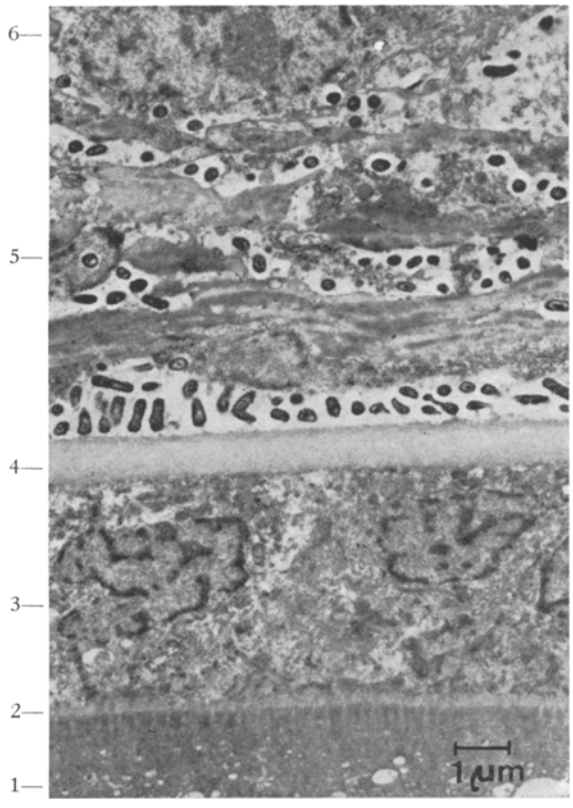

Dünnschnitt durch die peripheren Teile der Eizelle und die umgebenden Follikelschichten. *Zoarces viviparus*, ♀, 13 cm (Nr. 114; fixiert am 14.11.68. Glutaraldehyd, Vestopal W). 1. Oocytoplasma; 2. Cortex; 3. Follikelepithel; 4. Basallamelle; 5. Theca folliculi mit zahlreichen Bakterien in den Interzellularräumen; 6. Bindegewebe.

der Endphase absterbende Embryonen den Tod des Muttertieres bedingen. Ausserdem entwickeln sich im Ovar zahlreiche gramnegative Bakterien, für die die schleimige, mucopolysaccharidhaltige Ovarialflüssigkeit[2] ein günstiger Nährboden zu sein scheint. Interessant ist, wie weit die Bakterien zu den Eizellen vordringen. Jede Oocyte ist umgeben von einer Rindenschicht (Cortex; Figur). Darauf folgen nach aussen[3,4]: perioocytärer Interzellularraum, Follikelepithel, Basalmembran, Theca folliculi und weiteres Bindegewebe. Das Bindegewebe ist stark vaskularisiert und von erweiterten Interzellularräumen durchsetzt. In diesen flüssigkeitserfüllten Räumen dringen die Bakterien vor, und zwar bis an die Basalmembran. Diese fungiert also nicht nur als Ultrafilter, sondern auch als Bakterien-Barriere. Bakterien erzeugen zahlreiche Fischkrankheiten[5,6]. Ihr Einfluss auf den Verlauf der Oogenese bei *Zoarces* ist unbekannt. Bei freilebenden Tieren wurden sie bisher nicht beobachtet.

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Evidence of a Pollen Esterase Capable of Hydrolyzing Sporopollenin

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Summary. The activity of an esterase is associated with the germinal pore formation of the pollen grains of barley. Cytochemical evidence is given that the enzyme is capable of hydrolyzing sporopollenin. Some staining methods new to palynological studies were introduced.

Normally, the pollen grains of the graminaceous species carry one round germinal aperture, pore (Figure 1), the development of which was recently described[2]. The male sterile barley mutant, *msg6cf*, was shown to produce inaperturate pollen[3]. The cytochemical studies of the mutant and non-mutant microspores revealed different sites of esterase activity. One of them, surface esterase, appeared in the primexine and then continued in the exine almost through the entire period of exine growth. Another was localized on the underside of the aperture region. This enzyme has been characterized as late poral esterase. These enzymes will be discussed below in connexion with some new staining methods developed during the study.

Methods. Napthol esters were mostly used as substrates for unfixed, frozen sections (Table). The cytoenzymological method took advantage of the simultaneous coupling method with freshly made HPR, hexazotate of pararosaniline (p,p′,p″-triamino phenylmethylene HCl) as the coupler[4,5]. Cytological stainings were performed with unfixed frozen sections. Fast blue B salt (tetrazotized *o*-dianisidine) was applied as a filtered solution, concentration below 0.5% in 0.2 *M* phosphate buffer, at the final pH 8.0. Freshly made diazotate of *p*-nitroaniline was applied with the same method as HPR, but at a concentration 3 times higher, viz. 15 m*M*.

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Esterase activity during the later half of YSP examined with different substrates

Substrate and method of application	Incubation time (min)	Esterase activity	
		At pore	In cytoplasm of tapetum cells
1-Naphthyl acetate, Ac	10	+++	+++
1-Naphthyl acetate, Tgch	10	+++	+++
2-Naphthyl acetate, Ac	10	++	++
Naphthol AS-D acetate, Ac	20–30	+	+
5-Bromoindoxyl acetate, ethanol	30	+	+
1-Naphthyl propionate, Ac	10	++	++
1-Naphthyl propionate, Tgch	10	++	++
1-Naphthyl butyrate, Ac	20	(+)	+
1-Naphthyl butyrate, Tgch	20	−	−
1-Naphthyl valerate, Ac	30	−	+
1-Naphthyl caprylate, Ac	30	−	−
Naphthol AS nonanoate, Ac	30	−	−
Naphthol AS nonanoate, Tgch	30	−	−
Naphthol AS nonanoate, dimethyl acetamide	30	−	−
1-Naphthyl laurate, Ac	60	−	−
1- or 2-Naphthyl palmitate, Tgch	60	−	−

The activity was assessed from the cytological preparations processed by the simultaneous coupling method with HPR. The 'Naphthyl' substrates were applied in the equimolar amount (13 m*M*). The incubation solution had the final pH 7.7, except for naphthol AS-D acetate, 5-bromoindoxyl acetate, and naphthol AS nonanoate, the methods of which were given in detail formerly[4]. The substrates were usually dissolved in acetone (Ac), or dispersed with sodium tauroglycocholate (Tgch). All the substrates were purchased from Sigma Chemical Co. The strongest staining is indicated by +++, fainter shades by ++, + or (+), and preparations indistinguishable from the control, by −. The control sections were run omitting the substrate. The indication marks can be taken as rough estimates of esterase activity, especially those processed with the 'Naphthyl' esters as the substrate.

Results and discussion. The reddish-brown azo dye final product by the studied enzymes could be distinguished from the yellow staining of the exine with hexazotized pararosaniline. This staining is observable during a period referred to yellow staining period (YSP). YSP starts soon after the release of the microspores from the callose wall. Before the start of YSP, there is no evidence of an exine containing sporopollenin. YSP abruptly stops when the exine becomes two-layered with tectum and foot-layer and develops the relief typical of the mature pollen surface. Thus, YSP covers the growth period of the exine.

Late poral esterase appears soon after the first sign of YSP as an inner lamellate part of pore. The site of the enzyme rapidly expands to the extent of the aperture region (Figure 2). From the side view, it seems to occupy a thin layer just under the annulus and the operculum as well as the pore between them (Figure 3). With the TEM studies, that site appears to consist of a globular and membraneous structure (Figure 4). It seems that the esterase is localized in the pore proper in a position to admit sporopollenin depositing there. Consequently, the pore itself does not become covered (Figure 1). Sporopollenin is considered to be produced by the tapetum[4,6].

When trying different methods, it became evident that the structures containing sporopollenin, viz. the exine, the tapetum membrane, and the Ubisch bodies can be specifically stained with Fast blue B salt. The foot layer of the exine of a mature pollen grain stains pink, while the tectum is paler, pink to greenish yellow after adequate washing in tap water. The difference is best seen at the pore region, where the thickened foot layer of annulus is distinctly pink, and the operculum of the tectum colour. The different nature of the layers is also visible with the fluorescence microscope in the unstained and Fast blue B stained exines, or those stained with diazotized *p*-nitroaniline (see below). The UV-absorption of the tectum and foot layer was also found to be different in some other species[7]. The stronger staining of the pore site with Fast blue B is evident at, or little after, the appearance of late poral esterase. The pore proper remains unstained with Fast blue B, unlike the preparations processed for esterases. A thermal treatment (180 °C for 6 h or longer) changes the exine to a form which is weakly stainable with Fast blue B.

Also all the other diazonium salts tested dye the sporopollenin-containing structures, and are recommended for palynological studies. One of the dyes used was freshly made diazotized *p*-nitroaniline, a dyeing reagent of phenolic substances and coumarins[8]. It was applied at a concentration 3 times higher than that used of HPR. Therefore, they contained roughly the same concentration of azo groups. The staining with diazotized *p*-nitroaniline does not reveal the YSP evident with HPR, but dyes the exine yellow at all the stages. Thus, there is a change in the exine at the end of YSP. This change is evidently a polymerization process, rendering the sporopollenin into a state not admitting the larger and more complicated molecules of HPR any longer, but the sporopollenin continues to admit the smaller diazotized *p*-nitroaniline molecules and many other types of diazotates of a smaller molecular size. The polymerization has been recently discussed[9].

According to BROOKS and SHAW[10], sporopollenin is an oxidative polymer of carotenoids and carotenoid esters. It contains and can produce weak phenolic acids[9,10]. Sporopollenin is one of the most resistant biogenic sub-

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stances, slowly degraded by a few micro-organisms; but it has been suggested that plants lack enzymes capable of degrading sporopollenin[9]. Changes have been described on the pollen surface when placed on the stigmas of some species[12,13]. However, the degradation of sporopollenin has not been demonstrated so far, rather the changes can be ascribed to the release of proteinlike substances[14].

The following picture of the formation of exine and pore was outlined: YSP is a stage of sporopollenin deposition and occurs as an unhardened form of the exine. The first microspore wall, the primexine, of the graminaceous species is pierced by cavities, probacules. These are routes by which the pro-sporopollenin reaches the growing foot layer. A transport function for probacules was also suggested by FLYNN and ROWLEY[15]. The site of the primexine is finally detectable in the mature pollen of barley as the arcaded space between tectum and foot layer. At the pore, the membrane structure of late poral esterase directly admits the depositing pro-sporopollenin.

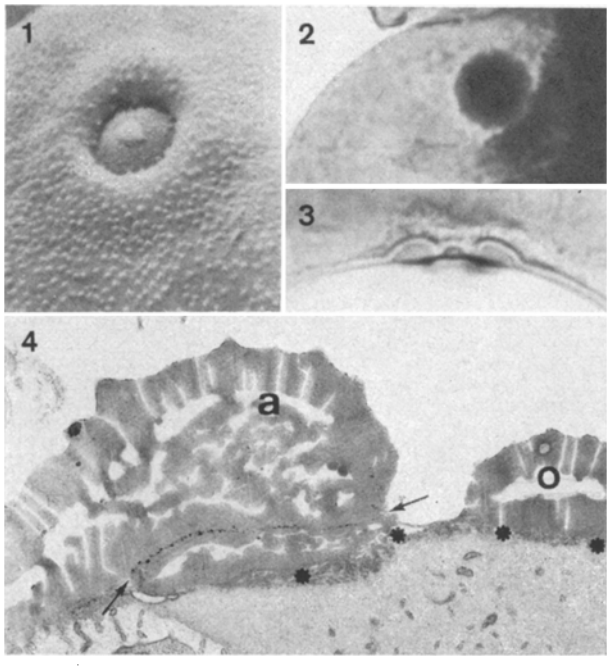

Fig. 1. A SEM view of the apertural site, where the annular thickening is evident. An operculum is visible in the middle of the pore, which is otherwise devoid of the exine coating. × 3,330.

Fig. 2. A top view of late poral esterase activity (the stained circle) seen with the light microscope approximately at the middle of YSP. The staining to the left indicates activity in the tapetum and the Ubisch bodies. The staining on the surface of the microspores suggests that the activity of surface esterase has a porous appearance. × 1,750.

Fig. 3. A side view of a sectioned pore of an almost mature pollen grain processed for esterase. The activity of late poral esterase is evident in the thin layer on the underside of the pore region. × 1,750.

Fig. 4. A TEM side view of annulus (a), operculum (o), and the pore proper between them. The globular and membraneous structure, the evident locality of late poral esterase, is distinguishable on the underside of the annulus and the operculum, and the space between them (asterisked). The thickened annular foot layer has an irregular appearance. The tectum is more regular than the foot layer, except for the operculum, where the layers take very similar appearance. The core membrane[2,4] is visible in the annulus (arrows). The cavea between the tectum and foot layer looks empty, but sparsely pierced by the bacules. Prepared as given elsewhere[4] of an almost mature pollen grain. × 25,500.

That is hydrolyzed and transferred to below the annulus, being responsible for the much thicker annular foot layer (Figure 4), which has a different appearance from that of the tectum.

The stronger stainability of the foot layer suggests an increased number of reactive sites for the dyeing reagent. Based on this differential staining, it seems that surface esterase affects the sporopollenin, which will contribute to the foot layer all over the pollen wall. A thermal dehydration of sporopollenin has been pointed out[16]. Thus, the disappearance of the stainability after the thermal treatment could be due the dehydration of the phenolic acids.

The function of late poral esterase is essential for the pore formation. Unlike surface esterase, late poral esterase remains active throughout the pollen maturation, and is detectable even in pollen grains at anthesis. It might have cutinase activity at the pollen germination. Late poral esterase is usually detectable also on the *msg6cf* mutant pollen grains, which seem to be devoid of an adequate early poral ontogeny on the primexine. The localization of late poral esterase seems to be confined to the structure under the aperture (Figure 4) and is thus unable to reach the tectum. This apparently accounts for the fact that the mutant pollen grains studied usually bore an irregular foot layer and an unaffected tectum at the apertural site[3,4].

Late poral esterase has a high affinity towards the substrate 1-naphthyl acetate. The affinity decreases when the carboxyl acid side chain of the substrate gets longer (Table). The enzyme cannot be considered to possess lipase activity. The indigogenic method with 5-bromoindoxyl acetate[4] revealed only cytoplasmic esterase. The optimum pH of late poral esterase lies between pH 6.5 and 8. The enzyme is substantially inactive at pH 5.0. None of the several inhibitors tested was considered to affect the enzyme. These envolved neostigmine bromide, a plant cholinesterase inhibitor[17]. Cholinesterase was expected to be present in pollen[6].

Sporopollenin contains carboxyl esters[16]. The esterases discussed must be capable of hydrolyzing sporopollenin, or its early form, pro-sporopollenin. This is suggested by the following points: a) The sporopollenin affected by the esterase is chemically changed judged from the differential staining. b) The appearance of the esterases approximately coincides with or closely follows the start of the deposition of sporopollenin. c) Late poral esterase is localized precisely to admit depositing sporopollenin to the pore proper, which remains 'open'. d) The pollen of the *msg6cf* mutant usually bears an irregular foot layer at the apertural site as evidence of the action of late poral esterase. An esterase with an activity fairly similar to the pollen esterases is remarkably abundant in the tapetum (Table), and at certain stages also in the Ubisch bodies. The esterase of the tapetum in its original state is considered to be active in the synthetic direction.

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