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Ultrastructure of the maturing embryo sac of *Lilium regale*

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A number of structural changes occur in the cytoplasm of the megasporocyte and embryo sac of *Lilium candidum*, associated with the differentiation and maturation of the cells (Rodkiewicz and Mikulska 1966). It appeared that, for certain development stages, not only the arrangement and number of cell nuclei are characteristic, but also the systems of cytoplasmic structures. The interpretation of their role was rendered difficult by the known fact that the flowers of *L. candidum* are sterile, since this seemed to suggest that degenerative processes occur in the development of the female gametophyte. *L. candidum* does not produce seeds when cultivated in normal conditions (Wóycicki 1949). The sterility of this species, however, similarly as that of the remaining lily species may be easily overcome. It is sufficient, according to the data of Gärtner (1849), to pollinate the flowers with pollen from other specimens. In *Lilium bulbiferum* setting of seeds was most successful when pollen from plants growing in a distant locality was used (Focke 1890). The point was here of course to get pollen from other clones of the vegetatively reproduced lily. The fact of successful pollination between these clones as well as of pollination with a mixture of the plant's own pollen mixed with that of other lily species has been described for *Lilium bulbiferum* (Sokołowska-Kulczycka 1965). The way of overcoming the sterility of *Lilium candidum* and of obtaining seeds from it has long been known. As early as 1577 Gessner demonstrated experimentally that lily inflorescences cut from that plant and placed in water continue to develop and produce viable seeds. This phenomenon has been repeatedly confirmed in this and other species of monocotyledonous plants (Lindemuth 1898). The own pollen of the self-sterile *Lilium candidum* does not germinate on the stigma if the inflorescence is attached to the bulb, but if it is cut off and the stigma is moistened with a 5-% fructose solution, some of the flowers form normal seeds (Nemec 1935).

It would result from the observations quoted that *L. candidum* must produce a fertilizable embryo sac, and that degenerative changes, occur, if at all, in the final stage of development or after maturation. Therefore, the cytoplasmic structures described by us are probably normal, although they are found in usually sterile generative organs. Neither have any microscopically noticeable deviations from the typical development of the embryo sac been revealed in *L. bulbiferum* which is also self-sterile (Sokołowska-Kulczycka 1965).

Embryo sacs of *L. regale* developing also according to the *Fritillaria* type were taken for comparison with the structure of *Lilium candidum*. *L. regale* is considered

as a species readily producing seeds when cultivated (Wóycicki 1949), although some of the specimens may be self-sterile (Stout 1922). The *L. regale* ovules fixed for examination in the electron microscope came from plants producing seeds. The results obtained so far concern only the later period of development beginning from the first four-nucleate stage and they refer mainly the cellular embryo sac. A similarity was found in the ultrastructure of both species. It consists in the occurrence of specific cytoplasmic bodies which in the course of development undergo hydrolysis, and of abundant concentric systems of cytoplasmic membranes (Rodkiewicz and Mikulska 1966; Mikulska and Rodkiewicz 1966). In each species, however, different features were noted which may be specific only for it, or have not been noticed in the second species on account of the fragmentary material examined. It would be extremely tedious to obtain a picture of the entire material, since in order to obtain a photograph of one cross-section of the lily emryo sac in a 10,000 magnification, it is necessary to take about 300 pictures.

MATERIAL AND METHODS

The lily (*L. regale*) ovules were fixed in 2 percent OsO_4 in veronal buffer at pH 7.4 and embedded in a mixture of methacrylates, they were cut in an ultramicrotome and contrasted with uranyl acetate. The preparations were examined with an Tesla BS 242 electron microscope.

OBSERVATIONS

The central cell

The appearance of the contents of the central cell changes as maturation progresses and the embryo sac ages. In the sedondary four-nucleate stage, a zone may already be distinguished in the embryo sac, which is later occupied by the central cell. One large vacuole bordered by a layer of parietal cytoplasm and separated by wide layers of cytoplasm from the micropylar and antipodal nuclei is mostly present in this zone. In the cytoplasm above the inner antipodal nucleus, numerous cytoplasmic membranes and fine-grained areas free of organoids and giving the impression of degenration are visible.

After formation of the central cell, two large cytoplasmic zones containing nuclei appear in it: one at the micropylar pole of the cell with a haploid nucleus, the other at the chalazal pole with a triploid nucleus. A large vacuole lies between these two zones. In the dense cytoplasm adjacent to the micropylar cells, numerous oval profiles of concentric cytoplasmic membrane systems are visible (fig. 5). Both the cytoplasm enclosed in the membrane systems and between them exhibited very few organoids, with the exception of rare mitochondria or Golgi structures. The optical density of the cytoplasm in this zone varied. On some photographs, the entire cytoplasm, both between the membrane systems, and within them was of more or less the same density. On other pictures lighter, optically less dense external layers of the membrane

systems were visible against the background of the remaining denser cytoplasm, or else the whole cytoplasm between these systems and their external layers was light, and denser cytoplasm was visible only in one, two or three layers of the membrane system.

Wide areas of the central cell cytoplasm are deprived of mitochondria which in other parts of the embryo sac are generally visible on each photograph. In some periods at any rate, the mitochondria of the central cell are agglomerated in large groups. So far such groups have been observed arranged near the embryo sac wall or the nucleus (probably haploid). Such a group (fig. 8) is several microns in diameter and on its cross-section profiles of about 100 mitochondria are distributed. Most of them have rounded outlines. The differences in size may partly result from the unequal cross-section planes of the particular mitochondria. Among the round mitochondria, some profiles of biscuit shape ones are seen. Between the mitochondria there are several osmiophilic lipid bodies, some vesicles and ground cytoplasm. The whole gives the impression of a separate, compact system.

At a later period, the cytoplasmic membranes zone of the central cell is optically less dense. The cytoplasm between the systems does not noticeably differ in density from the vacuoles, its cytoplasmic nature could be conjectured on the basis of the Golgi structures dispersed in it. Similarly, the cytoplasm in the external layers of the membrame systems is optically almost empty; against its background, denser central parts of the system are distinctly visible. Sporadically in the centres of multilayered systems, profiles of small stacks of membranes, resembling in structure crystalline protein, could be seen. Within the layered systems osmiophilic lipid bodies frequently occurred.

Photographs of other cross-sections show, against the background of a very rare central cell contents, systems of membranes with an appearance different than that of those formerly described. Beside small single or two-layer bodies with a dense content, and medium-sized ones consisting of several layers, there occurred bodies composed of a dozen layers or more reaching some dozen microns or so in diameter. In the giant and smaller bodies, the membranes lay close to one another separated by narrow bands of cytoplasm of optical density as low as that of the background (fig. 9). The central part of the system may have had a denser granular structure. Osmiophilic bodies and sometimes mitochondria were frequently observed in the systems. The membranes of these systems were double as seen most distinctly at the moment of membrane splitting.

Then the separating elementary membranes formed a number of connected larger and smaller vesicles reaching sometimes about 1 μ in diameter.

Fig. 13 gives a picture of far advanced disorganization of a part of the cytoplasm. Folded and stuck together membranes, vacuoles, coarse-grained material and lipid bodies are seen. In some places remnants of membranes are arranged in irregular piles, which may also be met in less disorganized parts of the central cell and in other cells of the embryo sac. The final picture of disorganization is shown in fig. 14 where a large osmiophilic lipid body surrounded by vacuole-like remnants of the membranes, forming as if a rosette, are seen.

Photographs of the embryo sac of the lily (*Lilium regale*) fixed in OsO_4

Plate I

1. Fragment of the chalazal pole of the eight-nucleate embryo sac. Two parts of the folded nucleus (*N*) with a deep-entering cytoplasm peninsula. Within the nucleus are small nucleoli and dark chromatin agglomerations. In the lower part of the photograph a layer of fine-grained substance (*P*) separating the neighbouring cells of the embryo sac is visible. × 6000

2. Cytoplasm of the chalazal part of the eight-nucleate embryo sac. Profiles of large bodies (*C*) are seen, some of them may be plastides and others cytoplasmic bodies, particularly those containing smaller oval structures (*C*); mitochondria *M*, osmiophilic lipids. On the sides edges of large vacuoles (V) are seen. × 9000

Plate II

3. Part of nucleus (*N*) and cytoplasm of two micropylar cells. In the cytoplasm numerous mitochondria, cytoplasmic bodies — some with optically empty spaces — and plastides undistinguishable from them are present. The wall (*P*) between the cells, embryo sac wall (*W*). × 9000

4. Cytoplasm of micropylar cell with cytoplasmic bodies (larger profiles) and mitochondria (smaller profiles). × 9000

Pla)te III

5. Dense cytoplasm with layered structures from the part of the central cell adjacent of the micropylar cells. *G* — Golgi structures. × 9000

6. Cytoplasm of micropylar cell; a body with dense granular of granular-vesicular structure of undetermined origin is visible as well as mitochondria, the profile represents probably remains of the membrane material, vacuoles (*V*). × 9000

7. Cytoplasm strand from an micropylar cell older than the one shown in figs. 6, 3 and 4. Cytoplasmic bodies with optically less dense contents, vacuoles (*V*), osmiophilic lipid body. × 9000

Plate IV

8. Central cell. Group of mitochondria at the embryo sac wall. Some mitochondria with constrictions (*M*). × 12 000

9. Central cell. Multilayered arrangement of membranes, next to them smaller systems against the background of optically rarefied cell contents. × 5000

Plate V

10. Fragment of antipodal cell from embryo sac older than in fig. 2. The contents of the cell is probably disorganizing: nucleus (*N*), vacuole (*V*), embryo sac wall (*W*), mitochondria at the wall. × 9000

11. Chalazal pole of embryo sac; embryo sac wall with black-staining plasmodesms, the cytoplasm of the antipode seems degenerated, × 9000

Plate I

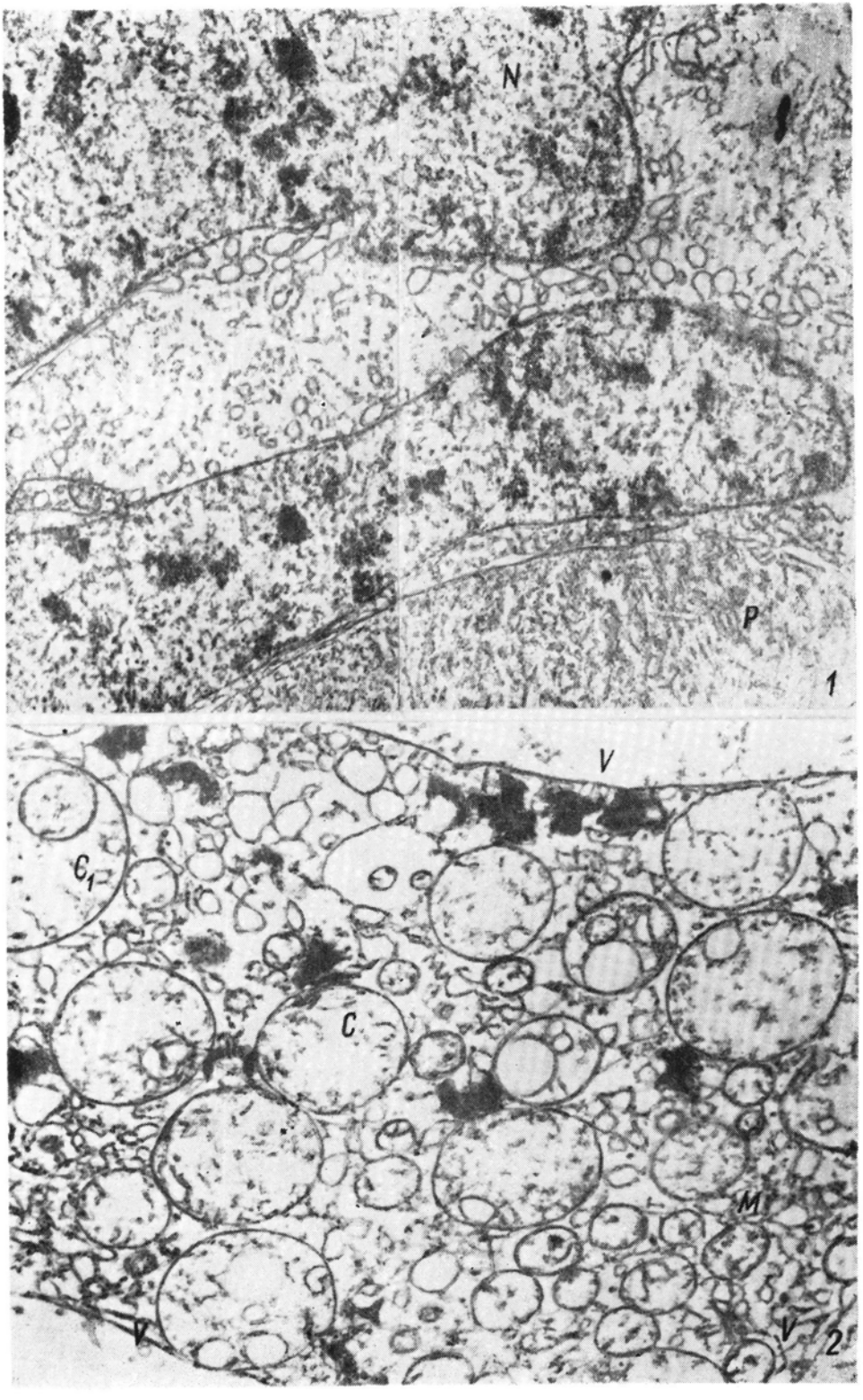


Plate II

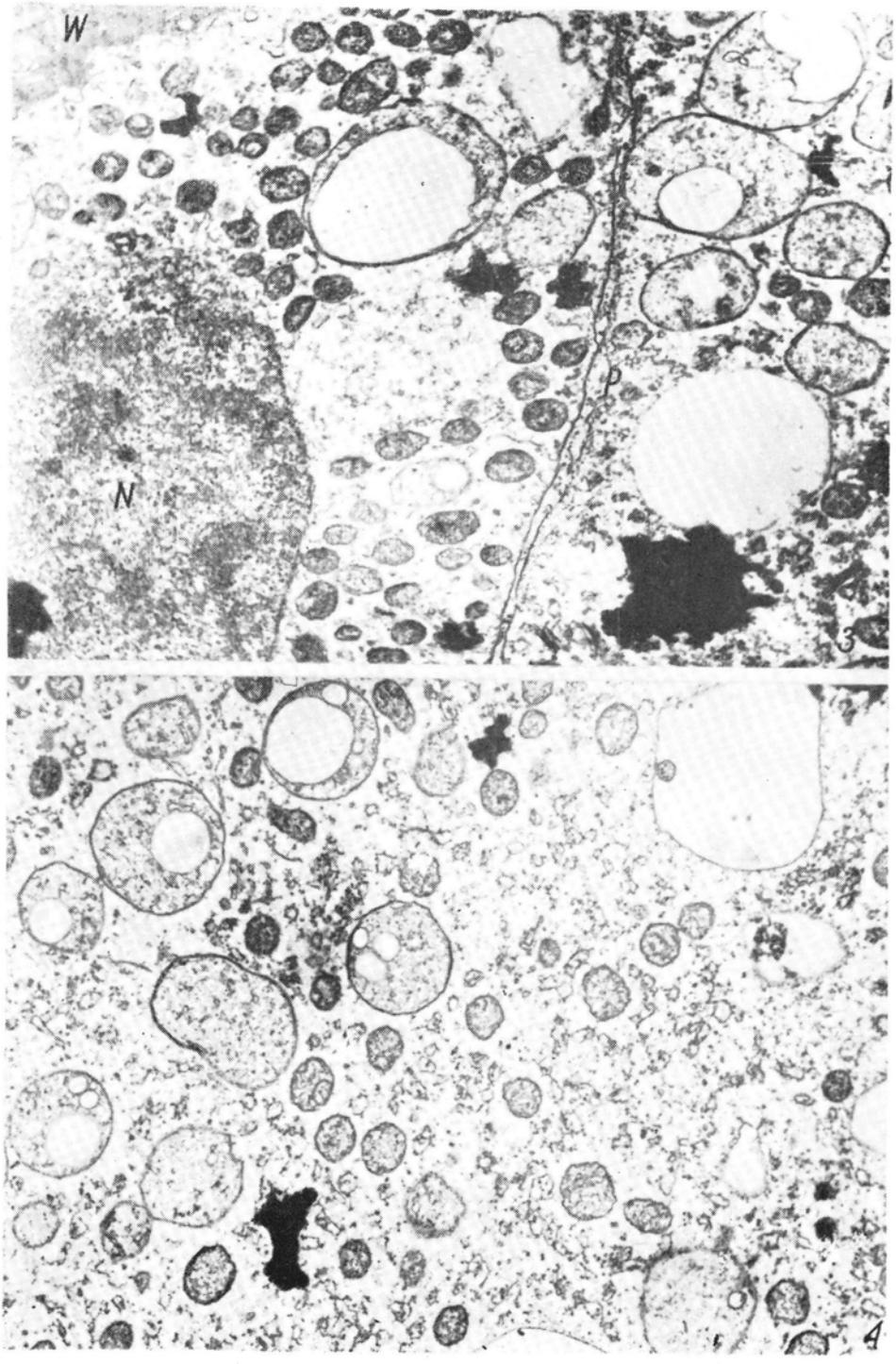
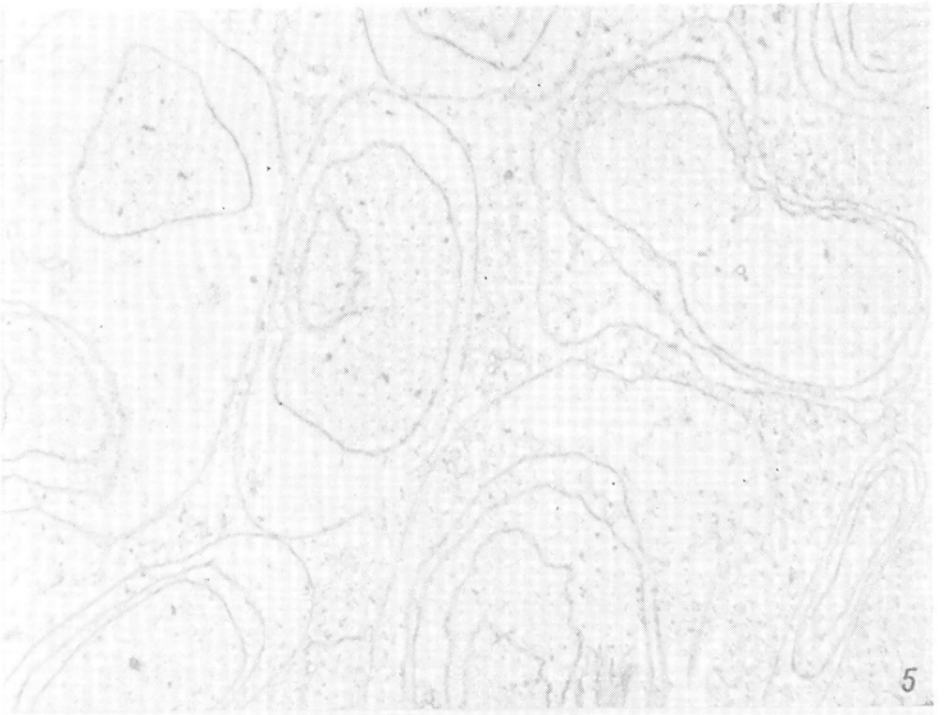


Plate III



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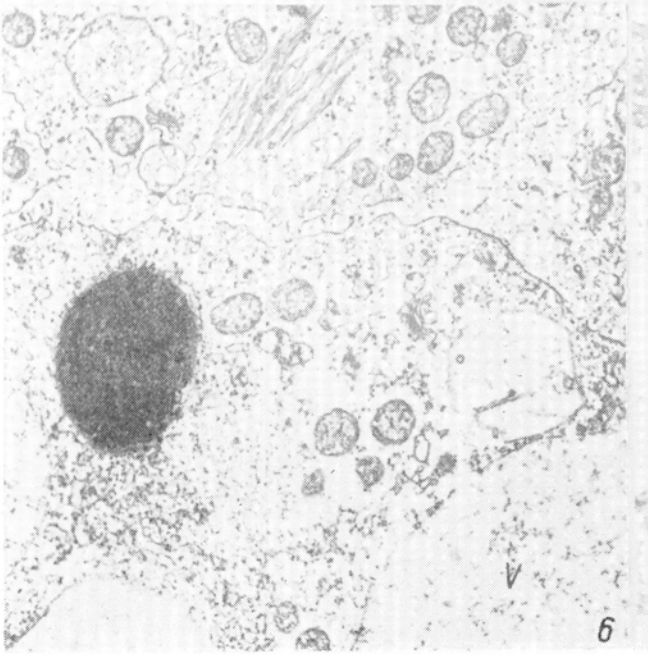

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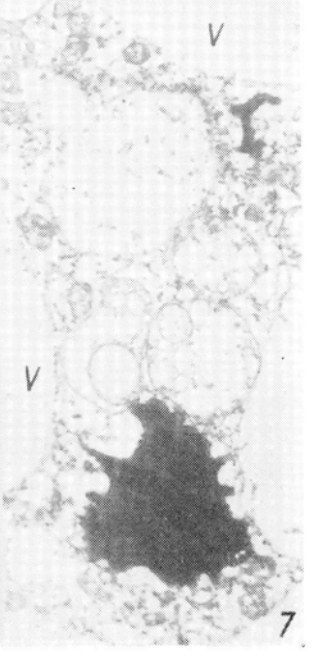

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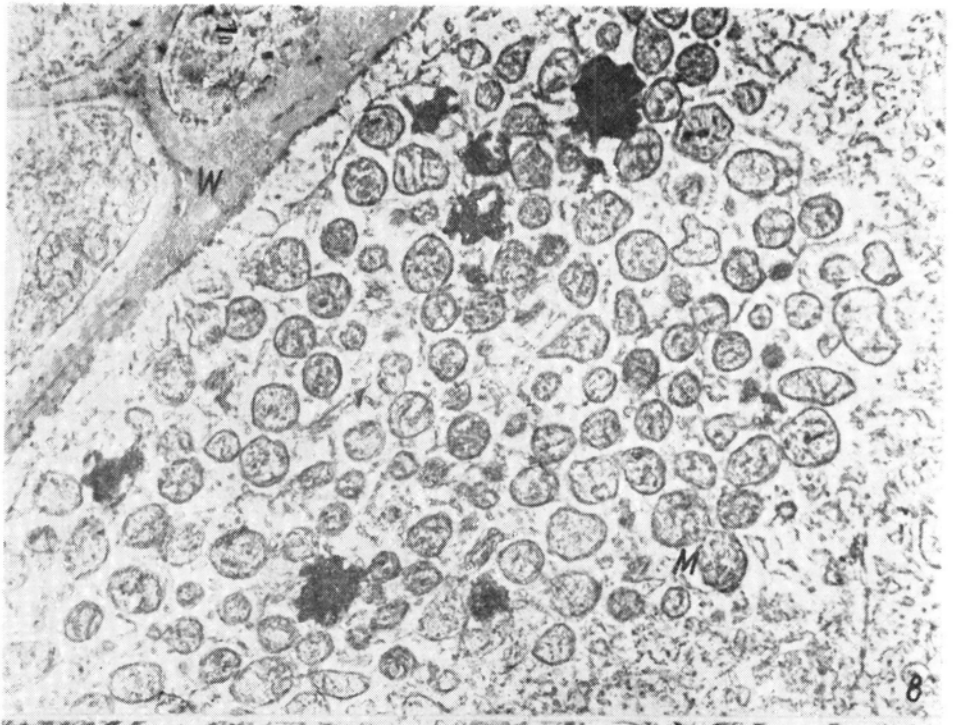
Plate IV



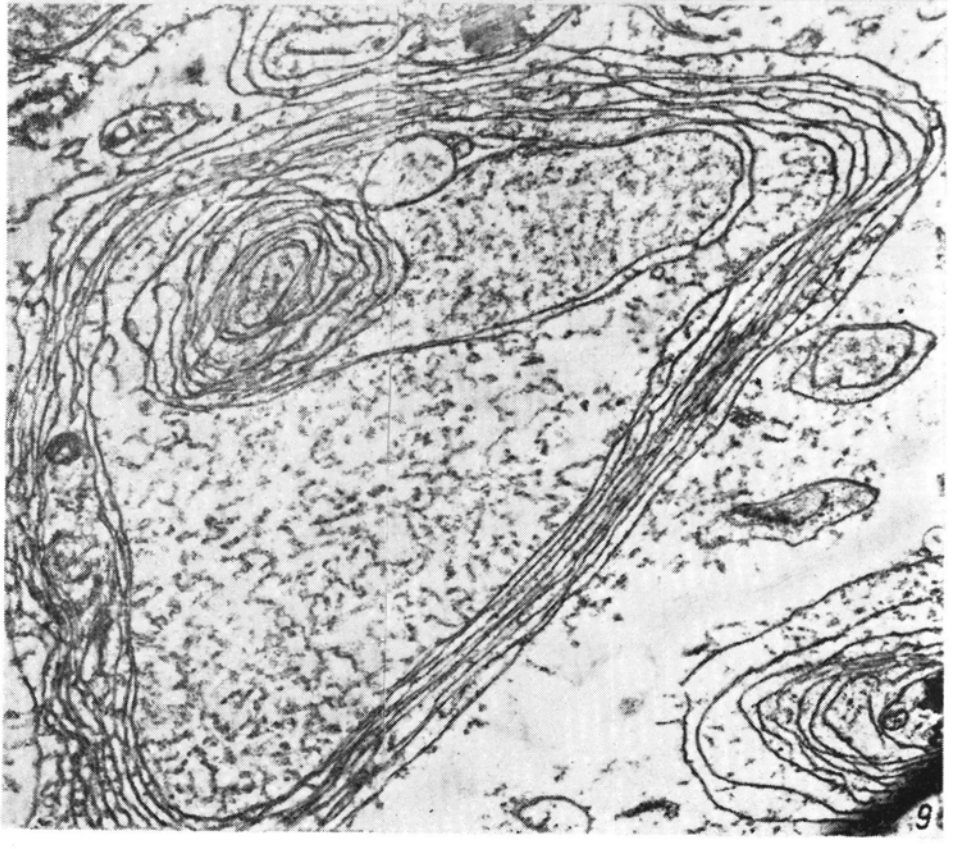
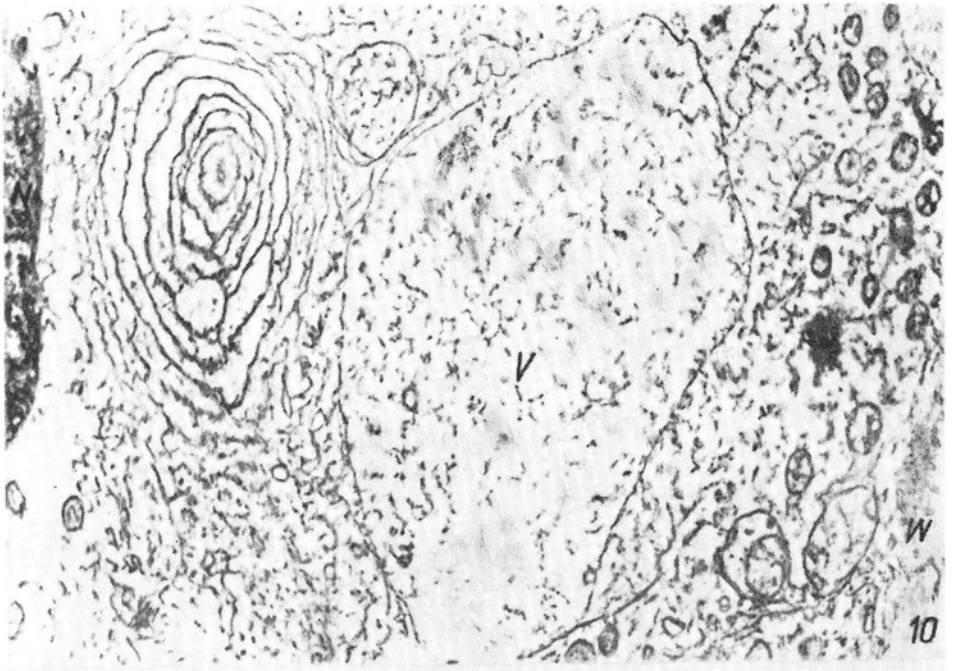


Plate V



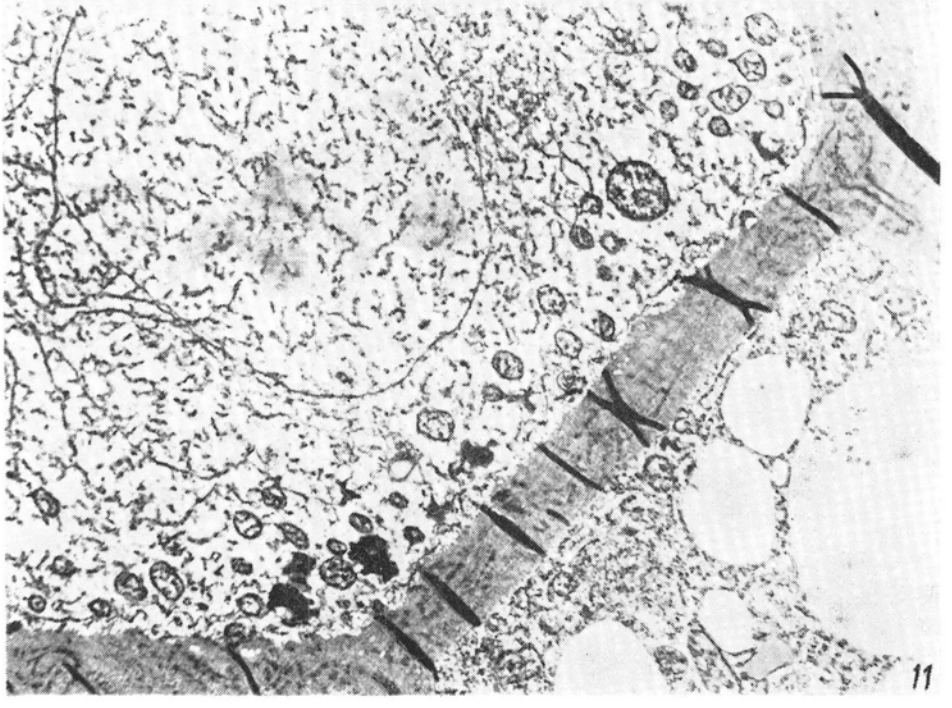
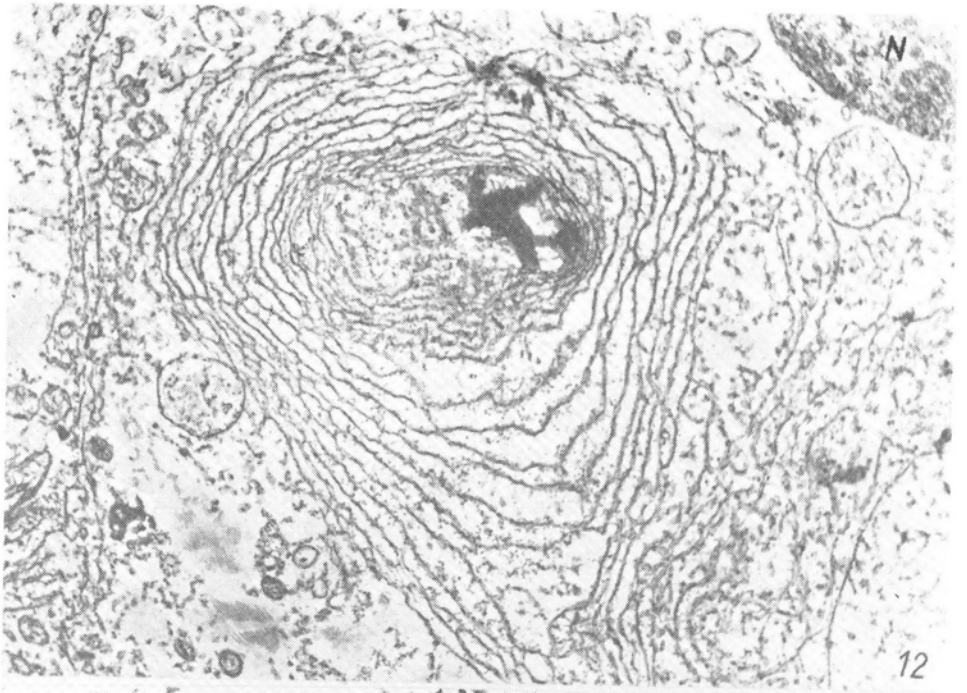
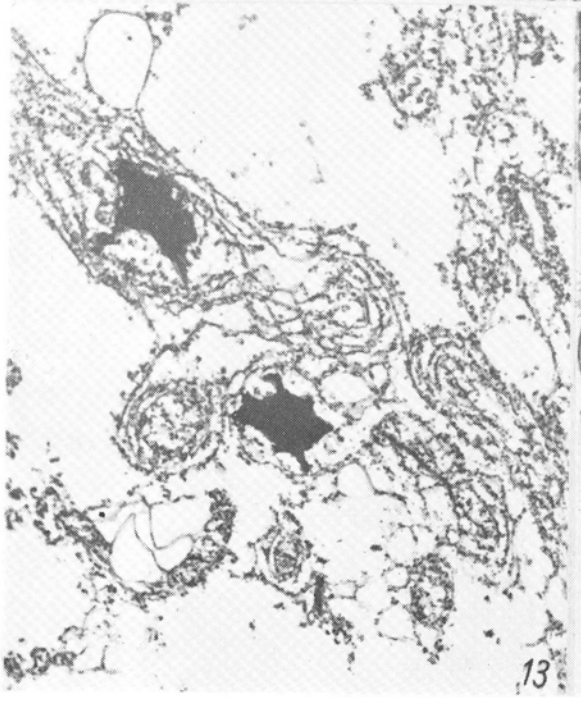


Plate VI





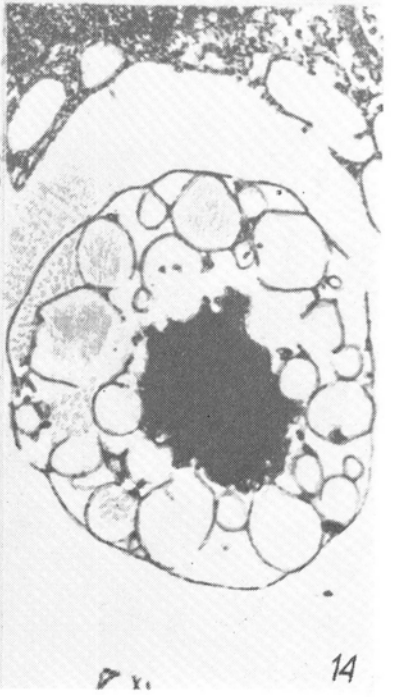
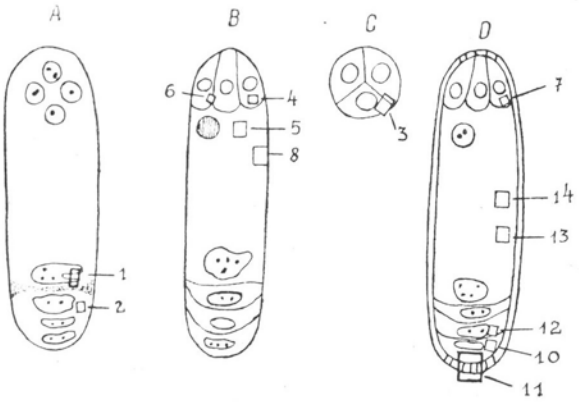


Plate VI

12. Multilayered arragement of membranes of ageing antipodal cytoplasm: nucleus (*N*), wall between antipodes (*P*). × 9000

13, 14. Structure visible in the peripheral part of the vacuole in the central cell, these shown in fig. 14 are constituting probably a further phase of destruction of the structures shown in fig. 13. × 9000

DIAGRAMS



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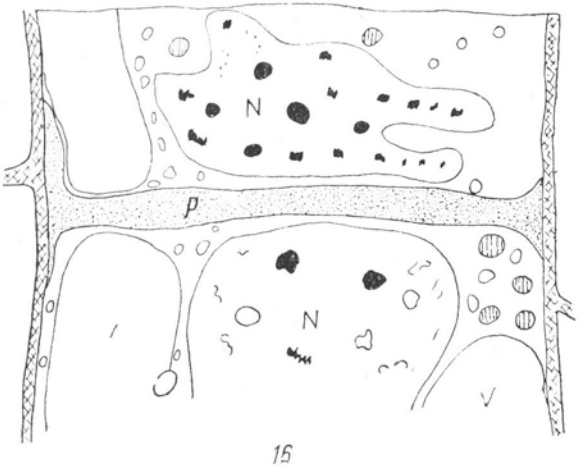

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15. Diagram' structure of lilium embryo sac, the figures denote the approximate places corresponding to the photographs. *A* — eight-nucleate phase, the wall between the cells at the chalazal pole is shown by dots. *B* — Embryo sac divided into cells. *C* — Cross-section through micropylar part. *D* — Mature embryo sac with plasmodesms visible on chalazal pole and the probable sites of plasmodesms on the micropylar pole

16. Diagramatic drawing of chalazal part of embryo sac with wall (*P*) formed between the newly formed cells: nucleus (*N*), vacuole (*V*). Comp. fig. 1.

Cell walls

The walls between the embryo sac cells form in the eight-nucleate stage. No stages of formation of the wall were observed. In their earliest stage, the micropylar cells were already completely separated by narrow walls about 0.5 μ thick (fig. 3). The walls between the three micropylar cells and between them and the central cell were optically empty or contained a certain amount of granular material. The most prominent structure of the wall on the photographs were the limiting cytoplasmic membranes of each of the neighbouring protoplasts. Apart from this most frequently observed picture of the wall, some others occur. The septa may be wider, with a large amount of granular material bordered, on the side of each protoplast, by a cytoplasmic membrane. In some places these membranes are bent or even torn, what should probably be considered as an artefact, although it may also mark the moment of formation of a continuous membrane from some smaller elements.

The structure of the broad wall formed in the initial phase of cell separation on the antipodal pole is much better visible. A fragment of this wall running close to the nucleus is shown in Fig. 1. This wall stretches between the chalazal nuclei reaching to the lateral walls of the embryo sac. It either separates the central cell from the antipode, or the two antipodes situated closer to the central cell. This wall 3—4 μ thick consists of fine-grained substance (fig. 16). On both sides the septum is bordered by cytoplasmic membranes of the adjacent protoplasts. The whole surface of the septum is lined with a cytoplasm layer, extremely thin in the places where the protoplasts are vacuolized. At a later stage much thinner walls are visible at the chalazal pole, almost deprived of optically opaque substance. In the period of antipodal cell degeneration the wall is narrow and partly destroyed (fig. 12).

Micropylar cells

No differences could be found in the structure of the micropylar cells. The cytoplasm of the younger ones contained numerous mitochondria, distinct minute Golgi structures and small vacuoles. The most salient feature were bodies with dense contents surrounded by a double membrane. Some of the latter structures have inside optically empty areas of varying size. Sometimes systems with an irregular profile, probably of structures formed of lamellar fragments (fig. 6) lay in the cytoplasm. Only a few times optically dense formations 2—3 μ in diameter were detected. They were highly osmiophilic and had an indefinite internal structure seemingly granular or granular and vesicular (fig. 4).

At a later stage, a highly advanced vacuolization is observed in all the micropylar cells. Large vacuoles are transected by cytoplasm strands with mitochondria, Golgi structures and lipid bodies, similarly as in the earlier period of development. On the other hand, no bodies with double membranes and dense granular contents are visible. In their place, structures of about the same size but with almost or completely optically empty contents appear (fig. 7).

Antipodal cells

The antipodal cells at the moment of wall formation exhibit similar cytoplasmic components as the micropylar cells, they are, however, more vacuolized than the latter. Noteworthy is also a population of bodies with dense granular cytoplasmic contents, mitochondria, osmiophilic lipid bodies, Golgi structures and minute vacuoles. In a somewhat later phase, changes are noted, above all in the bodies which exhibited dense contents which now is rarified and does not noticeably differ in density from the ground cytoplasm (fig. 2). Some of these bodies contain smaller structures surrounded by membranes and optically empty spaces. The ground cytoplasm contains numerous vacuoles reaching 0.5 μ in diameter.

The cytoplasm of older cells of the embryo sac from opened flowers seems to be in a state of violent decomposition (figs. 10—12). Within it, large vacuoles and concentric membrane arrangements with a structure similar to that of the final developmental phases of the central cell are seen. The numerous membranes are wavy, in some places stuck together, in others distended in the form of vacuoles. Between the successive double membranes there are narrow layers with dispersed minute granules or else they are optically quite empty. The structure of the cytoplasm, the nucleus and the cytoplasmic organoids gives the impression of being very badly fixed.

Plasmodesms

Pits and plasmodesms are generally considered as a common attribute of the cell wall, particularly of tissue-forming cells. In the archesporal tissue of the ovule of the orchid *Dendrobium*, the plasmodesms connect all the cells, but in the differentiating mother cell of the megaspore, at a certain moment the plasmodesms cease to be visible, although they remain present in the walls of other cells of the ovule (Israel and Sagava 1964). The megasporocyte is cut off from direct contact with the surrounding cell layer which rapidly undergoes degeneration. So far no data were available on the existence of pits and plasmodesms linking the embryo sac with the surrounding tissue. On the contrary, Jensen (1965) mentions that he has never observed pits in the embryo sac walls of the cotton plant. In observations of the successive development stages of the *Lilium candidum* embryo sac, beginning with the megasporocyte phase, pits were detected only once in the wall on the micropylar pole of the embryo sac in eight-nucleate stage. Inspection of embryo sac preparations of *L. regale* also revealed pits only once (fig. 11). They were found on the chalazal pole of a mature embryo sac. Pits were never noted in the lateral walls in the numerous preparations of both the lily species examined.

DISCUSSION

Observations of the ultrastructure of the female gametophyte have only been recently started and to date only a few papers on this subject can be quoted (Israel and Sagawa 1964; Sagawa and Israel 1964; Pluijin 1964; Jensen 1965 a, b;

Eymé 1965; Vazart and Vazart 1965; Diboll 1966 and our publications from 1963 on). It should be noted that the development as a whole is not described exhaustively in any of these papers. Their scope was limited mostly to one stage, or only one cell in this stage. This is not surprising if we consider the specific conditions of investigation, the time-consuming preparations for each picture and the necessity of taking a great number of them for each stage. About 300 photographs would be necessary to give the crosssection of one lily embryo sac at none too large a magnification. In theory, several hundred cross-sections could be obtained from one embryo sac, so that the number of pictures would increase to hundreds of thousands. The developing gametophyte undergoes complicated morphological transformmations, and to follow them it would be necessary to examine the cross-section of several score of the successive phases. Since such an exhaustive collection of material is not possible, thus rather fragmentary data have to be utilized, often difficult to interpret. Thus the reconstruction of the transformations and the structure of the embryo sac resembles the work of the palaeontologist who from several fragments of bones reconstructs the whole skeleton of some animal. This work is, moreover, complicated by the fact that more than a dozen types of development of the embryo sac have been distinguished, and in each of these types a number of specific variants or deviations.

Some processes already partly known from light microscope observations have been distinguished in the development of the megasporocyte and megagametophyte of *L. candidum* (Rodkiewicz and Mikulska 1966). In the period of mass increment of the megasporocyte its cytoplasm contains a large system of parallel endoplasmic reticulum membranes. Before the meiotic metaphase I, closed concentric membrane systems form from the elements (cisterns) of the reticulum, enclosing part of the cell cytoplasm together with mitochondria and other organoids. The multi-layered cytoplasmic bodies formed in this way transform subsequently, owing to the decomposition of a number of membranes, to single cytoplasmic bodies surrounded by one double membrane. The dense content of the cytoplasmic bodies disappears probably by hydrolysis and is utilized by the maturating embryo sac. The cytoplasmic bodies are most numerous at the poles of the embryo sac in the eight--nucleate phase.

The observations of Eymé (1965, 1966) on megaspores and embryo sacs of *L. candidum* and two species of tulips, agree basically with these data. In the light microscope the cytomembranes are visible in the megasporocyte cytoplasm, they can also be seen in the electron microscope. Their development reaches its peak in the period preceding meiosis. Some membranes form organized ergastoplasmic systems, the maximum number of which occurs in the prophase. Disintegration of the organized ergastoplasm and formation of structures called as cytoplasmic nodules containing isolated cytoplasm occur in the same phase.

Also Camefort (1965) describes structures very similar to the cytoplasmic bodies. Namely, in the cytoplasm of the oosphere in *Ginkgo*, cytoplasmic inclusions occur, called by the author "yolk inclusions". They are separated from the ground cytoplasm by a double membrane sometimes continuous with the endoplasmic reticulum. It

should be noted that this is not a single membrane as that surrounding vacuoles, the content of which may be precipitated rendering them thus similar to the inclusions described. The segments of endoplasmic reticulum, mitochondria and Golgi structures contained in them indicate their cytoplasmic, and not paraplasmic character. Thus, similarly as in the lily megasporocyte, two kinds of cytoplasm may be distinguished in the oosphere: the rather light ground cytoplasm, both in the light and in the electron microscope, and dense cytoplasm in distinct bodies surrounded by a double membrane originating from the endoplasmic reticulum. The bodies contain acid phosphatase and undergo hydrolysis (Camefort 1966). This author (1959, 1962, 1963, 1965) observed cytoplasmic inclusions in the oosphere of the pine, they were, however, not completely cut off from the ground cytoplasm. Other cytoplasmic inclusions consisted of a number of concentric cytoplasm layers set up by deformed plastides. In another gymnospermous plant, *Pseudotsuga*, numerous protein structures are visible which are utilized in germination (Ching 1965).

In the embryo sacs of *Lilium regale*, both in the four-nucleate and in the cellular stages, numerous bodies several microns in diameter were present, surrounded by a double membrane and filled with a dense cytoplasmic contents (figs. 3, 4). In the early cellular stage they were chiefly aggregated in the micropylar and antipodal cells where they constituted the most prominent element of the cytoplasm. As the cells aged, the contents of the cytoplasmic bodies became less dense (figs. 2, 7), this process seems to occurr earlier at the chalazal pole. In many cytoplasmic bodies, optically empty spaces are formed which seem to increase in size, and transformed the whole structure into a vacuole. Among these bodies, plastides could not be distinguished, though they should be present in the embryo sac cells (Vazart and Vazart 1965; Diboll 1966; Jensen 1965). In the secondary four-nucleate stage, various structures consisting of concentrically closed cytoplasmic membranes and a certain number of single cytoplasmic bodies were present in the area to be occupied by the central cell, and further in the cell itself. Some of the layered systems had centres corresponding in size and structure to the cytoplasmic bodies. Closed mitochondria and other cytoplasmic elements were also present there which may be associated with the formation and development of the cytoplasmic bodies. The multi-layered and single cytoplasmic bodies gave a positive reaction to proteins with bromophenol blue after Mazia et al. (1953), frequently also lipids occurred there staining with Sudans. These reactions were run under the light microscope. It is believed that similarly as in *L. candidum*, the cytoplasmic bodies in *L. regale* surrounded by a double membrane are formed in the period of the first meiotic prophase and constitute the specific cytoplasm utilized in later phases of development. Large parts of the cytoplasm and whole cells undergo degeneration and destruction in the course of development of the embryo sac. These processes take place on a large scale in the central cell, and before its formation in the central part of the embryo sac and in the antipodes where the entire structure of the protoplasm is destroyed. Figs. 5, 9—14 represent probably the cytoplasm in the course of decomposition and hydrolysis. It would seem that the systems of cytoplasmic membranes occurring at this period are associated with the process of disappearance of the ground cyto-

plasm. The numerous membranes of the endoplasmic reticulum in the form of whorls have been described in the central cell (Jansen 1965 b; Godineau 1966). The picture of the membrane whorls against the background of dense ground cytoplasm, and in the subsequent stage against a background of decreasingly dense cytoplasm is sufficiently suggestive.

It is probable that there occurs in the central cell a differentiation of cytoplasm into an area with numerous cytoplasmic membranes undergoing gradual rarefication and finally complete destruction, and a layer at the embryo sac wall and close to the nuclei where the cytoplasm remains to the end of the cell's lifetime. The cytoplasmic area with the membrane systems is almost completely free of mitochondria which are grouped in large aggregates (fig. 8) at the embryo sac wall and/or at the nuclei. This change in the distribution of the mitochondria which generally are disseminated in the cytoplasm should result in physiological changes in the corresponding zones of the cytoplasm. Probably also, the mitochondria shifted from the zones of the cytoplasm undergoing destruction are preserved and take part in the further development of the part of the embryo sac where the endosperm is formed.

SUMMARY

During the development of the embryo sac of *Lilium regale* observed from the four-nucleate stage to maturity in the electron microscope, a population of cytoplasmic structures 1—4 μ in diameter was revealed. The structures consisted of cytoplasm and were surrounded by double membranes. They were particularly numerous in the micropylar and antipodal cells. In the course of development the cytoplasmic contents of these structures became less dense and disappeared partly or completely.

In the central cell and in the antipodes, systems of cytoplasmic membranes are visible, probably associated with the processes of cytoplasm destruction very intensive in these cells. The rarefied cytoplasm was depraived of mitochondria which were aggregated in large groups at the nucleus and at the embryo sac wall.

At the chalazal pole of the embryo sac, broad plasmodesms were observed in the embryo sac wall, but not noticeable on any of the longitudinal walls.

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Ultrastruktura dojrzewającego woreczka zalążkowego Lilium regale

Streszczenie

W rozwoju woreczka zalążkowego *Lilium regale* obserwowanego od stadium czterojądrowego do dojrzałości w mikroskopie elektronowym zauważono populację struktur cytoplazmatycznych o średnicy 1—4 μ. Struktury te były zbudowane z cytoplazmy i otoczone podwójną membraną. Występowały one szczególnie licznie w komórkach mikropylarnych i antypodalnych. W czasie rozwoju cytoplazmatyczna zawartość tych struktur uległa rozrzedzeniu, całkowitemu lub częściowemu zanikowi.

W komórce centralnej i w antypodach widoczne były układy membran cytoplazmatycznych, które prawdopodobnie były związane z procesami niszczenia cytoplazmy intensywnie zachodzącymi w tych komórkach. Rozrzedzająca się cytoplazma komórki centralnej pozbawiona była mitochondriów, które były zebrane w wielkie ugrupowania przy jądrze i ścianie woreczka zalążkowego.

Na wierzchołku chalazalnym woreczka zalążkowego zaobserwowano w ścianie woreczka szerokie plasmodesmy, niewidoczne nigdzie na ścianach podłużnych.

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