excised pistils via the pedicel appears to be most effective for the study of the nutritional role of the pistil in the synthesis of pollen tube wall. Pollination does not have a significant influence on uptake and distribution or on incorporation of label into insoluble material.

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