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The localization of nonspecific esterase and cholinesterase activity in germinating pollen and in pollen tube of *Vicia faba* L. The effect of actinomycin D and cycloheximide

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Abstract

Two types of esterases, nonspecific esterase and cholin esterase have been distinguished in germinating pollen grains and in pollen tubes of *Vicia faba* using cytochemical methods. The localization of each of them was different. In the hydrated non-germinating pollen grain the nonspecific esterase was present in the cytoplasm and in the intine. The cholinesterase was localized mainly in the sexine and on the outside of the plasma membrane. A particularly large agglomeration of this enzyme was found in the aperture. During germination both types of extracellular esterases were released into the medium. In the pollen tube the activity of the enzymes studied was connected with the wall of its tip. The localization of both types of esterases in the germinating pollen grain and the growing tube were independent of actinomycin D. Cycloheximid prevented the cholinesterase localization at the pollen tube tip.

Introduction

The mature pollen grain is the place where numerous enzymes occur, which are released into the medium during germination. Various hydrolases, transferases, dehydrogenases, oxidases, lyases and ligases have been found in pollen diffusates (*cf.* Knox 1984, Heslop-Harrison 1987 for reviews).

One of the enzymes occurring in pollen diffusates is esterase (Heslop-Harrison 1987). Light microscope studies have revealed that in pollen grains esterase occurs in the spherosomes (Górska-Brylass 1965), in the peripheral layer of the cytoplasm, and in the intine and exine of the sporoderm (Vithanage and Knox 1976, Pettitt 1980). It has been biochemically demonstrated that pollen exterase is polymorphic in nature (Heslop-Harrison 1987). The role of pollen esterase is not clear. Its activity has been detected at the place of erosion of stigmal cuticle, which suggests that among the esterase isoenzymes there is cutinase (Heslop-Harrison 1987). The enzyme found in

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the spherosomes is probably associated with lipids metabolism (Górska-Brylass 1965).

An esterase that so far has not been localized in the pollen grains is cholinesterase. Eserine, an acetylcholin and cholinesterase inhibitor, is known to inhibit growth of pollen tubes (Martin 1972). Research on the effect of acetylcholin (ACh) on pollen germination *in vitro* has failed to bring explicit results. Chhabra and Malik (1978) found accelerated elongation of the pollen tube due to ACh, while Ghary's (after Hartmann and Gupta 1989) results proved the contrary. It cannot be ruled out that ACh, which has been found in all plants studied so far (Gupta 1983), also occurs in pollen. The function of ACh in plants is associated with signal processes, such as the phytochrome activity, the movement of leaves and flowering (Hartmann and Gupta 1989). The ACh-AChE system may also take part in the intercellular interaction (Raineri and Modenesi 1986).

Germination and growth of pollen tubes do not require new RNA transcription (Mascarenhas 1975, Tupý *et al.* 1986). It should be presumed that the activity of pollen enzymes associated with the pollen-pistil interaction also does not depend on the new transcription. It is known from studies carried out so far that the activity of the cutinase released from the pollen of *Tropaeolum majus* is insusceptible to inhibitors of RNA and protein synthesis (Shayk *et al.* 1977). On the other hand, growth of the pollen tube requires continous protein synthesis (Čapková *et al.* 1983). Inhibition of protein synthesis causes inactivation of dictyosomes and cessation of the pollen tube growth (Picton and Steer 1983). This suggests that the activity and/or release of proteins from the tip depends on the new translation.

Considering the above mentioned facts, the problems studied in the present work were: 1) the submicroscopic localization of nonspecific esterase and cholinesterase in germinating pollen grains and growing pollen tubes of *Vicia faba*, 2) the effect of transcription and translation inhibitors on the localizations of the activity of the enzymes under study.

Material and methods

The material for study were *in vitro* germinating pollen grains and tubes of *Vicia faba* L., cv. Nadwislanski (UMK Farm, Toruń-Piwnice).

Five mg of pollen from freshly opened flowers were placed in 0.5 cm^3 of liquid medium (0.003 % $CaCl_2$, 0.001 % boric acid and 10 % saccharose) and cultured on an *Elpan 357* shaker at 23 °C. After 5 min (non-germinating hydrated pollen), 30 min (germinating pollen) and 2 h (pollen with growing tubes) of culturing, the medium was removed and the fixative was added. The material was fixed for 1 h at 4 °C in 2.5 % glutaraldehyde in a cacodylate buffer pH 7.2 with 10 % saccharose. Then the material was washed for 1 h in three changes of a cacodylate buffer, pH 7.2 and placed in incubation solutions to reveal the activity of the enzymes. The activity of esterase was studied by the bromoindoxyl method (Pearse 1961) modified for electron microscopy. The incubation was run for 30 min at 37 °C in a medium: 0.1 cm^3 0.3 % 5-bromoindoxyl in ethyl alcohol. 0.5 cm^3 of 0.5 M $K_3[Fe(CN)_6]$, 0.5 cm^3 of 0.1 M $CaCl_2$, 1.0 cm^3 of Tris-HCl buffer pH 8.3, 2.3 cm^3 of H_2O. The activity of

(acetyl)-cholin esterase was studied by Karnowsky and Roots (1964) method described in the previous paper (Bednarska and Tretyn 1989). As control for both reactions the material was incubated in a medium (1) without substrate, (2) with 10^{-3} M NaF (nonspecific esterase inhibitor, after Walek-Czarnecka 1965), (3) with 10^{-3} M eserine (inhibitor of animal acetylcholin- and cholinesterase).

For electron microscopy the material was postfixed for 1 h in 1 % OsO_4 in cacodylate buffer pH 7.2, dehydrated in an alcohol series and embedded in *Epon 812*. Ultrathin sections were stained after Reynolds (1963). The sections were viewed and photographed in an electron microscope *Tesla BS 500*.

In order to study the effect of the inhibitors of RNA and protein synthesis on the localization of the activity of the enzymes under study, pollen grains were cultured in a medium containing actinomycin D (25 $\mu g\ cm^{-3}$) or cycloheximide (3 $\mu g\ cm^{-3}$). The culturing conditions, fixation and incubation for enzyme activity were the same as described above. The material (not embedded in *Epon 812*) was viewed and photographed using light microscope *Jenaval, K. Zeiss*, Jena.

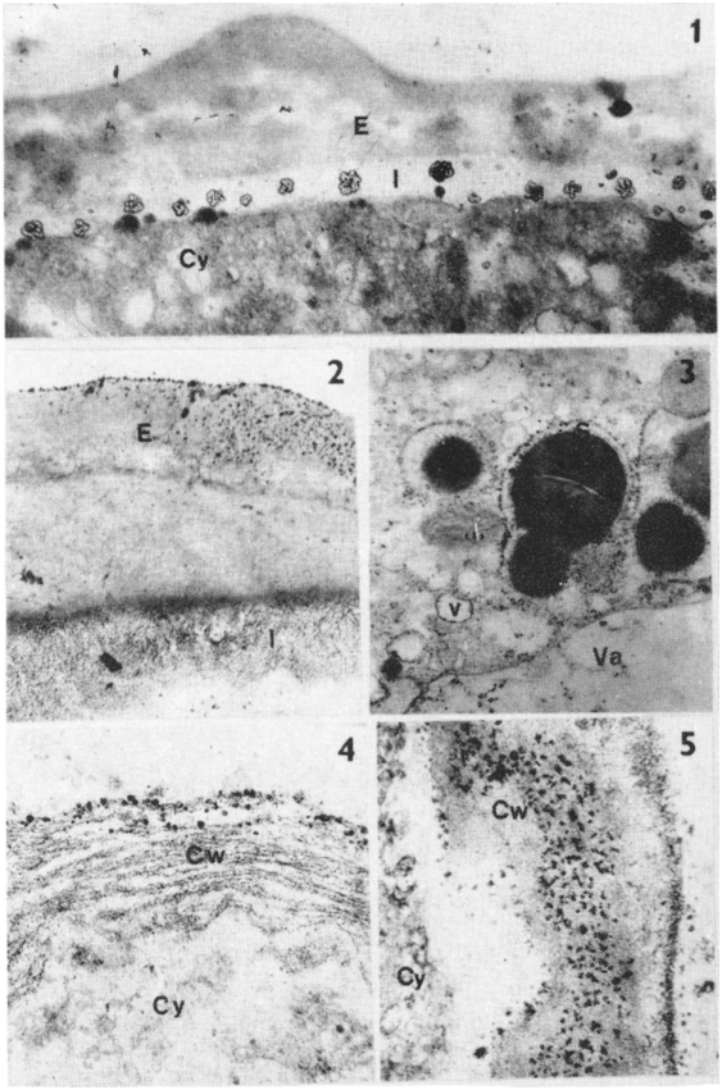
Results

Localization of nonspecific esterase activity. As a result of the method used, indigo crystals appeared at the place of activity of nonspecific esterase. At the light microscope level numerous precipitates were observed in the cytoplasm of the pollen in association with spheric structures, probably spherosomes, and in the sporoderm (Fig. 11a-c). In the pollen tube the reaction product was localized in the granulations of the cytoplasm and in the wall. In short pollen tubes the enzyme activity was found in the entire tube, whereas in long pollen tubes mainly in their tips (Fig. 12a-c). At the EM level in hydrated pollen grains, the activity of nonspecific esterase was found in the sporoderm and in the cytoplasm. In the sporoderm, electron-dense precipitates which agglomerated into rosette-like structures, occurred inside the intine (Fig. 1). In the germinating pollen, the precipitates no longer formed rosette-like agglomerations and were localized in the sexine and on the pollen surface (Fig. 2). This points to nonspecific esterase being released to the medium. In the cytoplasm, the reaction product occurred in association with spherosomes and in the vesicles (Fig. 3).

In the pollen tubes the precipitates were found in association with pectic fibrils in the wall of the tip (Fig. 4) and in the inner layer of the pecto-cellulose wall below the top of the tip (Fig. 5).

Incubation of the material in a medium without substrate and with NaF inhibited the formation of the enzymatic reaction product nearly completely (Fig. 10a-c). In the light microscope the material under study was completely devoid of blue colouring. Incubation in eserine, on the other hand, had no effect at all on the formation of the coloured product of enzymatic reaction.

Localization of cholin esterase activity. When using the Karnovsky and Roots (1964) method , red-orange precipitates were found in pollen grains and pollen tubes at the site of the enzyme activity. In nongerminating pollen enzymatic reaction appeared in the wall; a particularly great agglomeration of precipitates was observed in the

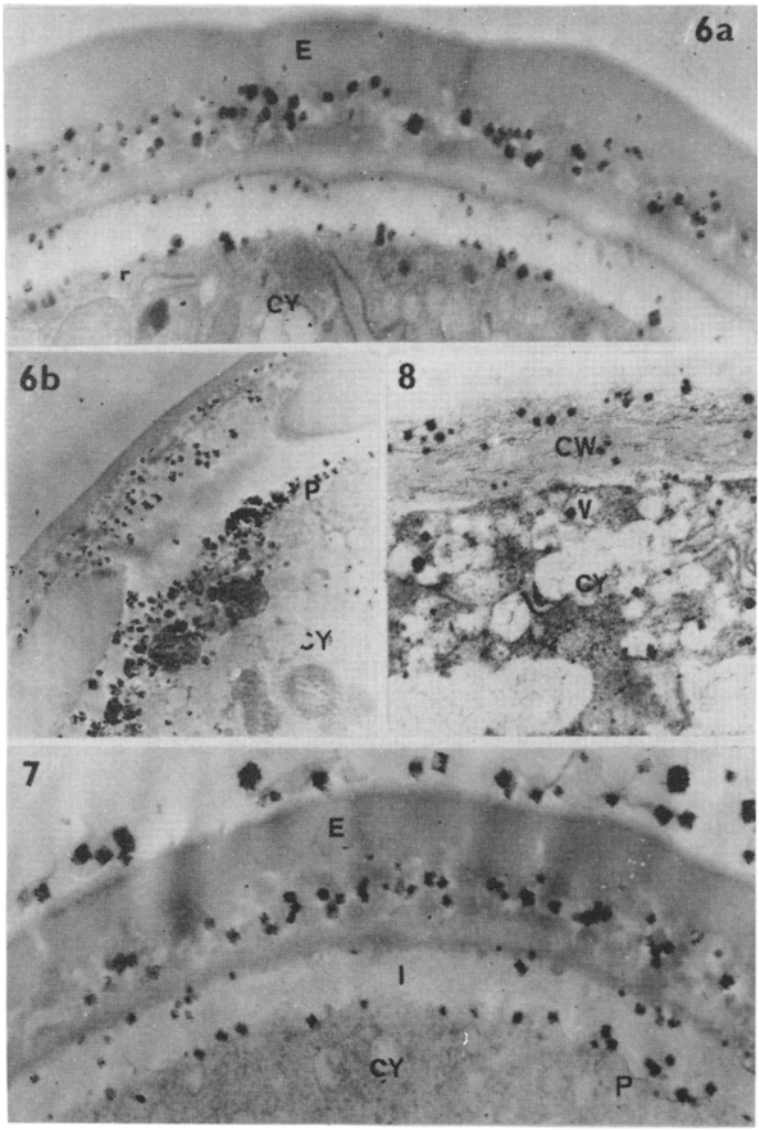


Figs. 1-3. Localization of nonspecific esterase in the pollen grain and pollen tube. - 1. Hydrated pollen grain - precipitates occur in rosette-like structures inside the intine. x 35 000. - 2. Germinating pollen grain - precipitates are present in the sexine and on the sporoderm surface. x 42 000, - 3. Cytoplasm of pollen grain - precipitates are localize round spherosomes and in vesicles. x 42 000.

Figs. 4-5. Localization of nonspecific esterase in pollen tube tip. - 4: At the top of the pollen tube tip the precipitates are associated with pectin wall fibrils. x 60 000, - 5: Below the top of the tip precipitates are present in the inner layer of the pecto-cellulose wall. x 36 000.

Abbreviations to all the figures: exine - E, intine - I, cytoplasm - Cy, cell wall - cw, aperture - A, spherosome - S, plasma membrane - P, vesicle - V, vacuole - Va, mitochondrion - M.

aperture (Fig. 13a-c). In the pollen tubes the enzyme activity was associated with their tips (Fig. 14a). In the short pollen tubes reaction product was localized round the entire tubes, while in the long ones only round their tips.



Figs. 6-7. Localization of cholin esterase in pollen grain. - 6a,b: In hydrated pollen grain precipitates occur in exine, on the plasma membrane, and some scare ones in the intine (- 6a), agglomeration of precipitates is visible in the aperture (- 6b), - 7. In germinating pollen grain precipitates are localized in the sporoderm and on the pollen surface. All Figs. x 36 000. For abbreviations see Figs. 1-5.
Fig. 8. Cholin esterase localization in pollen tube. Precipitates occur in vesicles. x 35 000.

In the electron microscope the reaction product appeared in the form of oblong precipitates. Their localization in the non-germinating hydrated pollen was different from that of indigo precipitates. The precipitates were present in the exine and in the intine area, mainly in association with the outer side of the plasma membrane (Fig. 6a). A particularly great agglomeration of precipitates was observed in the aperture (Fig.6b). No precipitates were found in the cytoplasmic organelles.

In germinating pollen the localization of precipitates was observed to change. They were found in the intine and exine of the sporoderm and on the surface of the pollen grain (Fig. 7). This points to the secretion of the enzyme to the medium.
In the pollen tube tip the precipitates were localized mainly in the external layer of the wall and on its surface (Fig. 8). Only a few precipitates appeared inside the tip wall. In the cytoplasm the reaction product appeared in vesicles 60-100 nm in diameter. The vesicles were often observed in contact with the plasma membrane, which points to exocytosis (Fig. 8).

Table 1. Localization of nonspecific esterase and cholin esterase in the pollen tube of *Vicia faba.*

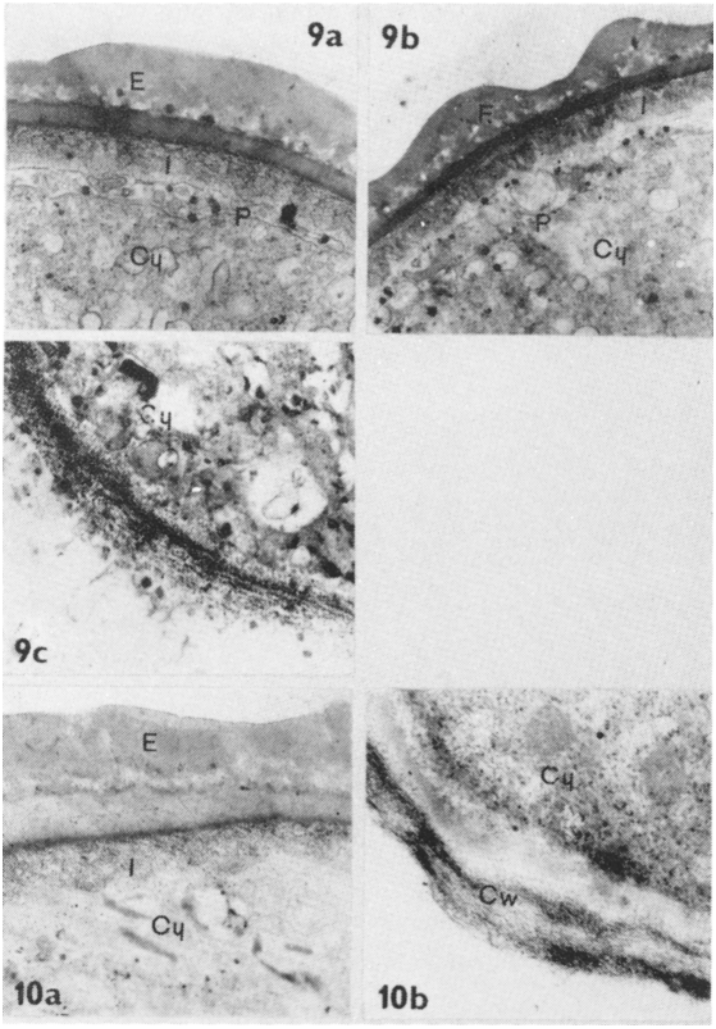
Localization of the enzyme	Nonspecific esterase	Cholinesterase
hydrated pollen grain	intine, vesicles, spherosomes	plasma membrane, exine, aperture
germinating pollen grain	exine, pollen surface	aperture, sporoderm,pollen surface
pollen tube	pectin and cellulose cell wall of the tip	vesicles in the tip, cell wall of the tip

The controls failed to give an explicit answer to the specifity of the enzymatic reaction. In the material incubated without substrate (Fig. 9a) or in the presence of eserine (Fig. 9b-c), the reaction was only partly prevented. Both in pollen plasma membrane and on the pollen tube tip precipitates did appear, but their number was lower than in the material incubated in the presence of ACh or without eserine. NaF which inhibited the reaction to nonspecific esterase, had no significant influence on the formation of the product of reaction to cholinesterase. The localizations of both esterase types are presented in Table 1.

The effect of actinomycin D and cycloheximide on esterases activity localization. Actinomycin D did not inhibit germination in pollen grains of *Vicia faba.* The pollen tubes grew somewhat more slowly, but the percentage of germinating grains was similar to that in the control. Cycloheximide, on the other hand, at the concentration used, caused a significant reduction of the percentage of germinating pollen (down to *ca.* 15 %) and a very pronounced reduction of the growth rate of the pollen tubes. After 2 h culturing, the tubes did not exceed 25 % the length of control tubes and did not grow any more.

In pollen grains whose hydration took place in the presence of actinomycin D or cycloheximide, the localization of nonspecific esterase was the same as in the control. Blue colouring was observed in the sporoderm (Fig. 11a-c). Likewise, actinomycin D and cycloheximide had no effect on the activity of the enzyme in the

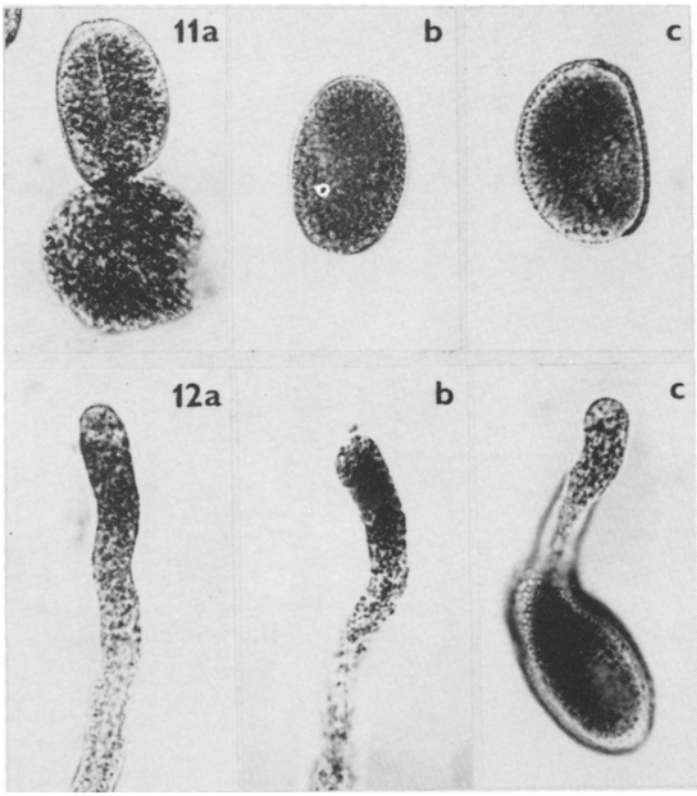
pollen tubes; the reaction product could be seen in the cytoplasm and in the tip wall (Fig. 12a-c).



Figs. 9a-c. Control of reaction to cholin esterase activity. - 9a: Pollen grain incubated without substrate - scattered precipitates are present on plasma membrane. - 9b,c: Material incubated in the presence of eserine - scattered precipitates on pollen plasma membrane (b) and in the wall of pollen tube tip (c) can be seen. All Figs. x 25 000.
Figs. 10a-b. Control of reaction to nonspecific esterase activity. A lack of precipitates in pollen wall (- 10a) and pollen tube wall (- 10b) incubated in the presence of NaF. x 25 000. For abbreviations see Figs. 1-5.

Neither of the applied inhibitor affected the localization of cholinesterase activity in non-germinating hydrated pollen grains. The reaction product occurred, like in the

control, in the sporoderm and mainly in its aperture (Fig. 13a-c). In growing pollen tubes the effect of actinomycin D and that of cycloheximide on the localization of the enzyme were different. In pollen tubes growing in the presence of actinomycin D the product of raction appeared outside the tip, *i.e.* like in the control (Fig. 14a-b); whereas in pollen tubes germinating and growing in the presence of cycloheximide, the enzyme activity did not appear in the tip. In most cases the product of reaction was observed in the aperture (Fig. 14c).

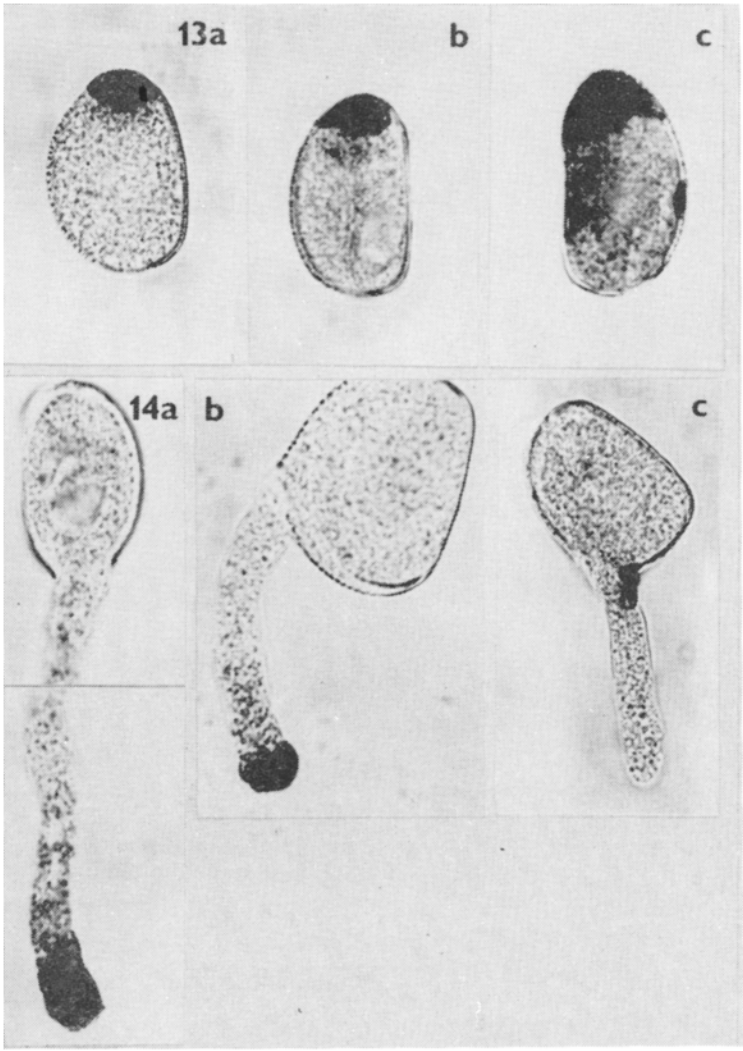


Figs. 11-12. Localization of nonspecific esterase in pollen grains and pollen tubes grown in standard medium (control), in the presence of actinomycin D or cycloheximide. x 1 200. - 11a-c: In hydrated pollen the enzyme occur in the sporoderm and in cytoplasm: 11a - control (cytoplasm lying beside injured pollen), 11b - actinomycin D, 11c - cycloheximide. - 12a-c: In pollen tubes nonspecific esterase occurs in cytoplasm and in tip wall: 12a - control, 12b - actinomycin D, 12c - cycloheximide.

Discussion

Localization of the enzyme. The present experiments permitted to distinguish in the pollen grain and pollen tube of *Vicia faba* two types on esterase, nonspecific esterase and cholin esterase.

In hydrated pollen grain nonspecific esterase is associated with the cytoplasmic organelles (spherosomes and vesicles) and with the intine. The presence of esterase in the spherosome membranes confirms the suggestion of Górska-Brylass (1965), that the activity of that enzyme is connected with lipid metabolism.



Figs. 13-14. Cholin esterase localization in pollen grains and tubes grown in standard medium and in the presence of actinomycin D or cycloheximide. x 1 200. - 13a-c: In hydrating pollen grain the enzyme is present in the sporoderm, a particular agglomeration is observed in the aperture: 13a - control, 13b - actinomycin D, 13c - cycloheximide. - 14a-c: In pollen tubes grown in control medium (- 14a) and in the presence of actinomycin D (- 14b) the enzyme is present in the tips, whereas in the presence of cycloheximide (- 14c) the enzyme does not occur at the tube tip, but in aperture place. For abbreviations see Figs. 1-5.

In the intine this enzyme is localized in rosette-like agglomerations. The occurrence of intine proteins in tubular or lamellar structures inside the polysaccharide matrix has been described in *Crocus* (Knox and Heslop-Harrison 1971).

During pollen germination the intine nonspecific esterase is secreted to the medium, probably by release of the enzyme from rosette-like intine structures. In germinating pollen the active enzyme is present in exine and on the pollen surface, but it was absent from the intine. In the pollen tube an activity of nonspecific esterase is present in the tube tip. The activity of the enzyme occurred in the cytoplasm and in the pectin and cellulose tip wall. The wall enzymes are probably connected with the construction of the pollen tube wall and are also secreted into the medium. The secretion of enzymes to the medium in the early germination period and during pollen tube growth is a well-known phenomenon and is connected with pollen-pistil interaction (Heslop-Harrison 1987).

The esterase found in the pollen grain and tube of *Vicia faba* using Karnovsky and Roots (1964) method shows properties which can be described for cholin esterase. This enzyme is able to use ACh as a substrate and eserine partly inhibits its activity. Its localization was different from that of nonspecific esterase. Moreover, NaF, which inhibited nonspecific esterase, did not affect the formation of the product of reaction to cholinesterase. Plant cholinesterases are not identical with animal acetylcholinesterases but their molecular and biochemical properties suggest that they perform the same functions in regulating the level of ACh in plants (Tretyn and Kendrick 1991). In hydrated pollen the activity of cholin esterase is present on the plasma membrane and in the exine. The source of precipitates occurring in the intine was probably the enzyme which was released from the plasma membrane during hydration of pollen. A particularly large accumulation of this enzyme was in the aperture. The occurrence of cholin esterase in the plasma membrane and in the wall of plant cells has been described in bean roots (Fluck and Jaffe 1974) and in wheat karyopses (Tretyn *et al.* 1986). During pollen germination in *Vicia faba*, the cholinesterase is secreted outside the pollen - its activity is present on the surface of the sporoderm.

In the pollen tube the cholin esterase is present in its tip. The enzyme was secreted to the medium by exocytosis. Its activity occurred mainly on the external side of the tip wall and in vesicles near the plasma membrane. The process of secretion of the cholin esterase into the medium by the germinating pollen grain and pollen tube in *Vicia faba*, demonstrates that this ezyme plays a part in the pollen-pistil interaction. A similar enzyme has been found on the surface of the receptive stigma in *Pharbitis nil* (Bednarska and Tretyn 1989). The participation of the cholinergic-like system in interspecific and intertcelular interaction in plants is suggested by the fungal components of lichen *Parmelia caperolia* (Raineri and Modenesi 1986).

The effect of inhibition of transcription and translation on the localization of the enzyme under study. The localization of activity of both esterases under study in pollen grains hydrating in the presence of actinomycin D or cycloheximide is the same as in the control. This shows that the enzymes are synthesised in the period preceding anthesis. This is in agreement with the results of studies on the genesis of the sporoderm proteins: the exine proteins are synthesised in the tapetum, the intine

proteins in the cytoplasm of the microspore or of the vegetative cell during pollen maturation (Knox 1984).

The localization of activity of cholin esterase in the growing pollen tube tip seems to depend on the new protein synthesis. In pollen tubes germinating in the presence of cycloheximide, no activity of cholin esterase was found in their tips but often, like in non-germinating pollen grains, in the aperture place. This may be due to inhibition of synthesis and/or to enzyme transport. The localization of cholinesterase in the tube tip is not affected by actinomycin D. This suggests that a new translation, which is probably necessary for the activity of the enzyme in the tube tip, is independent of the new transcription.

It is known that germination and growth of pollen tubes do not require new RNA synthesis (Mascarenhas 1975, Tupý *et al.* 1986). On the other hand, inhibition of translation causes inactivation of the pollen tube growth (Picton and Steer 1983).

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