MORPHOLOGICAL AND QUANTITATIVE CHANGES IN THE POPULATION OF CELL ORGANELLES DURING MICROSPOROGENESIS OF PINUS SYLVESTRIS L.
III. MORPHOLOGICAL CHANGES DURING THE TETRAD STAGE AND IN THE YOUNG MICROSPORE. A QUANTITATIVE APPROACH TO THE CHANGES IN THE POPULATION OF CELL ORGANELLES.

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SUMMARY

A description and discussion are given of the morphological changes of the cell organelles during the middle and late tetrad stage and the young microspore of *Pinus sylvestris* with special reference to the pollen wall formation.

In the middle tetrad stage the template of the pollen wall pattern appears to be the result of Golgi material excretion and callose wall formation. The fine fibrillar material from the content of the Golgi vesicles, consisting partly of a polysaccharide, forms the primesexine. During the late tetrad stage the sporopollenin originates from the microspore and is deposited on membranes mainly outside the cell. It penetrates into the primesexine. The starch in the plastids disappears and the lipid granules become voluminous; both elements are related to the pollen wall formation. The swelling of the sacci may be an osmotic process.

From the quantitative approach it appears, that the young microspore contains approximately one fourth of the number of cell organelles present in the cell during zygotene. The cell organelle population probably does not change in number during the meiotic stages.

A general survey summarizes the whole process of microsporogenesis till the young microspore stage. The control of pollen wall formation is also discussed.

1. INTRODUCTION

1.1. The pollen wall formation

The pollen wall formation takes place during the tetrad stage. The variability and specificity of the pollen wall have evoked many studies about its formation, patterning and chemical composition. Answers to questions about the control and origin of the pollen wall pattern, the formation of sporopollenin and its attachment to the microspore, have been proposed by various authors in different ways.

Although a sporophytic control (ROGERS & HARRIS 1969; MEPHAM 1970; FORD 1971) as well as a gametophytic control (LINSKENS 1969; ECHLIN 1971) have been suggested in the formation of the pollen wall pattern, this control is still questionable. With regard to this point the connection between cytoplasm

and the microspore wall pattern has been reported by HORNER, LERSTEN & BOWEN (1966), ROGERS & HARRIS (1969) and HESLOP-HARRISON (1971). FORD (1971) has concluded that the initiators for the elaboration of the exine pattern have to be present in the cytoplasm before the cell plate formation takes place.

Pollen wall formation starts within the tetrad which is surrounded by a special callose wall. A space between the plasma membrane and the callose wall appears. Thereby a retraction of the plasma membrane (LARSON & LEWIS 1962; SKVARLA & LARSON 1966; LEPOUSÉ 1971) or an extrusion of vesicles outside the plasma membrane have been supposed (ECHLIN & GODWIN 1968). The plasma membrane also shows many undulations or protrusions (SKVARLA & LARSON 1966; ECHLIN & GODWIN 1968; DICKINSON 1970). In the space fibrous material, the primexine appears consisting of cellulose (HESLOP-HARRISON 1968, 1968a). It seems reasonable to assume that it is derived from Golgi vesicles (ECHLIN & GODWIN 1968; DICKINSON 1970). LEPOUSÉ (1970) has demonstrated that Golgi vesicles in the tetrad of *Abies pinsapo* contain polysaccharides and excrete their content. Around the microspore and in the sacci of *Pinus banksiana* periodic acid Schiff positive (PAS +) material is present (DICKINSON & BELL 1970). First the probacula appear in the gaps of the primexine and often above a fold of the plasma membrane, followed by the elements of the tectum. Some relations between the appearance of the probacula and different types of cell organelles were described. A relation with endoplasmic reticulum (ER) has been supposed by HESLOP-HARRISON (1963) and SKVARLA & LARSON (1966); with mitochondria by VAZART (1970); with ribosomes and vesicular components probably derived from Golgi bodies, by HESLOP-HARRISON (1968); with microtubules by ECHLIN & GODWIN (1968); with Golgi vesicles directed by microtubules by DICKINSON (1970) and in *Pinus banksiana* by the position of large cytoplasmic vesicles at the cell surface (DICKINSON 1971).

During the formation of the probacula the callose wall of *Lilium longiflorum* does not change (DICKINSON 1970). In *Abies pinsapo* (LEPOUSÉ 1971) and *Pinus banksiana* (DICKINSON & BELL 1970) long protrusions are visible on the inner side of the callose wall. LEPOUSÉ (1971) showed that these protrusions consist of callose. WATERKEYN & BIENFAIT (1968, 1970) demonstrated a regular geometric pattern on the inner side of the callose wall in *Ipomoea purpurea*, which may be considered as a template for the first spore wall of primexine matrix. In *Styphelia viridis* and *S. triflora* FORD (1971) suggested, that the callose wall is necessary for the ectexine pattern formation.

Sporopollenin is considered to be the main chemical component of the exine. Its first appearance coincides with an increase of electron density of the exine and around the Ubisch bodies or orbicules or plaques in the tapetal fluid (HESLOP-HARRISON & DICKINSON 1969). The electron dense material appears always outside the cell (ECLIN & GODWIN 1968; HESLOP-HARRISON & DICKINSON 1969). The origin of sporopollenin in the microspore has been proved in *Tradescantia bracteata* (MEPHAM & LANE 1970), in the orchid *Eulophidium sandersianum* (CHARDARD 1971), in *Abies pinsapo* (LEPOUSÉ 1971), and has been supposed in *Pinus banksiana* (DICKINSON & BELL 1970) and *Podocarpus macrophyllus* (VASIL

& ALDRICH 1970). On the contrary, it has been supposed that in *Allium cepa* (RISUENO, GIMÉNEZ-MARTIN, LOPEZ-SAEZ & GARCIA 1969) and in *Epidendrum scutella* (COCCUCI & JENSEN 1969) the sporopollenin around the microspore originates from the tapetal cells. Although the origin of sporopollenin remains an open question, the precursors of sporopollenin, possibly without any electron density, have certainly to be synthetized in the microspore as well as in the tapetal cells (HESLOP-HARRISON & DICKINSON 1969). Granules of sporo-pollenin or prosporopollenin have been reported to be present in the cytoplasm of *Eulophidium sandersianum* (CHARDARD 1971). LINSKENS & SUREN (1969) demonstrated that lipid droplets in the cytoplasm of the pollen grain of *Asclepias curassavica* may contribute to the formation of the pollinium wall. More common is the observation of the presence of layers of sporopollenin around the microspore. In these layers on the base of the bacula and tectum, lamellae or laminae of unit membrane dimension have been found (ANGOLD 1967; ROWLEY 1967; LEPOUSÉ & ROMAIN 1967; DUNBAR 1968; ECHLIN & GODWIN 1969; MEPHAM & LANE 1969; HECKMAN 1970; DENIZOT 1971). It has been postulated that the deposition of sporopollenin on lamellae of unit membrane dimension should be a universal mode of sporopollenin deposition (ROWLEY & SOUTHWORTH 1967). Even DICKINSON & HESLOP-HARRISON (1968) extend this deposition of sporopollenin on lamellae to all parts of the exine of *Lilium longiflorum*. Therefore, in theory, the formation of the pollen wall pattern should be related to the plasmalemma and connecting cytoplasmic structures (HESLOP-HARRISON & DICKINSON 1969).

After the breakdown of the callose wall by enzymes in the surrounding tapetal fluid, the microspores are set free (ESCHRICH 1961; MEPHAM & LANE 1969; DICKINSON & BELL 1970). In *Pinus banksiana* the swelling of the sacci may be a result of osmotic forces or imbibition (DICKINSON & BELL 1970).

As mentioned in a previous description (WILLEMSE 1971), the formation of the pollen wall pattern in *Pinus sylvestris* is connected with the excretion of Golgi material simultaneously with a local callose wall formation, at the site of the contact between plasma membrane and callose wall. Within the microspore the sporopollenin originates from membranes. Below the terminology of the pollen wall stratification by ERDTMANN (1969) has been used.

1.2. Quantitative approach

Quantitative microscopical investigations on cell and cell organelles are not used generally (ROSS & JANS 1968), since they are apt to misinterpretation.

From results, obtained by means of a quantitative approach, it seems that in between the cell divisions the plastids divide (MICHAELIS 1962). In *Trifolium hybridum* the plastids are not distributed at random during the cell division (BUTTERFASS 1969). Sphaerosome aggregation and disaggregation during meiosis of *Lilium longiflorum* and *L. henryi* have been demonstrated by a quantitative analysis of electron microscopic photographs (HESLOP-HARRISON & DICKINSON 1967). During the interphase and telophase of meristematic cells of *Epilobium hirsutum* the plastids, mitochondria and Golgi bodies of the whole cell were

counted. Just before cell division the Golgi bodies augment, plastids and mitochondria divide during the interphase, while all cell organelles are unevenly distributed (ANTON-LAMPRECHT 1967). During division, changes in cell volume are related to changes in the spindle (BARLOW 1970). The present study deals with a significant change in the number of plastids and Golgi bodies during microsporogenesis of *Pinus sylvestris*. An increase of organelles after the meiotic divisions could not be demonstrated.

During the sporophytic-gametophytic transition a reorganization of orgaelles in the cytoplasm occurs. In *Pinus sylvestris* some of the changes in morphology could be compared with the phenomena, which are also described in mitotic cells. From zygotene till young microspore stage the changes in morphology and quantity of the organelles are not so intensive as has been found during microsporogenesis of other plants (HESLOP-HARRISON 1971b).

2. MATERIAL AND METHODS

2.1. Morphological investigations

The same material and methods for collecting, fixation, staining and embedding were used as is described previously (WILLEMSE 1971a, 1971b).

For freeze-etching the microspores of *Pinus sylvestris* were slowly centrifuged in water and a drop of water concentrated with microspores from the pellet was placed on a small copper disc, immediately frozen in liquid freon 22 and subsequently put in the liquid nitrogen. With a Balzers apparatus freeze-etching was carried out according to the procedures described by MOOR, MÜHLETHALER, WALDNER & FREY-WYSSLING (1961) and MOOR (1964).

2.2. Quantitative approach

Up to the young microspore stage the following stages of development can be distinguished: 1. early zygotene (EZ), 2. late zygotene (LZ), 3. pachytene (P), 4. diplotene (D), 5. diakinesis (DA), 6. metaphase, anaphase, telophase I (M I), 7. interphase II (I), 8. metaphase, anaphase, telophase II (M II), 9. early tetrad stage (ET), 10. middle tetrad stage (MT), 11. late tetrad stage (LT). The zygotene has been divided in two parts on the basis of the long duration of the stage and the morphology of the nucleus. According to different stages of wall formation the tetrad stage has been divided in three parts.

Countings were made on electron microscopical (EM) photographs of sections of complete cells. For this investigation distinct photographs of cell sections in the different stages of development were selected. In the cell section a large part of the nucleus was present. Only sections of totally different cells were chosen.

In each stage of development the areas of different numbers of cell sections were determined by counting the number of points of a grid with a mutual distance of 4,45 mm covering the section. (Test countings have shown that the error in this method is approximately 1 %.) This was done on EM photographs at a magnification of 7,200 ×. Both the total cell area without cell wall and the

area of the cytoplasm were measured in this way. Then the mean value and standard deviation of these areas (number of points) were calculated for each stage of development.

The areas of the largest section of intact cells were determined on LM photographs at a magnification of 240 × using the grid (test countings have shown that the error in the method used in this case is approximately 5%). Of these areas the mean value and the standard deviation were calculated.

From the cell sections of EM photographs at a magnification of 6,000 × the number of plastids with a starch granule, plastids, mitochondria, lipid granules and Golgi bodies were counted. The mean value and standard deviation of the numbers of the above mentioned cell organelles per unit of area of the cytoplasm (= 5,9 × 5,9 μm^2 = 100 points) were calculated. In the same cells the presence of the following organelles was noted: dumb-bell shaped plastids, dumb-bell shaped mitochondria, large vesicles with a clear membrane, dilated cisternae of smooth ER (SER) and Golgi vesicles, rough ER (RER), SER, ribosomes, polysomes and microtubules. For each stage of development and each type of the above mentioned cell organelle the number of cells containing that type of organelle is given.

2.3. Statistical analysis

For each stage of development the mean value and standard deviation of the number of counted cell organelles per unit of area (100 points) in the sampled cell sections were calculated. The one way analysis of variance was applied to these values. If the result was significant at the 5% level, it was investigated with SCHEFFÉ's (1959) test for multiple comparison for each pair of mean values whether they are significantly different from each other. It has to be remarked, however, that with Scheffe's test not such a pair can be distinguished, although the analysis of variance gives a significant result. The same test was applied to the data concerning the area and greatest section of cells. Moreover, the correlation between the mean value of the area measured with EM and the section with LM for the same stage of development was tested with Spearman's correlation coefficient.

3. RESULTS

3.1. Morphological observations

3.1.1. The middle and late tetrad stage

The middle tetrad stage starts after formation of the callose wall between the four microspores. When the footlayer or nexine I of the pollen wall becomes visible, the late tetrad stage commences.

During the middle tetrad stage the nucleus is situated near the exterior of the callose wall and a small strip of cytoplasm lies between the nucleus and the callose wall. The nucleus has a homogeneous nucleolus and the karyoplasm contains granules of approximately 30 nm (*fig. 1*). Sometimes the nuclear membrane shows invaginations, a phenomenon which has also been observed in the early

tetrad stage (WILLEMSE 1971b). In comparison with the preceding stages, some plastids have a small starch granule, while also dumb-bell shaped plastids are present. The mitochondria contain an electron transparent material, including a few cristae. Also lipid granules are present, some of them connected with an electron transparent vesicle, forming a "lipid complex". Vesicles with a clear membrane and an electron transparent content are dispersed in the cytoplasm. Just before the start of the pollen wall formation cup-shaped Golgi bodies are observed (*fig. 3*). Cisternae of SER, but few RER are present in the cytoplasm. Microtubules are absent. Ribosomes as well as polysomes have been observed.

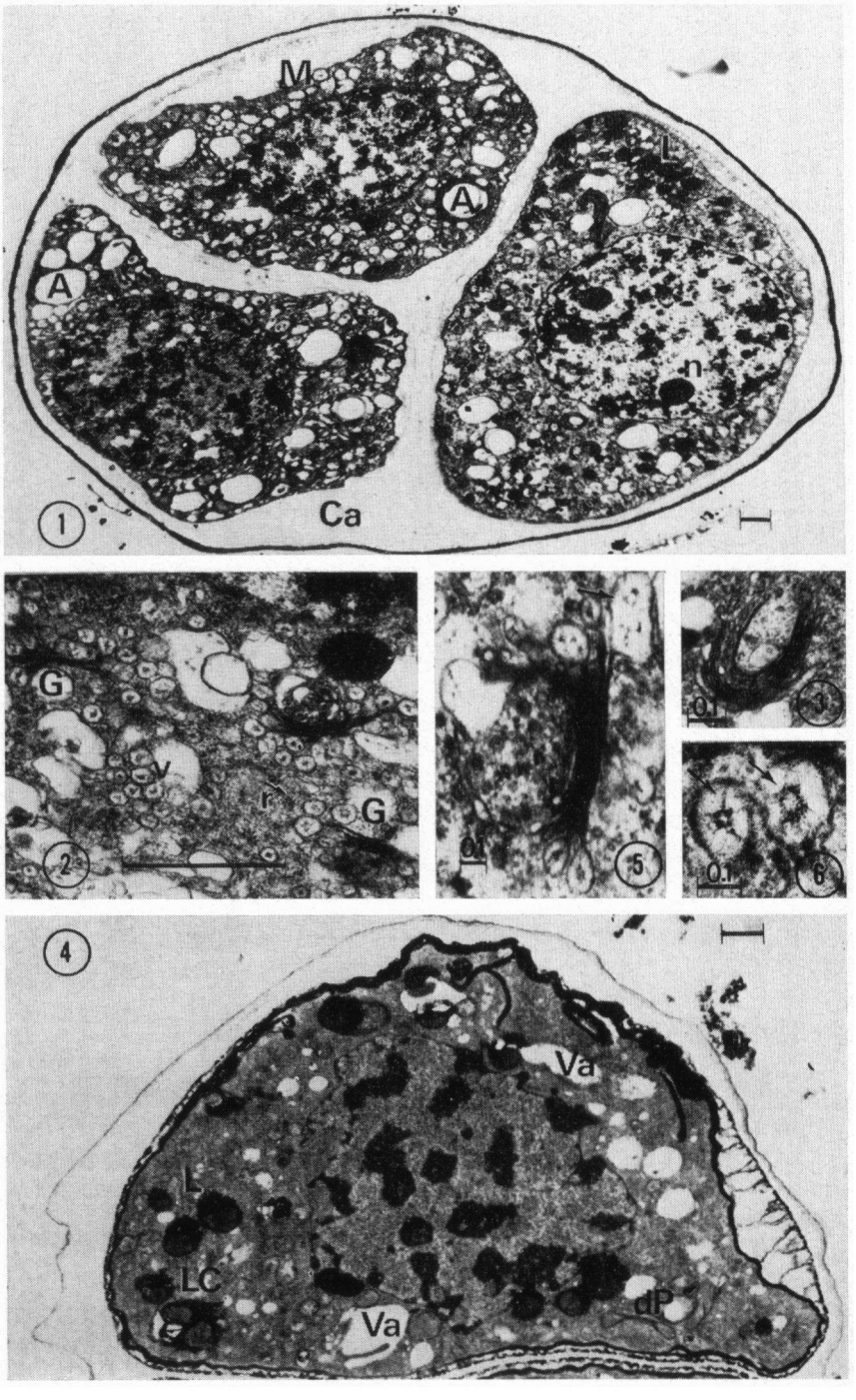
During the late tetrad stage the nucleus lies in the centre of the cell. The chromatin of the nucleus is somewhat contracted (*fig. 4*). The starch granules in the plastids have disappeared. The number and size of the lipid granules increase, while many granules are surrounded by black dots. They are connected with electron transparent vesicles and form the "lipid complex". The vesicles with a clear membrane grow in volume and the vacuolisation of the cytoplasm starts (*fig. 4*). The Golgi bodies disappear (*fig. 20*). Strands of RER have been observed more frequently (*figs. 13, 23*). The cytoplasm has many ribosomes; therefore, the polysomes are not clearly distinguishable, but they are present (*figs. 20, 23*).

3.1.2. Pollen wall formation

Golgi bodies in the cytoplasm produce a large number of vesicles (*fig. 2*). The Golgi vesicles with a unit membrane have an electron transparent content. In their central part the electron dense granular and fine fibrillar material makes contact with the unit membrane by means of the fine fibrils (*figs. 5, 6*). After lead citrate staining this inner structure becomes more distinguishable. The Golgi vesicles may fuse during their production on the Golgi body and during their transport (*figs. 5, 8*). During excretion of the Golgi material, the membrane of the vesicle makes contact and fuses with the plasma membrane (*figs. 7, 8*). The content of the Golgi vesicles is excreted between the plasma membrane and the callose wall (*figs. 8, 9*). At the point where Golgi material is excreted, the plasma membrane seems to be pushed away from the callose wall, due to the fusion with the membrane of the Golgi vesicles (*figs. 8, 9*). In this way, the plasma membrane becomes convoluted (*figs. 11, 12*).

Just before excretion of the Golgi material, the plasma membrane is situated against the straight callose wall (*fig. 10*). During excretion of the Golgi material, the contact between the plasma membrane and the callose wall persists locally (*figs. 9, 11, 12*). Here the callose wall formation is not blocked and there a protrusion on the callose wall in the direction of the cell develops. The inner side of the callose wall loses its evenness and gets many protrusions on regular distances (*figs. 11, 12, 17*).

Between the plasma membrane and the callose wall, the granular material originating from the Golgi vesicles changes gradually into a fine fibrillar network (*figs. 12, 14, 17, 18*). It precipitates against the callose wall initially between the protrusions and finally against the protrusions (*figs. 13, 17*). The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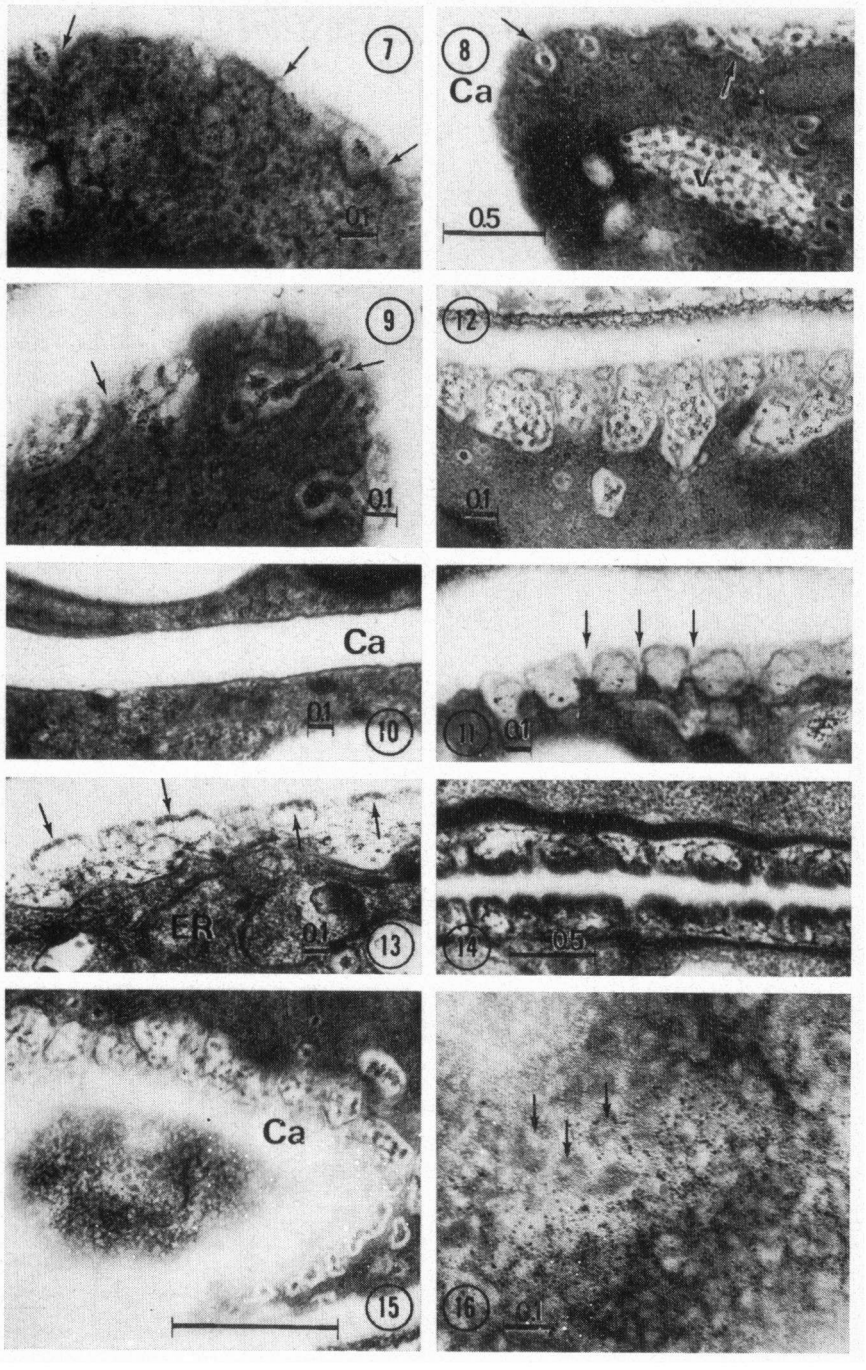
material between the protrusions becomes more electron dense, marking the first sign of the tectum (*figs. 13, 17*). Afterwards the bacula become visible as more electron dense structures. Gradually very electron dense material becomes visible on tectum and bacula. At the same time this material appears also locally along the plasma membrane, as the footlayer or nexine I (*figs. 14, 18*).

This process can be followed easily on those places where the sacci are formed. The protrusions on the callose wall and connected with the plasma membrane are branched and long with numerous contents of Golgi vesicles in between (*figs. 12, 15, 17*). The material of the Golgi vesicles becomes more fibrillar starting between the protrusions and precipitates against the callose wall between the protrusions. Above the plasma membrane the Golgi material remains a longer time more granular (*fig. 17*). In a tangential section the protrusions are round and situated at somewhat regular distance (*figs. 15, 16*). Finally, all the granular material from the Golgi vesicles changes into a fine fibrillar network (*fig. 18*). The fine fibrillar material of the Golgi vesicles precipitates and condenses against the callose wall and its protrusions (*fig. 18*). In these joint fibrils more electron dense material appears on the places of tectum, bacula and along the plasma membrane. The now completely fibrillar Golgi material makes contact with the footlayer, bacula and tectum (*fig. 18*).

The Golgi bodies now disappear in the cytoplasm and no contact exists between the plasma membrane and the callose wall (*figs. 14, 18*). No tectum and bacula are present in the region between the sacci, which is the future germination pole of the pollen tube. Under the plasma membrane a sheet of ER has not been observed here.

The very electron dense material, probably the sporopollenin, still increases in size in the sexine and nexine I. A number of long tapes is visible along the plasma membrane and on the boundary extending in the cytoplasm (*figs. 4, 19, 20*). In these electron dense tapes, lamellae of unit membrane dimension are present. These lamellae have not been observed in the tectum and bacula (*figs. 22, 28*). The plasma membrane follows the tapes deep into the cytoplasm (*fig.* 21). In the cytoplasm the tapes not always border on unit membranes (*fig. 24*). ER is sometimes found parallel to the tapes (*fig. 23*). Also a close contact with lipid granules or the "lipid complex" exists with the tapes (*figs. 20, 24*). In the cytoplasm centres of circular bowed membranes have been observed

Fig. 1. M. Tetrad stage: the nucleus with homogeneous nucleolus (n) is situated near the exterior of the callose wall (Ca). In the cytoplasm are visible plastids with a starch granule (A), mitochondria (M) and lipid granules (L), × 4,600.
Fig. 2. Detail cytoplasm with Golgi bodies (G), Golgi vesicles (v) and ribosomes (r), × 24,000.
Fig. 3. Cup-shaped Golgi body, × 54,000.
Fig. 4. Late tetrad stage: in the cytoplasm large lipid granules (L) and the "lipid complex" (LC) and a dumb-bell shaped plastid (dP). Vacuoles (Va) appear, × 6,200.
Fig. 5. Detail of Golgi body with a fused Golgi vesicle (arrow), × 37,000.
Fig. 6. Detail of granular and fine fibrillar content (arrow) of the Golgi vesicle, × 69,000.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(*figs. 21, 22*). Between and on their membranes the very electron dense material is present. Dark granular material is found in the bowed membranes (*figs. 25, 26*). The pollen wall sexine and nexine I are well developed before the microspores are set free from the callose wall (*fig. 29*).

3.1.3. The young microspore

Starting at the sacci, the callose wall and possibly the thin cell wall are partly broken down by external enzymatical degradation (*fig. 18*). In the liberated microspore the nucleus moves to the future germination pole and subsequently to the cell centre. In the cytoplasm many vacuoles appear and some plastids contain a starch granule. No further changes of cell organelles have been observed (*fig. 27*).

In the young microspore the tectum and bacula swell. The nexine I grows and the layers of the nexine II appear (*figs. 30, 31*). In the completely developed pollen wall the nexine II is not distinguishable from the nexine I (*fig. 32*). After the start of the formation of the intine the pollen wall formation stops, including the thickening of the tectum and bacula. The nexine is thicker on the germination side than on the opposite side (*fig. 27*).

The content of the sacci changes during the growth of the sacci. In comparison with a later stage of development, in freeze-etched pictures a cristallization pattern in the content of the sacci different from water has been found (*figs. 33*, 34).

3.2. Quantitative approach

Diagram I shows the changes of the largest cell section, the cell section area and the number of some cell organelles during the different stages of microsporogenesis up to the young microspore stage. The results are given of the determinations of the cell and cytoplasm area, obtained from EM photographs of cell sections and from LM photographs of intact cells. The mean values with standard deviation of the number of some cell organelles are noted per unit of area of the

Fig. 7. Excretion of the Golgi material: membrane of Golgi vesicle fuses with the plasma membrane (arrow), × 49,000.
Fig. 8. Fused Golgi vesicles in the cytoplasm (v); content of the Golgi vesicles is outside the cell (arrow), × 29,500.
Fig. 9. Plasma membrane shows undulations by its fusion with the membrane of the Golgi vesicle (arrow), × 46,000.
Fig. 10. Plasma membrane situated against the straight callose wall (Ca), × 36,500.
Fig. 11. Protrusions on the callose wall, note the contact with the plasma membrane (arrow), × 42,000.
Fig. 12. Formation of the callosic protrusions on the plasma membrane at the place of the future saccus, × 52,000.
Fig. 13. Between the protrusions the precipitation of electron dense material starts (arrow). Note the ER in the cytoplasm, × 35,000.
Fig. 14. Appearance of the footlayer along the plasma membrane, × 25,000.
Fig. 15. Local tangential section of the callose wall (Ca), × 24,500.
Fig. 16. Detail Fig. 15, arrows: arrangement of the round protrusions, × 73,000.

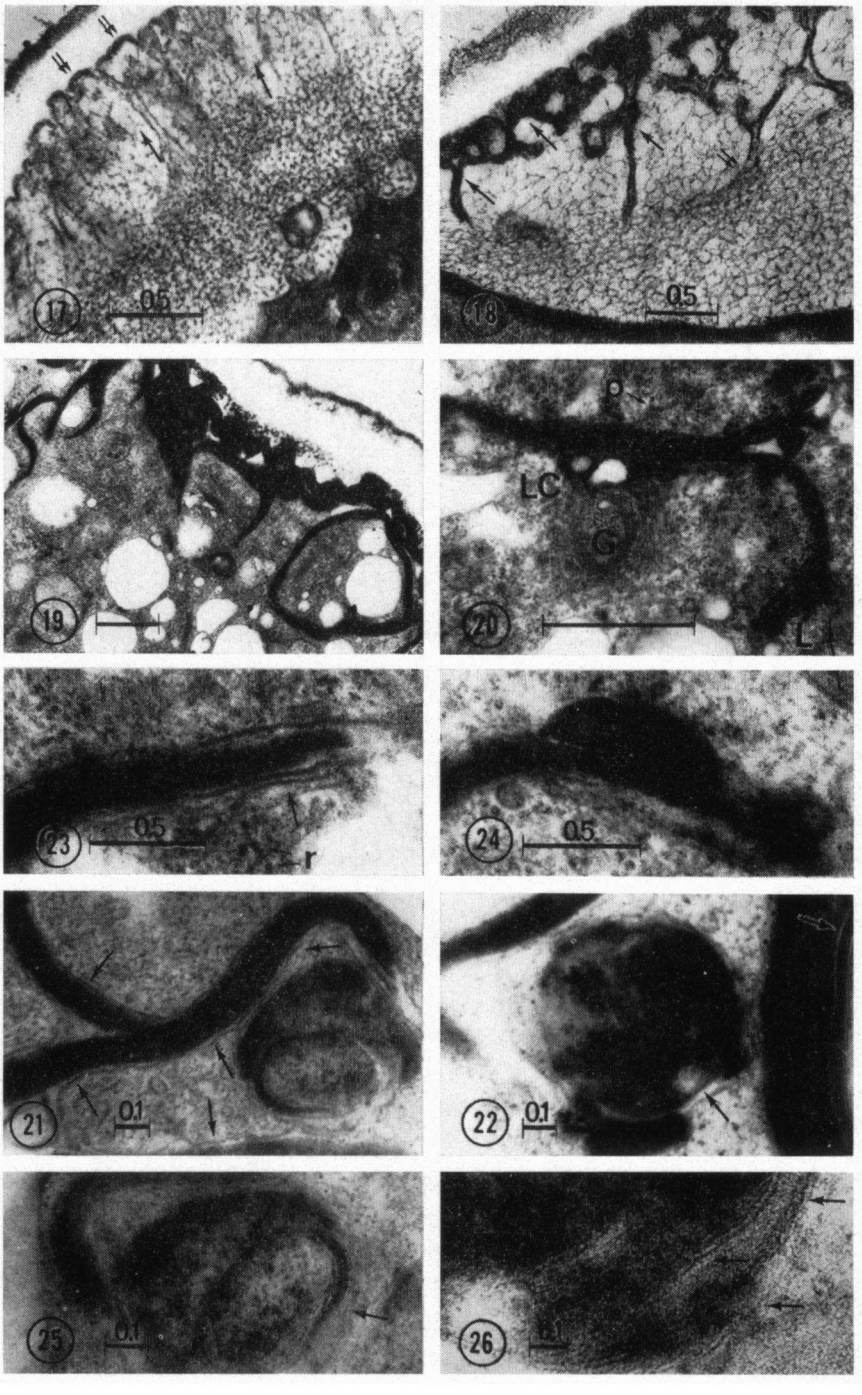
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Table 1. The portion of cell sections in which the indicated cell organelles are present during the subsequent stages of microsporogenesis.

	Stages: EZ	LZ	P	D	DA	MI	I	MII	ET	MT	LT
Total number of investigated cell sections	5	5	5	7	5	7	5	6	5	5	5
Dumb-bell shaped plastids	.8	.6	.6	.7	.6	.4	.4	.1	.8	1.0	.6
Dumb-bell shaped mitochondria	.2	.6	.2	.1	.0	.0	.2	.1	.0	.0	.2
Large vesicles	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
RER	1.0	.8	1.0	.1	.0	.0	.0	.0	.0	.2	.4
SER	.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.4
Ribosomes	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Polysomes	.2	1.0	1.0	1.0	1.0	.2	.0	.8	1.0	.2	.4
Microtubules	.2	.6	.8	1.0	1.0	1.0	.6	1.0	.2	.0	.2

cytoplasm (= 100 points). In the diagram the results of the Scheffé's test are also included.

Table I gives the numbers of cell sections in which some organelles are present during the different stages of development.

The results of the statistical analysis can be summarized as follows:

1. All measurements in the different stages by means of the one way analysis of variance for the numbers of cell organelles and for the cell area and largest section are significant at the 5% level.
2. Scheffé's test gives significant results for the plastids with a starch granule, plastids, Golgi bodies and the measurement of the largest section of the cell on LM photographs, as indicated in diagram I.

Fig. 17. Formation of the saccus. Long protrusions with fine fibrillar material (arrow) in between, against the plasma membrane a zone of more granular material. Electron dense material appears against the callose wall (arrows), × 27,000.

Fig. 18. Formation of the saccus. Against the callose wall and its protrusions electron dense material precipitates (arrow). All Golgi material is a fine fibrillar network now. Note the condensation of this material on the base of the protrusion (arrows) and the connection with the footlayer, × 20,000.

Fig. 19. Late tetrad stage: along the plasma membrane and in the cytoplasm electron dense tapes, × 9,200.

Fig. 20. Electron dense tape with lipid granule (L) and "lipid complex" (LC). Note the disappearing Golgi body (G) and polysomes (p), × 21,900.

Fig. 21. Detail electron dense tape in the cytoplasm. Note the plasma membrane along this tape (arrow), × 45,000.

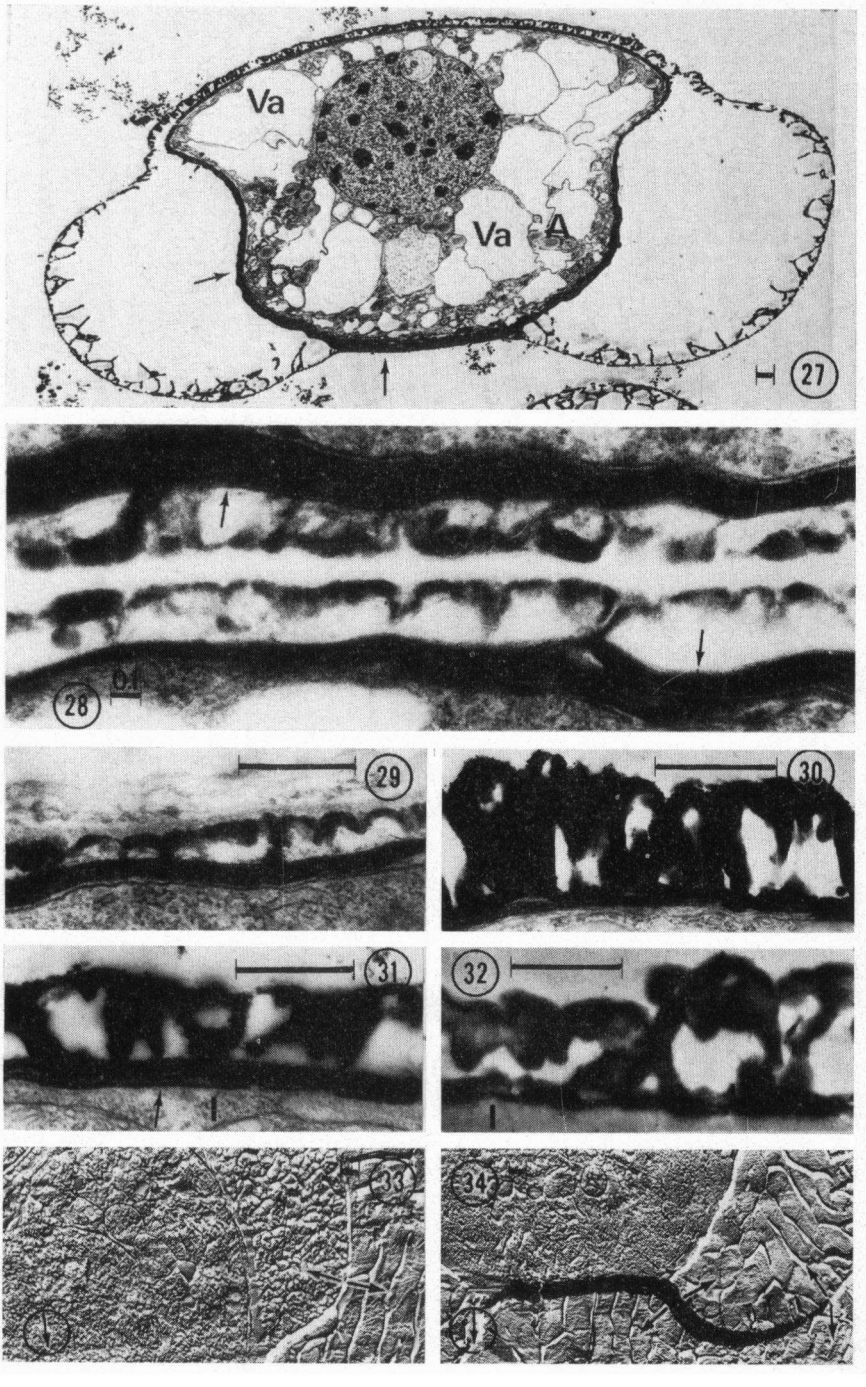
Fig. 22. Lamellae of unit membrane dimension in the electron dense tapes (arrow). Survey centre of circular membranes with electron dense material, × 43,000.

Fig. 23. ER along the electron dense tape (arrow); ribosomes (r) in the cytoplasm, × 34,500.

Fig. 24. Detail fig. 20, electron dense tape with lipid granule, × 34,500.

Fig. 25. Detail fig. 21, circular thin membranes (arrow), × 30,000.

Fig. 26. Detail fig. 22, membranes on which electron dense material appears (arrow), × 59,000.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3. A significant correlation exists between the mean values per stage of the cell section area measured on EM photographs and those of the largest section of the cell measured on the LM photographs (Spearman's coefficient was 0.83, $p < 0{,}005$).

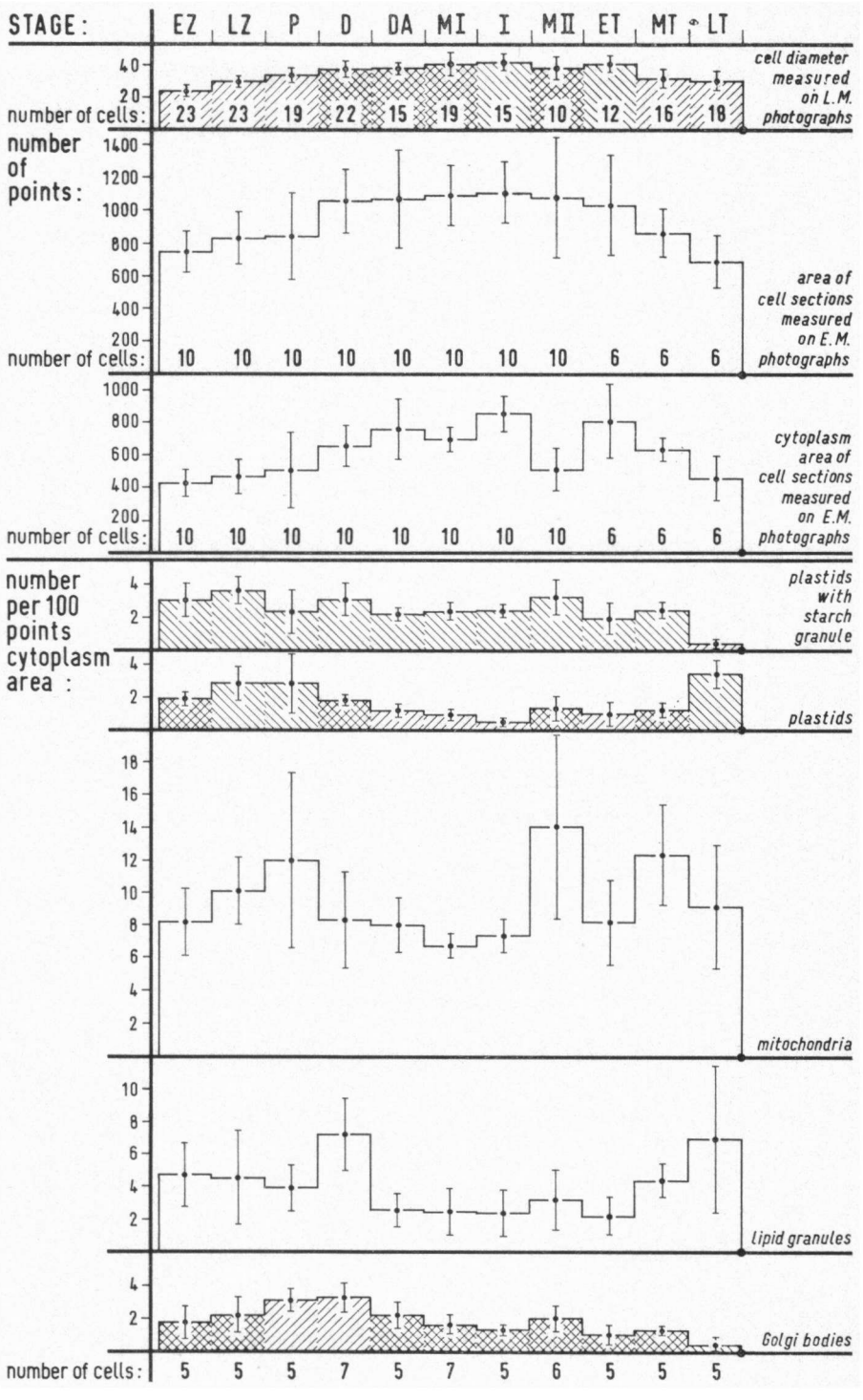
4. DISCUSSION AND CONCLUSION

4.1. Pollen wall formation: the formation of the sexine

In the pollen wall formation of *Pinus sylvestris* the template of the exine pattern is a result of excretion of Golgi material and a continued formation of callose on the plasma membrane between the excreted Golgi material. The more granular fine fibrillar Golgi vesicle content changes after excretion into a fibrillar network, which precipitates and condenses against the callose wall at first between the callosic protrusions and subsequently against the protrusions. The callose wall functions as a template. This is in accordance with the opinion of WATERKEYN & BIENFAIT (1970).

The Golgi vesicles contain the primesexine, the basic material for the tectum and bacula. This material consists of polysaccharides (LEPOUSÉ 1970) and may be hemicellulose and/or pectic-like (DICKINSON & BELL 1970). It seems to be sensitive to a poststaining with leadcitrate. From the response to uranyl acetate and leadcitrate HESLOP-HARRISON (1971a) suggests that it may contain a lipoprotein. However, the nature of the electron transparent content of the Golgi vesicle remains unknown. As soon as the content of a Golgi vesicle has been excreted, it causes a break of the contact between plasma membrane and callose wall. The plasma membrane is locally replaced by fusion with the membrane of the Golgi vesicle. The plasma membrane does not retract. The undulations of the plasma membrane are caused by the excretion of Golgi material as has been suggested by ECHLIN & GODWIN (1968). They are no artifacts as HESLOP-HARRISON (1971a) suggested. In the present author's opinion,

Fig. 27. Young microspore; cytoplasm has vacuoles (Va) and plastids (A) with a starch granule. Note the thickness of the nexine (arrow), × 2,300.
Fig. 28. Late tetrad stage: lamellae of unit membrane dimensions are present only in the nexine I (arrow), × 46,000.
Fig. 29. Pollen wall of a microspore just breaking out, tectum and bacula are small, × 17,200.
Fig. 30. Tectum and bacula are thickening in a young microspore, the formation of the nexine II starts, × 18,200.
Fig. 31. The nexine II (arrow), thickening of the tectum and bacula stops, the intine formation starts (I), × 18,000.
Fig. 32. Pollen wall of a ripe pollen grain: nexine I and II are both one layer, size of the tectum and bacula corresponds with those of fig. 30 and 31, × 17,300.
Fig. 33. Freeze-etched microspore before the growth of the sacci. Note the crystallization pattern in the saccus and the surrounding water (arrow), × 1,980.
Fig. 34. Young microspore: the sacci have been developed, the content of the saccus is the same as the surrounding water (arrow), × 1,980.
Unless mentioned otherwise, the line on the figures represents a length of 1 μm.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cell diameter measured on L.M. photographs
number of cells:
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number of points:
area of cell sections measured on E.M. photographs
number of cells:
10 10 10 10 10 10 10 10 6 6 6
cytoplasm area of cell sections measured on E.M. photographs
number of cells:
10 10 10 10 10 10 10 10 6 6 6
number per 100 points cytoplasm area :
plastids with starch granule
plastids
mitochondria
lipid granules
Golgi bodies
number of cells:
5 5 5 7 5 7 5 6 5 5 5

it seems not reasonable to support DICKINSON's (1970) view that a considerable pressure may exist in the space between plasma membrane and callose wall.

The localization of the protrusions is already determined during the very first excretion of the Golgi material. The Golgi vesicles are not directed by microtubuli during their transport to the plasma membrane. In the first instance they are juxtaposed outside the plasma membrane. Between the excreted Golgi material the contact between plasma membrane and callose wall persists; here the origin of a protrusion may be found. The contact between plasma membrane and callose wall seems necessary for the formation of the callosic protrusions.

The excretion of Golgi material probably represents a rapid process (ECHLIN & GODWIN 1968; BROWN 1969). In pine the excretion of Golgi material occurs simultaneously with the formation of the protrusions on the callose wall. This does not correspond with the theory that the whole onus of pattern establishment could be placed upon the plasmalemma and that it is associated with cytoplasmic structures including ER (HESLOP-HARRISON & DICKINSON 1969; HESLOP-HARRISON 1971a). The observations in pine do not corroborate the hypothesis of DICKINSON (1970) that after formation of the primexine the origin of the plasma membrane protrusions should be connected with Golgi vesicle excretion, which is directed by microtubules.

4.2. The formation of the nexine

In pine the sporopollenin is produced on membranes of the microspore. During the last stages of primesexine formation electron dense material appears first at the future place of the tectum, then at the bacula and finally along the plasma membrane. An opposite sequence is more generally found: first the appearance of the probacula and thereafter of the tectum (SKVARLA & LARSON 1966; ECHLIN & GODWIN 1968; DICKINSON 1970). In *Pinus sylvestris* the sporopollenin of the nexine I and II is produced on the plasma membrane or on membrane structures in the cytoplasm. However, no lamellae of unit membrane dimension in the material of the sexine have been observed. This is contrary to the suggestion of HESLOP-HARRISON & DICKINSON (1969) that in all elements of the exine the sporopollenin always is deposited on lamellae of unit membrane

Diagram 1. The mean values and standard deviations of the cell section area and the largest section of the cell (= cell "diameter") are given in numbers of point per stage of development.

The mean values and standard deviations of the number of organelles per 100 points cytoplasm area are given for each stage of development. The numbers of investigated cell sections or cells ("number of cells" on the diagram) are stated on the basis of the diagram.

The results of the Scheffé's test are indicated by means of an orientated shading. Double shading indicates that the corresponding mean values do not differ significantly from any other mean value in the same diagram. If two or more columns in the diagram have the same type of shading in one direction the corresponding mean values do not differ significantly.

dimension. The electron dense material of the sexine, probably sporopollenin, appears before the formation of the nexine I. It is transported from the microspore cytoplasm to the tectum and bacula, or along the fibrillar network of the Golgi material (*figs. 14, 18*), or as an electron transparent precursor. After formation of the footlayer, the transport of electron dense material to the elements of the sexine may be blocked. But after the breakdown of the callose wall the tectum and bacula are still thickening. Sporopollenin produced by the tapetal cells is possibly absorbed now in the sexine. As has been described elsewhere (WILLEMSE 1971), the tapetal cells produce globules on which sporopollenin is present. In that case an electron transparent precursor of sporopollenin may exist in the tapetal fluid. Another possibility would be that the transport of electron dense material to the sexine is not blocked by the nexine I. In that case all sporopollenin for the pollen wall may originate from the microspore.

The long tapes of nexine I are mainly observed outside the plasma membrane, but in pine some tapes seem to be situated partly or completely in the cytoplasm. The bundles of unit membranes observed in the cytoplasm on which electron dense material appears, may possibly be considered as the origin of the tapes consisting of sporopollenin. These unit membranes are connected with electron dense granular material, possibly originating from the lipid granule. A connection of lipid granules or the "lipid complex" with the tapes has also been observed. A relation may exist between the lipid granules, which are very voluminous in this stage, and sporopollenin production, as may occur in *Eulophidium* (CHARDARD 1971) and *Asclepias* (LINSKENS & SUREN 1969). The deposition of sporopollenin on membranes occurs in *Pinus sylvestris* inside as well as outside the cytoplasm. The high number of ribosomes in the cytoplasm and the decrease of starch in the plastids may also be related to the pollen wall formation.

A difference exists in the composition of the sexine and the nexine. The sexine, partly derived from material of the Golgi vesicles, contains more polysaccharides than the nexine. The callose of the protrusions is broken down during the enzymatical removal of the callose wall.

In pine the pollen wall formation may be analogous to the callose wall formation (WILLEMSE 1971b). Golgi material forms after excretion a network of fine fibrillar material, probably consisting of hemicellulose and/or pectic substances, which becomes impregnated by callose or sporopollenin. These wall substances originate from and are orientated by means of membranes, mainly outside the microspore.

4.3. Formation of the exine pattern and the swelling of the sacci

The formation of the exine pattern in pine depends on the quantity of excreted Golgi material. The first steps of Golgi material excretion are very important for the localization of the protrusions on the callose wall. In the area between the sacci the nucleus lies near to the callose wall. Here the small strip of cytoplasm contains few Golgi vesicles; protrusion formation lacks and no sexine is formed. The formation of the sacci is related to a high number of Golgi vesicles. The nexine is formed and orientated by membranes, mainly the plasma mem-

brane. During the pattern formation the number of Golgi vesicles, the content of which locally has been excreted, corresponds to the local quantity of Golgi vesicles in the cytoplasm and the localization of the nucleus. An analogous situation during cell wall formation in *Pleurochrysis scherffelii* has been reported by BROWN (1969).

In *Pinus banksiana* the content of the sacci gives a PAS+reaction (DICKINSON & BELL 1970). In *Pinus sylvestris* the network of fine fibrills in the sacci disappears. During a short time the sacci contain material, which is chemically different from water. The cytoplasm and the sacci of the microspore probably swell osmotically by uptake of water.

The reappearance of starch in the plastids and the vacuolisation of the cytoplasm of the microspore may be a consequence of an uptake of water and possibly of sugars from the tapetal fluid. No imbibition occurs as may be concluded from the simultaneous swelling of the whole microspore, the disappearance of the fine fibrillar network in the sacci and the watery content of the just swollen sacci.

4.4. Quantitative approach

The number of some types of cell organelles are determined per unit of area of the cytoplasm. This density of organelles of each stage of development can be compared in relation to each other and a quantitative survey of a part of the microsporogenesis may be given. A determination of the absolute number of some types of cell organelles demands a complete series of subsequent cell sections. This is not practicable at a high number of different stages of development.

The one way analysis of variance gives significant results in all series. This means that during microsporogenesis significant changes in cell volume and in number of some types of cell organelles occur. However, the measured mean values and standard deviations of the number of cell organelles are very fluctuating. A clear regular or systematic increase or decrease of numbers of organelles could not be stated.

These fluctuations may be due to the different reaction of the cell on the same preparation treatment, dependent on the stage of development, or to an influence of micro- and macro-climatological conditions. Of more influence is the low number of investigated cells and the possible polarity of some cell organelles in the cell, which cannot be excluded. Therefore, the results of this quantitative approach are not completely reliable.

One regularity in the results may be remarked: the density of cell organelles decreases or increases when the volume of the cell changes. From diakinesis till the early tetrad stage an increase of cell volume and cytoplasm volume and a decrease of the density of cell organelles takes place. During the second division stage the volume of the cytoplasm decreases and the density of cell organelles increases (see diagram I). This is an indication that no increase or decrease of the cell organelles may be expected. This is in agreement with the observation that chondriosomes, sphaerosomes and proplastids do not change in number during the meiosis in microsporogenesis of *Impatiens glandulifera* (STEFFEN & LANDMANN 1958).

On the basis of the quantitative results it is not reasonable to make a difference between the early zygotene and late zygotene. Differences in the number of lipid granules, plastids and Golgi bodies are demonstrated between the early tetrad, middle tetrad and late tetrad stage. This subdivision of the tetrad stage seems reasonable.

4.5. The cell size

The size of the cell increases from zygotene till interphase II and decreases subsequently after the early tetrad stage to approximately the same size as in zygotene. This is shown both by the mean values of the area and, according to Scheffé's test, of the largest section of the cells in the different stages of development and is affirmed as a positive correlation between the size and the largest section. It may be concluded that the cell volume changes during microsporogenesis. The increase of volume is not only the result of the enlargement of the nucleus and of the nuclear region during the cell divisions, but also of the increase of the cytoplasm area as is shown in diagram I. Only during the second division stage the area of the cytoplasm is compressed.

A relation to the changes in the spindle may exist, because the increase of volume occurs during the division stages (BARLOW 1970). But from prometaphase till telophase, the number of Golgi vesicles and dilated SER in the cytoplasm increases also. An osmotical change in relation to the contraction of the chromosomes may not be excluded as a cause of an increase of volume.

4.6. The cell organelles

The mean values of the number of mitochondria show a considerable fluctuation during all stages of development. In the series no differences could be given by the Scheffé's test. Therefore, it is impossible to conclude here whether the observed dumb-bell shaped mitochondria are dividing mitochondria.

The number of lipid granules increases during the diplotene and the late tetrad stage, being stages of wall formation.

The fluctuation in the number of mitochondria and lipid granules is partly due to the changes in the volume of the cytoplasm.

The number of Golgi bodies is large during the pachytene and diplotene stages of callose wall formation. From diakinesis till the late tetrad stage the number of Golgi bodies decreases, during the late tetrad stage the number is very low, the Golgi bodies disappear. It may be possible, that in the different cell processes not the same population of Golgi bodies functions. During cell plate formation a polarity of Golgi bodies cannot be excluded.

Till the late tetrad stage the number of plastids with a starch granule shows less differences. During this stage the starch in the plastids disappears.

The mean values of the number of plastids show a large fluctuation during all stages of development. During the late zygotene and the diplotene the mean value of the number of plastids and also their standard deviation are large, the reason of these high numbers is not clear. The number of plastids increases during the late tetrad stage according to Scheffé's test. This is a result of dis-

appearance of the starch in the plastids. The sum of the mean values of the plastids with a starch granule and the plastids of each stage of development shows that during microsporogenesis from the zygotene till the young microspore stage the fluctuation decreases. It can be concluded that the whole population of plastids in the cell shows no differences in number during this part of the microsporogenesis.

Although dumb-bell shaped plastids are present in all stages, also during the division stages in which plastids are not supposed to divide (MICHAELIS 1962; ANTON-LAMPRECHT 1967), it is difficult to conclude whether they are dividing plastids. A degeneration of the plastids has not been observed.

Vesicles with a clear membrane, SER and ribosomes are present in all stages. The presence of microtubules is related to the division stages. RER is absent during the division stages and changes during diplotene in SER. RER has been observed again in middle tetrad stage. Polysomes are present before and during the callose wall formation and disappear during diakinesis. A new population is observed in interphase II and possibly also in telophase II.

4.7. Survey of the quantitative approach

As mentioned above, the analysis of variance gives a significant result, but no clear systematical increase or decrease of the density of cell organelles could be found. In the description of the counted cell organelles the increase or decrease in a distinct stage could be stated, in particular in relation to different cell processes.

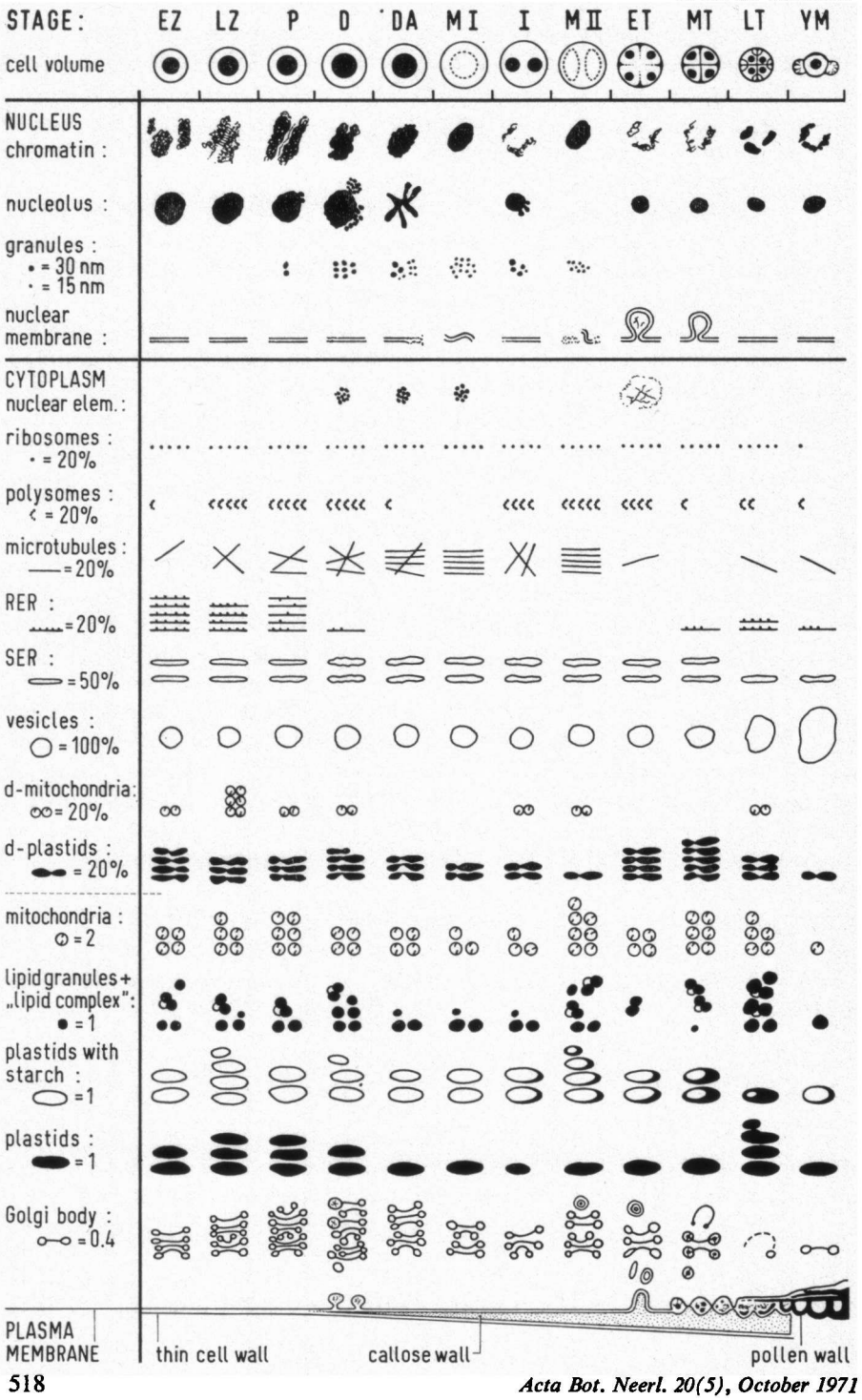
For this reason, although based on cell sections, the conclusion may suggest that starting in zygotene, the density of the cell organelle population in the cytoplasm does not change during the microsporogenesis of *Pinus sylvestris* till the young microspore stage. The RER, microtubules, polysomes and the Golgi bodies are possibly exceptions, but the quantitative changes of these organelles could be related to the process of cell division or cell wall formation. If a numerical stability of most cell organelles exists during microsporogenesis, it means that in the young microspore about one fourth part of the cell organelles of the diploid zygotene cell is present in each young microspore. The haploid nucleus of the young microspore is situated in cytoplasm of mainly diploid origin.

5. SURVEY AND SUMMARY OF THE QUANTITATIVE AND MORPHOLOGICAL CHANGES DURING MICROSPOROGENESIS OF PINUS SYLVESTRIS L.

In diagram II a survey is given of the most important quantitative and morphological changes of the cell organelles and the cell nucleus.

5.1. The cell size

During the meiotic divisions the cell size increases. The nucleus as well as the cytoplasm enlarge. Therefore, the density of cell organelles per unit of cytoplasm changes also.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cell volume
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chromatin :
nucleolus :
granules :
• = 30 nm
· = 15 nm
nuclear membrane :
CYTOPLASM
nuclear elem.:
ribosomes :
· = 20%
polysomes :
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microtubules :
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RER :
= 20%
SER :
= 50%
vesicles :
= 100%
d-mitochondria:
= 20%
d-plastids :
= 20%
mitochondria :
= 2
lipid granules+
„lipid complex":
• = 1
plastids with
starch :
= 1
plastids :
= 1
Golgi body :
= 0.4
PLASMA
MEMBRANE
thin cell wall
callose wall
pollen wall

Diagram 2. Survey of the morphological and quantitative changes during the microsporogenesis of *Pinus sylvestris*. The results of the quantitative approach are also drawn. In the margin above the dotted line the figures represent also the % of cell sections, in which the organelle is present (compare with table I). Under the dotted line the mean value of some counted organelles is approximately given per 100 point cytoplasm area (compare with diagram I). In the young microspore (YM) no quantitative result, but only the presence of the organelle is given. d-Plastids, d-Mitochondria mean dumb-bell shaped plastids, mitochondria. Nuclear elem. means nuclear elements in the cytoplasm derived from the nucleus.

5.2. The nucleus

In late zygotene and pachytene the synaptinemal complex has been observed, the karyoplasm of the late zygotene nucleus shows many "membranes". Till diplotene the nucleolus appears homogeneous, thereafter it produces granules, while dense bodies of nucleolar material are found in the cytoplasm. In diakinesis the nucleolemma is the last stage before which the nucleolus disappears. The granules of the nucleolus are mixed with the cytoplasm during the telophase and are supposed to be precursors for ribosomes and polysomes. This phenomenon is repeated in the second meiotic division. This delivery of granular material and parts of the nucleolus has also been described for mitotic cells. Before the divisions the nuclear membrane is partly broken down. The nuclear membrane shows invaginations during early tetrad and middle tetrad stage, which transport chromatin-like material to the cytoplasm. This action of the nuclear membrane has only been reported during microsporogenesis.

5.3. The cytoplasm

In the cytoplasm ribosomes are always present. During diakinesis and metaphase I there may be a lower number. A high number of ribosomes is observed in the centre of the cell after anaphase I and II, which are derived from the granular karyoplasm and change into polysomes. Polysomes may be related to the callose wall formation and possibly the pollen wall formation.

In the cytoplasm the microtubules appear in zygotene and are orientated in prometaphase I when the nuclear membrane is partly resolved. In interphase II up to the early tetrad stage they are found again.

RER, lying in packets, changes into SER during pachytene and appears again in the middle tetrad stage. During late tetrad stage electron dense material is found along a group of membranes. SER is observed in all stages, but it diminishes in the middle tetrad stage. SER is difficult to distinguish from the long flat cisternae produced by the Golgi body.

Vesicles with a clear membrane remain present in the cytoplasm, which may be considered as small vacuoles, since the increase in volume during the late tetrad stage takes place before vacuolisation of the cytoplasm of the young microspore.

Mitochondria do not change in morphology. The number of mitochondria is

fluctuating, mainly due to the changes in the cytoplasm volume.

Lipid granules increase in volume during the middle tetrad stage. Their number increases during diplotene and the late tetrad stage. The "lipid complex" appears during zygotene and pachytene and after telophase II till the young microspore stage, when callose and pollen wall formation starts. Lipid granules are possibly related to the formation of the sporopollenin.

The population of all types of plastids is constant in number. The plastids with a starch granule start to disappear in interphase II and their number reaches the minimum during the late tetrad stage. The decrease may be related to the callose wall and pollen wall formation.

The number of the Golgi bodies and the morphology of their vesicles change. Many vesicles of different shape are produced during diplotene, early and middle tetrad stage. This production is related to the formation of the fine fibrillar material for the callose wall and the pollen wall. Golgi bodies are possibly partly renewed during the second meiotic division. The nuclear membrane is partly rebuilt by fusion of Golgi vesicles.

The plasma membrane is involved in the formation and orientation of callose and sporopollenin.

From zygotene till the young microspore stage, the Golgi bodies, microtubules, polysomes and RER show the most changes. Vesicles with a clear membrane, SER and probably mitochondria show less variations. These results in *Pinus sylvestris* could not be compared with the results described in *Tradescantia* (MARUYAMA 1968), due to the great difference in appearance and morphology of these microspores.

5.4. The control of the pollen wall formation

After the meiotic divisions every microspore contains probably one fourth part of the organelles, which are present in the zygotene cell, except the Golgi bodies, RER, polysomes and microtubules. This means that a haploid nucleus functions in a mainly diploid cytoplasm. Golgi bodies, plastids, lipid granules, polysomes, ER and the plasma membrane are involved in pollen wall formation. Before the separation of the four nuclei in the early tetrad stage, nuclear material is transported by means of the invaginations of the nuclear membrane to the cytoplasm. This process may have a function in the control of the pollen wall formation, since the invaginations persist during the middle tetrad stage. This signal, or possibly another kind of signal, of the haploid nucleus on the not yet separated and mainly sporophytic cytoplasm may also explain the findings of ROGER & HARRIS (1969). They postulate a sporophytic control of pollen wall formation on base of the formation of a normal exine around a miniature pollen grain with an incomplete chromosome number.

The mainly sporophytic cytoplasm executes the orders of the haploid nucleus. In pollen wall formation there may be a haploid nuclear control and simultaneously a sporophytic influence via the cytoplasm and the cell organelles.

In *Pinus sylvestris* the patterning of the pollen wall depends at first on callose wall formation, simultaneous with Golgi vesicle material excretion. The position

of the nucleus in the cell, determined by the spindle, is also important. Thereafter, the production and orientation of the sporopollenin, mainly on the plasma membrane, complete the pollen wall pattern. All these elements are related to the cytoplasm, mainly of sporophytic origin, but the start of this process is initiated in early tetrad stage by the haploid nucleus, possibly by invaginations of the nuclear membrane (VASIL & ALDRICH 1970) before the separation of the four microspores (compare with FORD 1971).

These conclusions about the control of pollen wall formation are partly in agreement with the opinion recently published by HESLOP-HARRISON (1971a).

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