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Production of a Novel Extracellular Cutinase by the Pollen and the Chemical Composition and Ultrastructure of the Stigma Cuticle of Nasturtium (*Tropaeolum majus*)[1]

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ABSTRACT

Germinating nasturtium pollen (*Tropaeolum majus*) is shown to excrete an enzyme(s) which hydrolyzes all types of monomers from biosynthetically labeled cutin and *p*-nitrophenyl esters, which are model substrates for fungal cutinases. The pollen cutinase showed an optimum pH near 6.5 and was inhibited by thiol-directed reagents such as *p*-hydroxymercuribenzoate and N-ethyl maleimide but not by diisopropylfluorophosphate, an "active serine"-directed reagent indicating that the pollen enzyme is an "–SH cutinase" unlike the fungal enzyme which is a serine cutinase. Excretion of the pollen cutinase into the extracellular fluid was complete within 4 to 6 hours at 30 C. Since actinomycin D and cycloheximide showed little effect on the level of cutinase excreted, it appears that cutinase is an enzyme synthesized prior to germination. Release of cutinase into the medium did not require germination. Electron microscopy revealed the presence of a continuous cutin layer on mature stigma with extensive folds, which are proposed to play a role similar to that played by the cellular papillae found in the stigma of other plants. Chemical analysis of stigma cutin by depolymerization and combined gas-liquid chromatography and mass spectrometry showed that this cutin consists of mainly the C_{16} family of acids. The major (70%) components were dihydroxy C_{16} acids which consisted of 10,16- (64%), 9,16- (16%), 8,16- (12%), and 7,16- (8%) dihydroxy palmitic acid. Deuterium-labeling studies showed the presence of 16-oxo-9-hydroxy C_{16} acid and 16-oxo-10-hydroxy C_{16} acid in this cutin. The biochemical and ultrastructural studies indicate that the pollen tube may gain entry into stigma using cutinase excreted by the pollen.

The surface of at least the so-called dry stigmas is covered by a cuticle (3, 6, 8-11, 16-18). Since pollen tube must penetrate this barrier in order to gain entry into the stigma on its way to the ovary, it was suspected that the germinating pollen might use an enzyme which catalyzes the hydrolysis of cutin, the structural component of the cuticle. Since the incompatibility reaction in some plants appeared to involve the lack of penetration of pollen tube through the cuticle of stigma, cutinase was suggested to be involved in incompatibility (3). The experimental evidence for the presence of cutinase in germinating pollen consisted of the demonstration that addition of cutin preparation to germinating pollen caused a slight increase in titratable acidity (7, 18). With this increase as a measure of cutinase, it was found that the presence of cutinase in germinating pollen was a characteristic of plants which have "cutinized" stigma. Since fungal extracts also catalyzed release of titratable acids in the presence of cutin, including a cutin preparation from stigma surface, it was suggested that cutinase of germinating pollen was "identical with" cutinase detected in molds (7). Cutinase from neither molds nor pollen had been isolated or characterized and, therefore, it was not possible to compare the enzymes from the two sources. Recently, fungal cutinases, isolated in homogeneous form, have been characterized (22, 23, 25). The products released from cutin by the germinating pollen were also not characterized and therefore it was difficult to determine whether the pollen enzyme released all types of monomers of cutin or only the peripheral fatty acids.

In this paper we demonstrate, with the use of radioactive cutin, that germinating pollen from nasturtium excrete an enzyme(s) which releases all types of monomers from apple cutin. Results which suggest that the appearance of this cutinase activity in the medium does not require protein synthesis are also presented. Preliminary characterization of the pollen cutinase strongly suggests that the pollen enzyme catalyzes hydrolysis of cutin by a mechanism quite different from that involved in cutin hydrolysis by purified cutinases previously isolated from fungi. Electron microscopic examination of the ultrastructure of the continuous cuticular layer on the stigma and the first chemical characterization of stigmatic cutin are also reported.

MATERIALS AND METHODS

Plants. Nasturium plants (*Tropaeolum majus*) were grown from seeds (Chas. H. Lilly Co.) in a sand-perlite-mica peat (1:1:1) mixture under Gro-lux lights (~1,200 ft-c) supplemented with incandescent lights with 16-hr days. These plants produced flowers within 2 months of germination. Pollen were collected routinely with an aspirator and kept frozen until used.

Reagents. The following reagents were purchased from Sigma Chemical Company: *p*-nitrophenyl palmitate (PNP), *p*-nitrophenyl butyrate (PNB), iodoacetamide, N-ethyl maleimide, *p*-hydroxymercuribenzoate, actinomycin-D, cycloheximide, dithioerythritol, diisopropylfluorophosphate (Dip-F), *Aspergillus niger* cellulase, and fungal pectinase. Omnifluor and Aquasol were from New England Nuclear Corp.

Enzyme Preparation. Nasturtium pollen were suspended and

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germinated in a previously described (2) nutrient medium containing 0.5 mM dithioerythritol for 6 hr at 30 C in a gyratory shaker water bath. After this period pollen were removed by centrifugation at 12,000*g* for 10 min, the supernatant was filtered through glass wool, and the extracellular fluid was used as the enzyme source. A pentaerythritol-containing medium (5), which did not allow germination of the pollen, was used for comparison.

Preparation of Substrates. For measuring cutin hydrolysis, apple fruit cutin, biosynthetically labeled by incorporation of [1-^{14}C]acetate, was prepared as described before (21). The purification of the labeled cutin was monitored by hydrogenolysis ($LiAlH_4$) of samples followed by analysis of the lipid products for radioactivity as described elsewhere (15). If significant amounts (more than a few per cent) of ^{14}C were present in the water-soluble products, the samples were retreated extensively with cellulase and pectinase. The label in the final cutin preparations, which were used for cutinase assays, was present almost entirely in the hydroxy fatty acids of the polymer. Labeled discs of apple cutin thus obtained were homogenized in distilled H_2O using a TenBroeck glass homogenizer, centrifuged, and the pellet was resuspended in distilled H_2O. This procedure was repeated twice with water, three times with acetone, and three times with 0.1 M Na-phosphate (pH 6.5). The washed pellet was finally suspended in fresh phosphate buffer just before use in the assays.

To 50 ml of water 24 mg of *p*-nitrophenyl palmitate (or 19.6 mg of *p*-nitrophenyl butyrate) and 160 mg of Triton X-100 were added and the mixture was heated until the ester became liquid. The mixtures were then sonicated with the large probe of a Biosonic III for 1 min at 30% of the maximum power. This procedure gave a clear dispersion of the *p*-nitrophenyl esters.

Enzyme Assays. Appropriate aliquots of the enzyme were usually incubated in a total volume of 1 ml of 0.1 M Na-phosphate buffer (pH 6.5) with 1.75 mg of labeled (70,000 cpm) cutin in a gyratory shaker water bath at 30 C for various periods of time. At the end of the incubation, the reaction mixture was centrifuged at 12,000*g* for 10 min and the supernatant was filtered through a glass wool plug placed in a Pasteur pipette. An aliquot of the filtrate was assayed for radioactivity in either Aquasol or Omnifluor. Values obtained with identical reaction mixtures containing either no enzyme or boiled enzyme were subtracted from the experimental values to give the rate of enzymic hydrolysis.

p-Nitrophenyl esterase assays were done spectrophotometrically as described before (21). The hydrolysis was followed by measuring the *A* at 405 nm with a Gilford spectrophotometer and linear rates were observed for at least 10 to 15 min.

Determination of Radioactivity. Radioactivity in liquid samples and in thin layer chromatographic fractions was determined with a Packard 3003 liquid scintillation spectrometer. Aliquots of soluble products or silica gel from thin layer chromatograms were assayed for radioactivity in 15 ml of either Aquasol or Omnifluor. A 0.4% (w/v) solution of Omnifluor in toluene containing 30% ethanol gave a counting efficiency of 12% for ^{3}H and 76% for ^{14}C. All counting was done with a standard deviation of less than 3%.

Protein Determination. Protein concentrations were estimated by the method of Lowry *et al.* (19) after precipitation with 10% trichloroacetic acid. The precipitate was washed with 1% trichloroacetic acid and dissolved in 0.1 N NaOH before assaying for protein.

Isolation of Products of Enzymic Hydrolysis of Labeled Cutin. The mixture of enzyme and labeled cutin in a total volume of 3 ml was incubated for 6 hr at 30 C in a gyratory shaker. The reaction was stopped by the addition of 0.2 ml of 6 N HCl and the labeled cutin was removed by centrifugation for 10 min at 10,000*g*. The supernatant was filtered through glass wool, and an aliquot was assayed for radioactivity. The remaining supernatant was extracted three times with chloroform using a separatory funnel. The combined extract was evaporated to dryness and the residue was refluxed overnight with $LiAlH_4$ in 30 ml of tetrahydrofuran. The excess $LiAlH_4$ was decomposed by slow addition of the reaction mixture into water, and after acidification with HCl, the reduced products were extracted with chloroform (3 × 100 ml). After the chloroform was evaporated off under reduced pressure, the residue was dissolved in 0.1 ml of a 2:1 mixture of chloroform and methanol and subjected to TLC.

Chromatography. TLC was done on 1-mm layers (20 × 20 cm) of Silica Gel G activated at 110 C overnight. Thin layer plates were developed with ethyl ether-hexane-methanol (8:2:1). The chromatograms were sprayed with a 0.1% ethanolic solution of 2′,7′-dichlorofluorescein and then visualized under UV light.

Determination of Chemical Composition of Stigma Cutin. Stigma was excised from a large number of flowers and stored at −20 C until analysis. About 0.5 to 1 g of stigma was ground in a TenBroeck homogenizer with 10 ml of methanol, the homogenate was centrifuged, and the residue collected was repeatedly extracted with solvents as described before (15). The final residue was depolymerized by transesterification with 14% BF_3 in methanol (14), or by hydrogenolysis with $LiAlH_4$ or $LiAlD_4$ in tetrahydrofuran (27). The lipid products were isolated and analyzed as their trimethylsilyl ethers by combined GLC and MS as described elsewhere (14, 27).

Electron Microscopy. Stigmas and styles were collected from flowers from nasturtium plants grown as indicated above and were fixed in 2% glutaraldehyde in 0.1 M K-phosphate (pH 7.3) for 1 hr. After postfixation in 2% aqueous OsO_4 for 1 hr, the tissue was dehydrated in a standard ethanol series. The specimens were infiltrated and embedded in Spurr's plastic with propylene oxide as a diluent. Sections were cut with a Du Pont diamond knife and a Sorval MTB-2 ultramicrotome. The sections were stained in 2% uranyl acetate in absolute methanol for 6 min and then examined with a Zeiss 10 electron microscope. For scanning electron microscopy, specimens were fixed, postfixed, and dehydrated as indicated above. After the absolute ethanol step, the specimens were subjected to critical point drying using Freon TF followed by Freon 13 in a Bomar 1500 critical point drier. Gold and platinum coating was done in a Technics Hummer and specimen were examined using an ISI-60 instrument at 10 kv.

RESULTS

Hydrolysis of Cutin by an Extracellular Cutinase from Pollen. In order to examine whether germinating pollen excrete cutinase we searched for a medium which would allow *in vitro* germination of nasturtium pollen. No germination of nasturtium pollen could be observed with a pentaerythritol-containing medium (5), which allows rapid germination of lily pollen. In a medium containing sucrose and methionine (2), usually about 80% of the pollen germinated within a few hr and, by about 6 hr, all of the viable pollen appeared to have germinated.

If germinating pollen utilize a cutinase in gaining entry into stigma, this enzyme would be expected to be an extracellular enzyme. Therefore we examined the medium, in which nasturtium pollen germinated, for cutinase activity. Since exogenous [1-^{14}C]acetate is incorporated into all types of cutin monomers in apple fruit, we used such a biosynthetically labeled cutin preparation as the substrate in the search for a cutinase. Incubation of the medium, in which pollen had germinated, with the finely powdered labeled cutin, showed that about 10% of the label was released into the medium from the polymer in about 4 hr. In order to determine whether all types of monomers

are released by the enzyme preparation, the aqueous medium was extracted with chloroform. Virtually all the radioactivity contained in the medium could be recovered in the choloroform extract. Hydrogenolysis ($LiAlH_4$) of the material recovered from the chloroform extract followed by TLC of the recovered products showed that all types of monomers were released from cutin by the germinating medium of the pollen (Fig. 1). Boiling of the medium strongly inhibited (≃90%) cutin hydrolysis indicating the heat-labile nature of the cutinase activity.

Effect of Enzyme Concentration and Time on Hydrolysis of Cutin and Model Substrates. The amount of label released from the biosynthetically labeled cutin depended on the amount of extracellular fluid added. However, the release of ^{14}C was not measurable up to about 0.025 ml (≃75 μg of protein) of the medium; but further increase in protein resulted in dramatic increase in the release of label from the polymer (Fig. 2a). Higher (above 0.1 ml) protein concentrations gave a linear increase in the amount of ^{14}C released. The reason for this unusual protein concentration dependence is not clear. It is possible that at the extremely low concentrations the enzyme is inactive due to dissociation, adsorption at the surface of the polymeric substrate, or other reasons. Since the assay involves only the release of ^{14}C into the medium, all bond cleavages catalyzed by the enzyme might not be measured with such an assay. The enzyme preparation also catalyzed hydrolysis of certain model substrates, which are known to be substrates for purified fungal cutinases (22, 23, 25). With these model substrates, namely *p*-nitrophenyl esters of fatty acids, the rate of hydrolysis increased in a linear fashion with increasing protein concentrations in the range which did not give linear response with the assays using labeled cutin (Fig. 2b). Thus, it appears that the nonlinear increase in the rate of release of ^{14}C from cutin by increasing protein concentrations might be caused by some physicochemical feature of the assay with the insoluble polymer. However, similar assays with cutinase from fungal sources did not show any unusual concentration dependence (22, 25).

The time course of release of ^{14}C from labeled cutin by the extracellular cutinase of nasturtium pollen is shown in Figure 3. The amount of labeled soluble products released from the polymer increased with time but not in a strictly linear manner. Since the substrate was biosynthetically labeled, the radioactive fatty acids and hydroxylated derivatives would be expected to be incorporated into an unlabeled polymer present at the beginning of the incorporation period. Since the biosynthesis of this polymer is known to involve a stepwise transacylation (4),

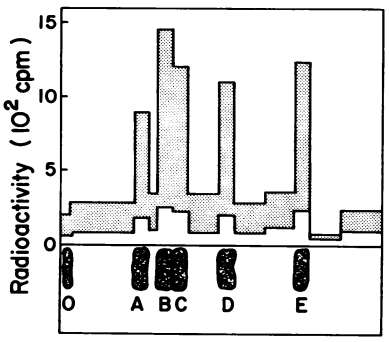

FIG. 1. Radio TLC of the hydrogenolysate ($LiAlH_4$) of products of enzymic hydrolysis of biosynthetically labeled apple cutin by extracellular fluid of germinating nasturtium pollen. Chromatography was on a 1-mm Silica Gel G layer with ethyl ether-hexane-methanol (8:2:1) as solvent. Origin (O); C_{18}-1,9,10,18-tetraol (A); C_{16}-1,7,16-triol (B); C_{18}-1,18-triol (C); alkane-α,ω-diol (D); primary alcohols (E). Lower unshaded bar graph represents products from boiled enzyme; and shaded bar graph represents products from enzymic hydrolysis.

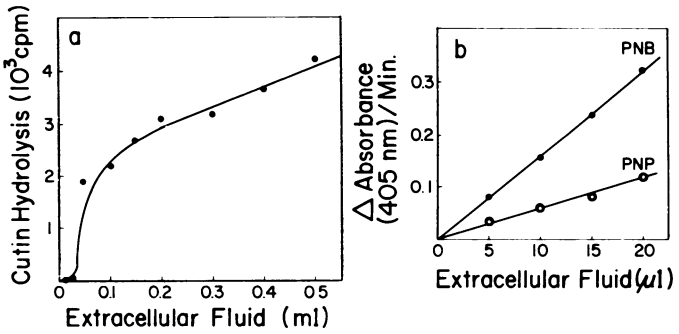

FIG. 2. Effect of enzyme concentration on hydrolysis of labeled apple cutin (a) and *p*-nitrophenyl esters (b). Each reaction mixture in (a) contained the indicated amount of extracellular fluid from germinating nasturtium pollen and 0.2 ml of a suspension of labeled cutin (7×10^4 cpm, 1.75 mg) in a total volume of 2 ml of 0.1 M Na-phosphate (pH 6.5) containing 0.5 mM DTE. All assays were done for 4 hr at 30 C. Spectrophotometric assays in (b) were done as described in the text.

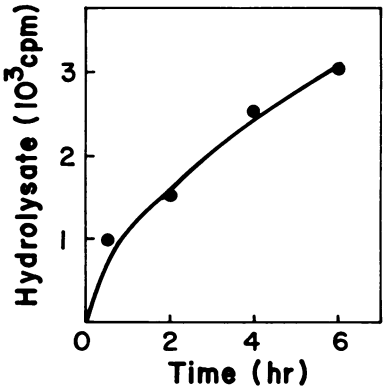

FIG. 3. Time course of release of soluble labeled products from biosynthetically labeled apple cutin by the extracellular fluid from germinating nasturtium pollen. Assays were done as described in the text with 0.5 ml of extracellular fluid.

the peripheral acyl moieties would be expected to contain more label than the monomers in the interior of the polymer. Considering this possibility the gradual decrease in the rate of release of labeled soluble material from the polymer noted in Figure 3 is expected. Another possible reason for the observed time course might be that during the latter periods of incubation the enzyme might be catalyzing the hydrolysis of soluble oligomers generated during the earlier periods. Production of oligomers during the early periods and their disappearance during the latter periods have been demonstrated with a purified fungal cutinase (22). Time course of hydrolysis of the model substrates by the fungal enzyme was strictly linear until subsaturating substrate concentration was reached. Until the enzyme is purified to homogeneity, cutinase activity has to be measured directly with the polymer substrate because of the possibility that the model substrates might also be hydrolyzed by enzymes other than cutinase in the extracellular fluid.

Effect of pH on Cutinase Activity. The effect of pH on the release of ^{14}C from labeled cutin by the germinating medium of nasturtium pollen was quite different from that observed with cutinases obtained from certain *Fusarium* species. With the pollen enzyme, just as in the case of the fungal enzyme, very little cutinase activity could be detected at pH 5 or below (Fig. 4). With the pollen enzyme there was a dramatic increase in enzymic activity as the pH was raised to 5.5 and subsequent increases in pH up to 7 caused only small changes in enzymic activity. Activity declined sharply as the pH increased from 7 to 8, and at pH 9 or higher measurable activity could not be

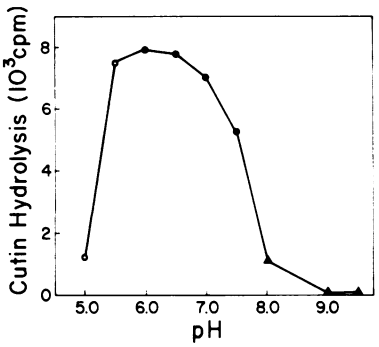

FIG. 4. Effect of pH on enzymic hydrolysis of labeled apple cutin by extracellular fluid of germinating nasturtium pollen. Reaction mixtures contained 0.5 ml of the extracellular fluid and 0.2 ml of a suspension of labeled cutin in a total volume of 3 ml of 0.1 M citrate phosphate buffer (○), phosphate buffer (●), or glycine-NaOH buffer (▲). Reaction mixtures were incubated at 30 C for 4 hr. Other experimental details are described under "Materials and Methods."

found. This pattern is in sharp contrast with that observed with the *Fusarium* enzyme which showed optimal activity near pH 10 (22, 25). With the fungal enzymes substantial activity could be observed only above pH 7 and the major increase in activity was observed as the pH changed from 7 to 10. On the other hand, a crude cutinase preparation from *Botrytis cinerea* was reported to show an optimum pH of 5 (24).

Effect of Inhibitors on Cutinase Activity. The sharp difference between the pH optimum of the pollen cutinase and that of fungal cutinases suggested that two different types of enzymes are involved in the hydrolysis of cutin. This difference might be an indication of a basic difference in the mechanism of catalysis of the two enzymes. In order to test this possibility the effects of some reagents, which might covalently modify the active site of cutinase, were examined. Since "active serine" is known to be involved in catalysis by the fungal cutinases (22, 25) diisopropylfluorophosphate, an active serine-directed reagent, was tested with the pollen enzyme. Preincubation with even up to 30 mM Dip-F[3] failed to give measurable inhibition of the cutinase activity of the germinating medium of nasturtium pollen (Table I) whereas 2 μM Dip-F severely inhibited the purified *Fusarium* cutinase, and even with the crude enzyme from the fungus, only less than 10 μM Dip-F was needed to inhibit cutinase activity almost completely. The insensitivity of the pollen enzyme to Dip-F was demonstrated with the model substrates as well as with labeled cutin. These observations very strongly suggested that active serine is not involved in catalysis by the pollen cutinase. Thiol-directed reagents, on the other hand, severely inhibited the pollen enzyme (Table I); *p*-hydroxymercuribenzoate (0.1-0.5 mM), iodoacetamide (1-5 mM), and N-ethyl maleimide (1-5 mM) severely inhibited hydrolysis of labeled cutin and the model substrates. Therefore, it appears probable that −SH groups are very important to the activity of this enzyme. On the other hand the fungal cutinases thus far examined are insensitive to −SH-directed reagents (22, 25). In fact, the fungal cutinase from *F. solani pisi* contains one disulfide but not free −SH group (T. S. Lin and P. E. Kolattukudy, unpublished results). Thus, cutinases follow the analogy of proteases in that the plant enzyme is an −SH enzyme whereas the fungal hydrolases are of the active serine type. It is quite obvious that the conclusion drawn previously that pollen cutinase is identical with mold cutinases (7) is not valid. Purification of pollen cutinase is needed before a detailed comparison between the two classes of cutinases can be made.

[3] Abbreviation: Dip-F: diisopropylfluorophosphate; PNP: *p*-nitrophenyl palmitate; PNB: *p*-nitrophenyl butyrate.

Evidence that Cutinase Is Formed Prior to Pollen Germination. In order to determine the time course of production of cutinase the extracellular fluid was examined with PNB and PNP as model substrates and with the biosynthetically labeled cutin (Fig. 5). Low levels of hydrolytic activities were found in the medium as soon as the fluid could be physically removed after suspending the pollen in the germinating medium. The amount of activity in the extracellular fluid increased up to 4 to 6 hr and some decrease was observed thereafter. PNP hydrolase activity was much smaller than the PNB hydrolase activity at all times. However the time course of appearance of the hydrolase

Table I. Effects of various inhibitors on the hydrolysis of labeled cutin and p-nitrophenyl esters by cutinase from nasturtium pollen.

An aliquot (0.9 ml) of the extracellular fluid was preincubated with the inhibitors at the indicated concentrations at room temperature for 30 min. Spectrophotometric assays with this preparation were done as described in the text. Each reaction mixture used for cutinase assay contained 0.75 ml of the preincubated extracellular fluid and 0.25 ml of a suspension of labeled cutin (1.75 mg) in a total volume of 1 ml of 0.1 M sodium phosphate buffer, pH 6.5. The reaction mixture was incubated for 4 hr at 30 C and other experimental details are given in the text. The averages of the values obtained in two experiments are shown; in both experiments about 10% of the label in cutin was released when no inhibitor was present.

Treatment	Concentration mM	Inhibition %		
		^{14}C-Cutin	PNP	PNB
p-Hydroxy mercuribenzoate	0.1	19	39	42
	0.2	43	69	73
	0.5	92	93	91
Iodoacetamide	1.0	34	15	18
	5.0	61	59	65
N-Ethyl maleimide	1.0	63	80	76
	5.0	81	89	85
Diisopropylfluorophosphate	1.0	0	0	0
	5.0	0	0	0
	10.0	0	0	3

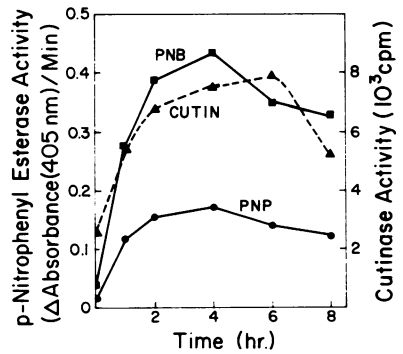

FIG. 5. Production of cutinase as measured by hydrolysis of labeled apple cutin, *p*-nitrophenyl butyrate (PNB), and *p*-nitrophenyl palmitate (PNP). In separate flasks 0.2 g of pollen was suspended and germinated in 2 ml of the nutrient medium containing 0.5 mM DTE at 30 C. After 0, 1, 2, 4, 6, and 8 hr of germination, pollen were removed by centrifugation and aliquots of the supernatant were assayed with the appropriate substrate as indicated under "Materials and Methods."

in the medium was quite similar irrespective of the substrate used.

The appearance of cutinase in the extracellular fluid might represent either synthesis of the enzyme or simply excretion and/or activation of preexisting enzyme. In order to distinguish between these two possibilities the effects of actinomycin D and cycloheximide on the appearance of extracellular cutinase were determined with PNP, PNB, and labeled cutin as substrates (Table II). Up to 4 hr there was no inhibition of appearance of cutinase in the extracellular fluid. A slight inhibition was noticed after 6 hr of incubation in the presence of the inhibitors. Inasmuch as it is known that the maximal levels of cutinase are found in the medium within 4 to 6 hr, it appears that these inhibitors do not significantly affect the appearance of extracellular cutinase. The syntheses of nucleic acids and proteins do not seem to be involved in the appearance of cutinase and therefore we conclude that a preexisting enzyme(s) is activated and/or simply excreted during the incubation of the pollen in the germinating medium.

If the time course of appearance of cutinase represents excretion of the preexisting enzyme or simple diffusion from the intine layer, a homogenate of ungerminated pollen might show as much cutinase as that found in the extracellular fluid after 4 to 6 hr of germination. In fact with PNB as substrate the homogenates of ungerminated pollen contained nearly as much hydrolase activity as the total activity found in the extracellular fluid of 6-hr germinated pollen and the homogenate of the germinated pollen (Table III). The extracellular fluid of germinated pollen contained over 95% of the hydrolase activity and the homogenate of the 6-hr germinated pollen contained less that 5% of the activity. With cutin or PNP as the substrate, the extracellular fluid contained twice as much hydrolase activity as that observed with a homogenate of the ungerminated pollen. It is possible that cutinase is present in the polymer matrix of the intine layer, and in the homogenate of the ungerminated pollen the enzyme is still entrapped in this matrix. If so the model substrate, being a small molecule, might be accessible to the enzyme whereas the insoluble polymer, cutin, would not be expected to be accessible to the entrapped enzyme. If this explanation is valid it might be possible to release the entrapped cutinase by incubating the homogenate of ungerminated pollen for a few hr. Such an incubation (6 hr) resulted only in a small decrease in cutinase activity (measured using either the model substrate or cutin) probably due to degradation or inactivation of the enzyme by the pollen homogenate. The possibility that activation of a preexisting enzyme occurs during germination cannot be ruled out. Excretion of cutinase by pollen may not depend on germination *per se*. In fact incubation of pollen in a pentaerythritol-containing medium, which did not allow germination of nasturtium pollen, resulted in the excretion of as much cutinase as that found in the medium which allowed germination of the pollen (Table III).

Table II. Effect of cycloheximide and actinomycin D on the production of extracellular cutinase by germinating nasturtium pollen.

The pollen were germinated for the indicated periods in the germinating medium containing 20 µg/ml of cycloheximide or actinomycin D and the extracellular fluid was assayed with PNP, PNB, and labeled cutin as substrates as described in the text. Values obtained with no inhibitor are shown in Fig. 5.

Time (hr)	Enzymatic Activity Measured with (% of Control)					
	Cycloheximide			Actinomycin D		
	PNP	PNB	Cutin	PNP	PNB	Cutin
1	90	101	112	109	104	113
2	110	95	102	112	103	94
4	93	97	109	107	108	90
6	72	88	76	61	84	87

Table III. Cutinase activity from germinated and ungerminated pollen.

In all cases equal aliquots were assayed for the enzymatic activity as described under materials and methods. Nasturtium pollen (400 mg) was incubated for 6 hr at 30 C in 5 ml of either the germinating medium (1 and 2) or in the medium which does not allow germination (line 5); in each case 0.5 mM DTE was included. The germinated pollens were washed and homogenized with the same medium, centrifuged at 10,000*g* and the supernatant was used for enzyme assays. For experiments 4 and 5 the same amount of fresh pollen was homogenized in the germinating medium. The amount of total activity in the extracellular fluid of germinated pollen is arbitrarily taken as 100 for comparison.

Enzyme Source	Enzyme Activity Measured with		
	PNB	PNP	Labeled Cutin
1. Extracellular fluid from germinated pollen	100	100	100
2. Homogenate of germinated pollen	4	3	5
3. Fresh homogenate of ungerminated pollen	83	55	42
4. Homogenate of ungerminated pollen incubated for 6 hr at 30 C	70	40	29
5. Extracellular fluid from ungerminated pollen	88	70	113

Ultrastructure of Stigma. In some plants, as the stigma matures, the cuticle ruptures and is sloughed off as flakes by the time of pollination (8, 26). In such plants the pollen tube would not require cutinase to gain entry into stigma. With the finding that nasturtium pollen excrete cutinase it was necessary to determine whether the mature stigma of this plant is protected by a cuticle. Electron microscopic examination of cross-sections showed a continuous layer of cuticle on mature stigma of nasturtium (Fig. 6). The ultrastructure showed elongated cells and intercellular spaces containing electron-dense exudates (arrows in Fig. 6) similar to those observed previously in certain other plants (16). Chloroplasts and other organelles similar to those previously observed in stigma cells were also found in the present case. No papillate hairs similar to those observed in other plants were observed either in the transmission electron micrographs or in the scanning electron micrographs (Fig. 7). However, the surface of the stigma showed ridges formed by folds in the cuticle (Figs. 6 and 7). These ridges and the flat space between them were much larger in the flat region at the tip of the stigma when compared to the stilar region. The dimensions of these areas suggest that the ridges might prevent pollen from sliding off the stigma (Fig. 7) and thus these ridges might serve the function of cellular papillate hairs found in other plants. In the stilar region the cuticle showed numerous folds and appendages formed by large folds in the cuticle (Figs. 6 and 7). Early in the growth of the stigma the cuticle was probably in close contact with the epidermal wall and as the rate of cutin synthesis exceeded the rate of enlargement of the tissue, folds must have developed in the cuticle. As the cuticle separated off the wall region some carbohydrate material might be expected to be physically carried with the cuticle and in fact carbohydrate material appears to be present even within the

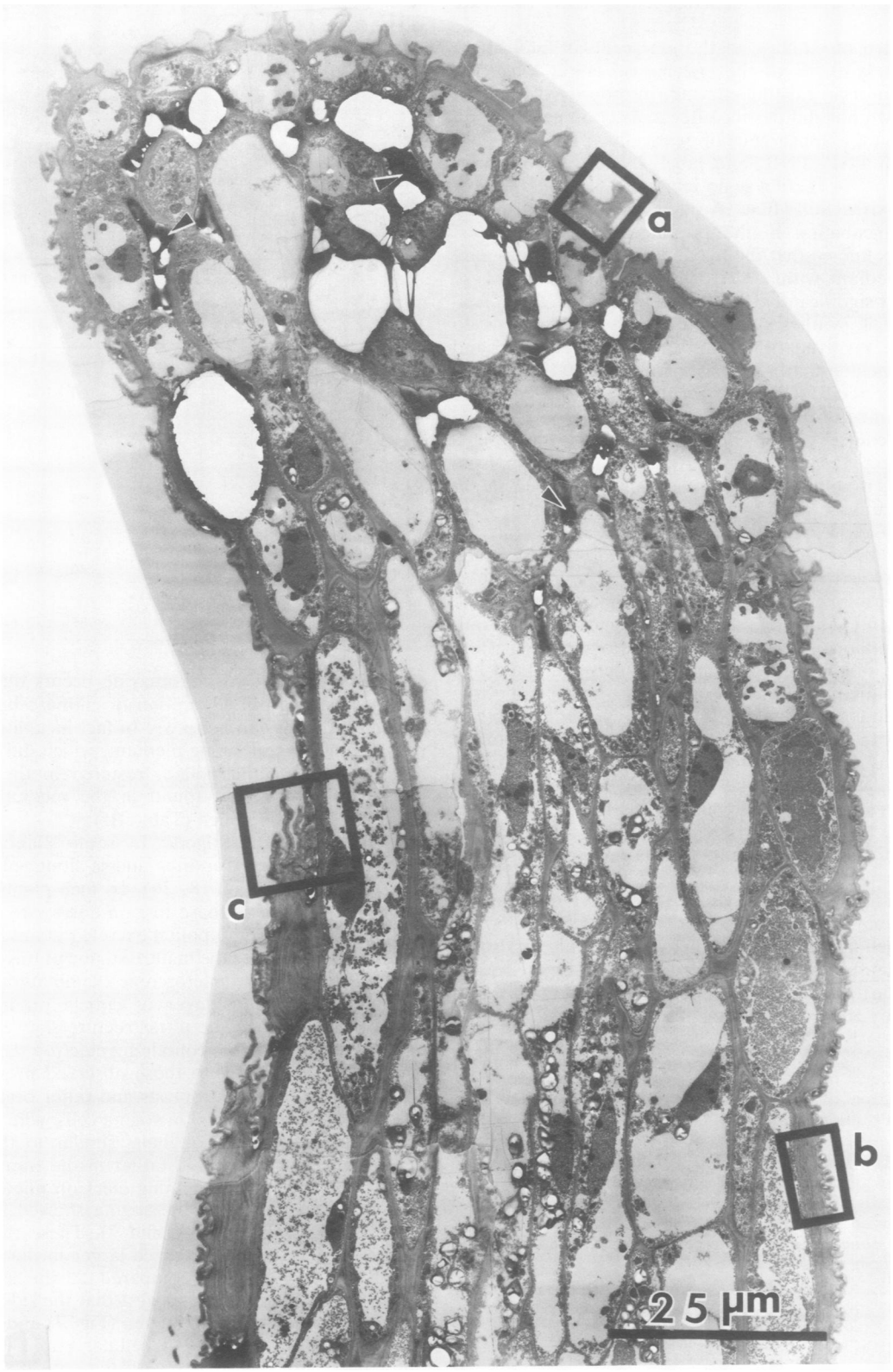

FIG. 6. Electron micrograph of cross-section of mature nasturtium stigma.

extended folds in the cuticle (arrows in Fig. 8). Within the large folds such as those found at the tip of the stigma what appears to be amorphous carbohydrate material is present. In addition what appear to be oil bodies were found in the space between the cuticle and the epidermal wall. These bodies were not electron-dense and might constitute cuticular waxes. Further tests of these tentative conclusions concerning the ultrastructure are needed. In any case it seems clear that the mature stigma of nasturtium is covered by a continuous layer of cuticle and therefore penetration of this layer is a prerequisite for the entry of the pollen tube into the stigma.

Chemical Examination of Stigma Cutin. Since the cutin from

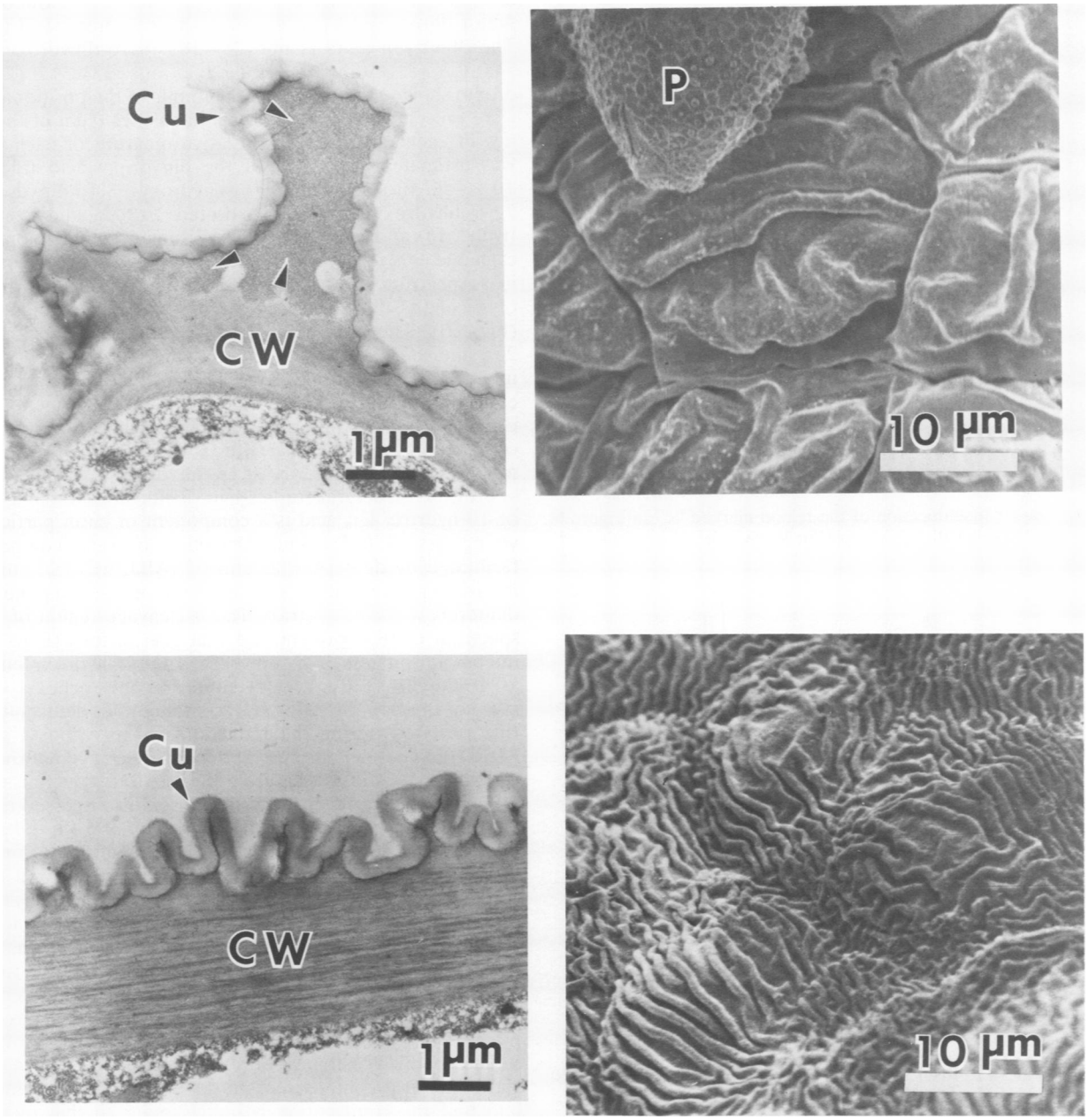

FIG. 7. Higher magnification of regions marked "a" (top left) and "b" (bottom left) in Figure 6 and scanning electron micrographs of stigma surface at the flat top region (top right) and the surface of the lower stylar region (bottom right). P: pollen; Cu: cuticle; CW: cell wall.

stigma has not been heretofore analyzed it was necessary to determine whether components previously found in plant cutin could be obtained from stigma. In order to verify the ultrastructural evidence concerning the presence of cutin on stigma surface, the insoluble polymeric material obtained from stigma was subjected to chemical depolymerization and analysis of the monomers by combined GLC and MS. When hydrogenolysis ($LiAlH_4$) was used as the depolymerization technique, the gas-liquid chromatograms of the chloroform-soluble monomers (as trimethylsilyl ether derivatives) showed one major component which constituted about 70% of the total monomers (Fig. 9, component 5). Retention time of this component was identical to that of hexadecane-1,7,16-triol previously identified in cutin hydrogenolysates of other plants (15). The mass spectrum of this component showed ions corresponding to M^+, M^+-CH_3, M^+-$(CH_3)_3SiOH$, and M^+-CH_3-$(CH_3)_3SiOH$ as previously observed in the case of the C_{16} triol. Intense ions representing α-cleavage on either side of the in-chain substituent were observed at m/e 275, 289, 303, and 317 showing that the C_{16} triol was a mixture of hexadecane-1,7,16-triol and hexadecane-1,8,16-triol, which are probably derived by reductive cleavage from 10,16-hydroxy-C_{16} acid and its positional isomers present in cutin (Fig. 10). A minor (3.5%) component (Fig. 9, component 4) was identified by its retention time and mass spectrum as hexadecane-1,16-diol as described before (15). Component 2 (6.4%) and component 3 (12%) were identified by their retention time and mass spectra as C_{16} alcohol and C_{18} alcohol, respectively. The mass spectrum of the latter fraction showed that it contained saturated as well as mono-, di-, and triunsaturated alchols; the unsaturated components constituted the major portion (>80%) of the C_{18} alcohol fraction. The C_{16} alcohol fraction, on the other hand, contained only saturated alcohol. Another minor component (peak 1, Fig. 9, 8%), did not give a readily interpretable mass spectrum, although it appears to be a trimethylsilyl ether of a alcohol (M^+ = 290). Injection of the hydrogenolysate in much larger quantities than that used in

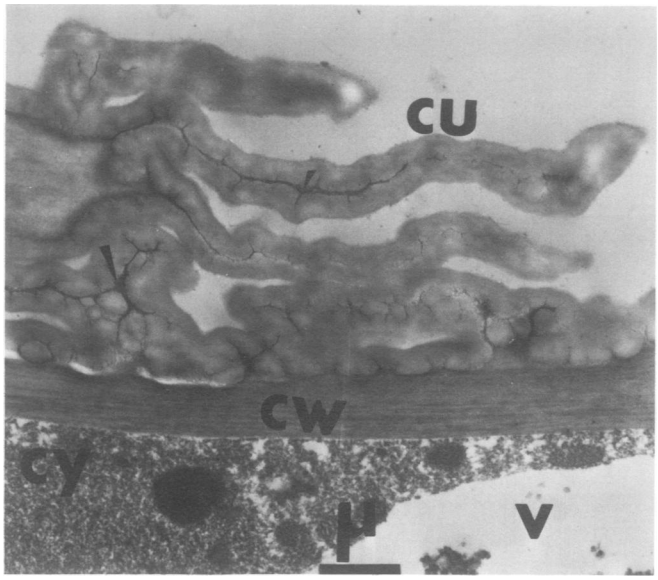

FIG. 8. Higher magnification of the region marked "c" in Figure 6. Cu: cuticle; CW: cell wall; Cy: cytoplasm; V: vacuole.

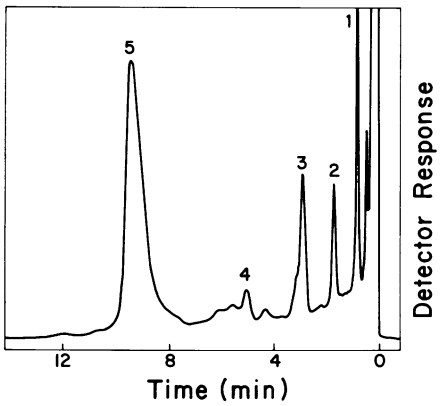

FIG. 9. Gas chromatogram of trimethylsilyl derivatives of hydrogenolysate of cutin from nasturtium stigma. Gas chromatography was done with coiled glass column (183 × 0.32 cm) packed with 5% OV-101 on Gas-chrom Q 80 to 100 mesh, kept at 250 C.

Figure 9 showed minor components which emerged from the gas chromatograph after the C_{16} triol. Their mass spectra showed that they were saturated and unsaturated C_{18} triol and C_{18} tetraol similar to those previously identified as major components of the hydrogenolysates of cutin from some plants (27). Thus, 18-hydroxy-9,10-epoxy C_{18} acid and 9,10,18-trihydroxy C_{18} acids are probably minor components of the cutin of nasturtium stigma.

Results discussed above show that the major component of the stigma cutin of nasturtium is dihydroxy C_{16} acid. However, the mass spectrum of the C_{16} triol derived from the dihydroxy acid does not reveal the positional isomer composition of the hydroxy C_{16} acid because the reduction of the carboxyl group converts it to a primary hydroxyl function, which is indistinguishable from the alcohol function at the ω-carbon. Therefore, a transesterification with methanol (containing 14% BF_3) was used to depolymerize the stigma cutin. GLC analyses of the methyl esters as their trimethylsilyl ether derivatives showed that the major component had a retention time identical to that of the methyl ester of dihydroxy C_{16} acid. The mass spectrum of this component showed the expected ions in the high mass region (14). The α-cleavage region of the spectrum revealed that the dihydroxy acid constituted a mixture of positional isomers. The major ion pairs at m/e 273,275 showed that the major dihydroxyl C_{16} acid was 10,16-dihydroxy acid (Fig. 10). The other ion pairs at m/e 259,289 (9,16-dihydroxy), at m/e 245,303 (8,16-dihydroxyl), and at m/e 231,317 (7,16-dihydroxy) showed that significant amounts of the positional isomers were present. Using the relative intensities (sum of the intensities of each pair) as a quantitative measure of each isomer it was estimated that 64% of the dihydroxy acid fraction was 10,16-dihydroxy-, while 9,16-dihydroxy-, 8,16-dihydroxy-, and 7,16-dihydroxy isomers constituted 16, 12, and 8%, respectively. From such a positional isomer composition we can calculate the relative intensities of the α-cleavage ions in the mass spectrum of the triol expected to be produced by hydrogenolysis of the dihydroxy acid during $LiAlH_4$ treatment of cutin (Fig. 10). Thus, the sum of the intensities of the ions at m/e 275 and m/e 317 was expected to be 72% of the sum of the intensities of the ions at m/e 275, 289, 303, and 317. The experimental value of 80% was in fair agreement with the expected value. Thus, the general conclusion that the major component of the stigma cutin is dihydroxy C_{16} acid and the major positional isomer is 10,16-dihydroxy C_{16} acid is valid.

Previous structural studies had demonstrated that 16-oxo-, 9 or 10-hydroxy C_{16} acid is a component of cutin particularly of young tissues (13). Since detection of such components is facilitated by deuterium labeling, $LiAlD_4$ was used to depolymerize stigma cutin. As expected, the major component of the deuterolysate was C_{16} triol. The α-cleavage region of the mass spectrum of this triol showed four sets of ions (Fig. 11). The intense ion at m/e 275 represented the ω-hydroxy end of the 10,16-dihydroxy C_{16} acid of cutin and the carboxyl end gave rise to the ion at m/e 319 containing 2 deuterium atoms (incorporated during the reduction of the carboxyl group by $LiAlD_4$). The ion at m/e 290 contained 1 deuterium atom which was incorporated during the $LiAlD_4$ reduction of the ω-oxo group of 16-oxo-9-hydroxy C_{16} acid as discussed elsewhere (13). The observation that the ion at m/e 303 (in the spectrum of hydrogenolysate) has been replaced by an ion at m/e 305 is in accordance with the conclusions that it originated from the carboxyl end of the ω-oxo acid. The presence of an ion at m/e 276 (corrected for the isotopic contribution from the ion at m/e 275 as indicated elsewhere [13]) strongly suggests that the 10-hydroxy isomer also has undergone further oxidation to 16-oxo-10-hydroxy C_{16} acid. From the relative intensities it was estimated that the 10-hydroxy isomer of the oxo acid was approximately twice as much as the 9-hydroxy isomer.

The chemical composition of the stigma cutin of nasturtium shows that it is composed of mostly the C_{16} family of monomers and that the composition is quite similar to that of the cutin young *Vicia faba* leaves. The present paper constitutes the first report on the composition of stigma cutin and the results of the

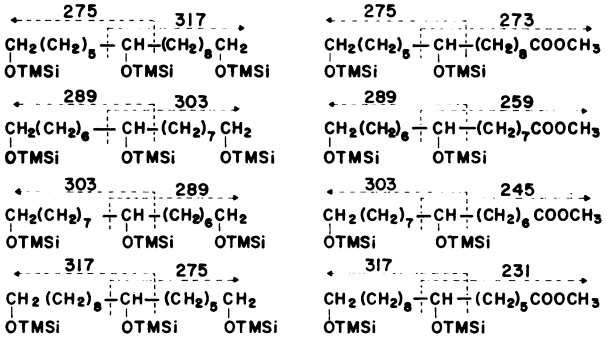

FIG. 10. Major α-cleavages undergone by main positional isomers of methyl esters of dihydroxy C_{16} acid found in cutin (right) and dominant α-cleavages of their hydrogenolysis products (left); TMSi: trimethylsilyl.

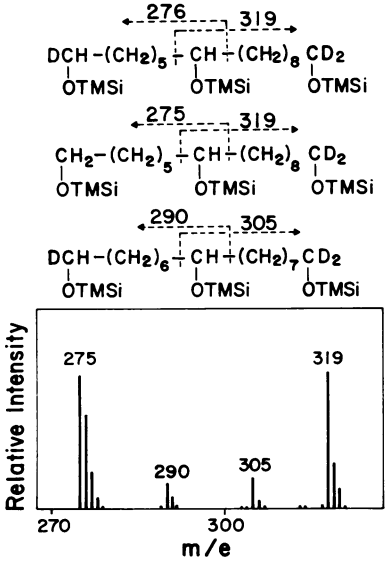

FIG. 11. Partial mass spectrum of trimethylsilyl derivative of C_{16} triol derived by deuterolysis ($LiAlD_4$) of stigma cutin from nasturtium pollen (bottom) and most likely structure of major ions (top).

structural studies support the ultrastructural study.

DISCUSSION

In spite of repeated suggestions that cutinase is probably involved in the penetration of the pollen tube into the stigma, at least in the dry stigma types, little is known about such an enzyme from pollen. The only experimental evidence available for the occurrence of such an enzyme is the demonstration of small increases in titratable acidity observed when germinating pollen were mixed with cutin preparations (7, 18). Using this test germinating nasturtium pollen were reported to contain cutinase. The results in the present paper provide direct evidence which show that nasturtium pollen excrete an enzyme which catalyzes hydrolytic release of all types of monomers from cutin. This observation strongly supports the conclusion that germinating pollen may use cutinase to gain entry into stigma. The electron microscopic examination showing that a continuous cuticular layer is present in mature stigma and the results of the chemical examination of stigma cutin also support this hypothesis.

Since the excretion of cutinase does not appear to require transcriptional and translational processes, a rapid excretion in the early stages of pollen germination is feasible. Such a rapid excretion would be required if this enzyme is to participate in the penetration of the pollen tube into stigma. Many proteins are known to be excreted into the aqueous medium quite readily by pollen (12, 20, 26) and obviously one of these is cutinase. From the rate of release of cutinase into the medium, it appears probable that the site of occurrence is not the exine, because proteins held in this region are released more rapidly (9, 18, 20, 26). Even though no direct evidence is available, it is possible that cutinase is present in the intine layer. A large portion of the enzymic proteins released by pollen into the medium appears to be derived from intine locations (12). Using cytochemical techniques and artificial substrates, esterases have been shown to be released by pollen (12, 20, 26). One or more of these esterases previously observed might have been cutinase, as it is known that fungal cutinases also hydrolyze a variety of esters (22, 25). The so-called poral esterase (1) observed in barley pollen was found in a structure under the aperture and it was suggested that this esterase might be cutinase. If cutinase, in fact, is localized in such structures it is possible that the enzyme would be released slower than the surface esterases and the rate of release of cutinase observed in the present study could be consistent with the above suggestions.

Even though it has been repeatedly postulated that lack of stigmatic activation of pollen cutinase might be involved in certain incompatibility problems no such activation has been demonstrated. The results presented here might be viewed as a starting point for further investigations to determine whether stigmatic components play a regulatory role on pollen cutinase. Our preliminary efforts failed to show any activation of pollen cutinase by a buffer extract of stigma surface of nasturtium and apple. However, further direct experimental tests need to be performed before valid conclusions concerning the role of stigmatic components on pollen cutinase and possible involvement of such interactions in incompatibility can be drawn.

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