ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XLII — nr 2
1973

# Double fertilization in *Helianthus*

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(Received: February 10, 1973.)

Abstract

After controlled pollination of *Helianthus annuus* L. florets, the whole course of fertilization is described and documented on 24 microphotos. The timing of events is evaluated. The average cell cycle in the proembryo is 2 hours and the nuclear cycle in endosperm — 60 minutes.
Plasmoptysis is suggested as the mechanism of pollen tube opening in the synergid. The structure of the thread-like sperm nucleus is interpreted as an end to end union of chromosomes, and the morphological changes of the sperm nuclei are explained as folding and coiling, based on a spiralization mechanism of chromosomes. Cytochemical observations indicating ribosome formation in the course of the nuclear cycles in the endosperm are described. The mechanisms accelerating nuclear cycles in the endosperm are discussed.

## INTRODUCTION

The first studies on the double fertilization process in angiosperms have shown that the fusion of male nuclei with female ones may occur immediately after entrance of the sperm nuclei into the female cells, as in *Helianthus* (Nawaschin 1900), or during the first mitosis in the fertilized cells, as in *Lilium* (Nawaschin 1898). These two opposed types of nuclear fusion are classified by Gerassimova-Navashina (1957) as premitotic and postmitotic types of fertilization. They correspond to the sea urchin type and the *Ascaris* type in animals. Among animals, postmitotic fertilization is widespread. It occurs also in mammals (Wilson 1928). For most angiosperms, however, premitotic fertilization is typical. It occurs in extreme form in *Compositae*, as shown by Gerassimova's (1933) detailed studies on *Crepis*.

It seems worth-while to re-examine thoroughly this extreme form of the premitotic fertilization process in the light microscope in an another and typical representative of *Compositae*, *Helianthus annuus*.

## MATERIAL AND METHODS

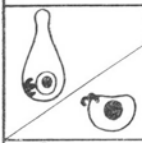
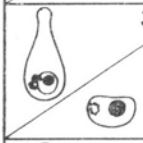
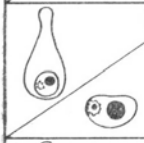
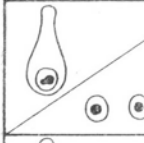
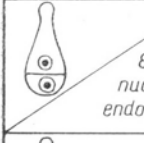
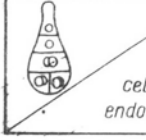
The capitulum of *Helianthus annuus* L. contains numerous florets, sourrounded by an involucre. The marginal ray florets are zygomorphic with ligular corolla. They are female and usually sterile. The central disc florets are actinomorphic with tubular corolla. They are hermaphrodite and fertile. The androecium consists of five stamens, the anthers are fused laterally to form a tube. They are intorse, shedding the pollen into the anther tube. The gynoecium is bicarpellary and syncarpous. The style is filiform and the stigma is bifid. The stigmatic surface, on which the pollen germinates, lies on its ventral side. The outer surface is covered with bristles. After anthesis the style elongates and pushes up the pollen from the anther tube. This facilitates cross pollination of other florets. The ventral stigmatic surfaces are never in contact with the own pollen of the florets. The flowers are thus cross-pollinated by visiting insects. The ovary is inferior, unilocular, containing a single anatropous ovule with basal placentation. The main axis of the ovary coincides with the axis of the anatropous ovule and with that of the embryo sac. This facilitates embryological investigation on the embryo sac, as well as fertilization and post-fertilization events. The disc florets are arranged spirally forming false whorls. The development and the opening of florets occurs centripetally.

The plants were grown in the garden. The flowers were hand-pollinated and fixed at various intervals after pollination in the laboratory. One or two days before anthesis in the first whorl of disc florets, the capitula were cut off and put separately into glass vessels with tap water in the laboratory at 24°—27°C, and under daylight. The next day at noon, after full anthesis in the first and second whorls of disc florets, the newly expanded stigmas were amply pollinated by means of a brush with pollen mixture from another capitula. The pollinated capitula were divided into 4—8 sectors. The ovaries excised from each sector were fixed separately at various intervals after pollination (from 15 min to 8 h). This enables examination of successive stages of the fertilization process from the entry of the pollen tube into the embryo sac to the first division of the zygote nucleus. In laboratory conditions, at 24°—27°C, the pollen tube reaches the embryo sac 45—60 min after pollination.

On the 161 best slides selected for analysis of the fertilization process, 108 embryo sacs were found to be fertilized with one pollen tube, and 53 contained additional pairs of sperm nuclei introduced by additional pollen tubes. Table 1 shows the incidence of the successive stages of fertilization at various time intervals after pollination.

Ovaries, incised after excision, were fixed during 24—36 hours in the modification of Navashin fixative CrAF 0.4-0.5-20, CrAF 0.8-1-20 and CrAF 0.8-0.75-20. The figures denote the final concentration (%) of

Table 1

| Fertilization and postfertilization stages | Time after pollination | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 45′ | 50′ | 55′ | 60′ | 1h 10′ | 1h 15′ | 1h 30′ | 2h | 4h | 6h | 8h |
|  3 | 1 | 1 | | | | 1 | | | | | |
|  6 | | 1 | | 1 | 1 | 3 | | | | | |
|  16 / 16 | | 2 / | 2 / 3 | 6 / 7 | 3 / 2 | 3 / 4 | | | | | |
|  39 / 44 | | | 3 / 2 | 15 / 18 | 6 / 4 | 15 / 20 | | | | | |
|  31 / 22 | | | | 6 / 2 | 4 / 3 | 10 / 2 | 3 / 2 | 2 / 4 | 6 / 9 | | |
|  10 / 2 | | | | | | | / 1 | 2 / | 8 / 1 | | |
|  3 / 8 | | | | | | | | | 2 / 7 | 1 / 1 | |
|  4 / 9 *8 or 16 nuclear endosperm* | | | | | | | | | 1 / 3+1 | 3 / 3+2 | |
|  3 / 3 *32 cellular endosperm* | | | | | | | | | | | 3 / 3 |

Timing of fertilization stages in *Helianthus annuus* after controlled pollination at 24°—27°C, based on 108 ovules examined.
For each stage, the number of cases recorded at various times is given.

$CrO_3$ (W/V), Acetic acid (V/V) and Formaline (V/V) in the fixative. The best proved to be CrAF 0.4-0.5-20. It is obtained by mixing immediately before use equal volumes of the following stock solutions:

| | |
|---|---|
| A) $CrO_3$ | 0,8 g |
| glacial acetic acid | 1 ml |
| distilled water up to | 100 ml |
| B) neutralized formaline | 40 ml |
| distilled water | 60 ml |

Sections, 7—10 $\mu$ thick, were mostly stained with 0.2% fuchsin at pH 2.6 by the following ad hoc elaborated procedure:

1. Bring paraffin sections to distilled water.
2. Hydrolyse in N HCl at 60°C, 12—15 min.
3. Rinse in distilled water.
4. Rinse in 0.5 M citrate phosphate buffer, pH 2.6.
5. Stain in 0.2% basic fuchsin solution in 0.5 M citrate-phosphate buffer, pH 2.6.
6. Wash in 0.5 M buffer, pH 2.6, 3 changes of 5 mins. each.
7. Rinse in distilled water.
8. Pass quickly through an ethanol series, 20%, 40%, 60%, 80%, 96%, absolute. To 96% ethanol add 0.2% fast green if a counterstain is desired.
9. Pass through xylene and mount in balsam.

The method is based on the reaction between the cations of fuchsin and the dissociated free anionic groups of the cellular structures and on the reaction between the undissociated molecules of fuchsin and the free aldehyde groups.

At pH 2.6 nearly all the carboxyl groups of proteins and polysaccharides are undissociated, but a great part of the free phosphate groups of nucleic acids are dissociated at this pH. In most plant cells, there is no other group of substances in the cellular structures with free anionic groups at pH 2.6 with the exception of phospholipids. The phospholipids, however, are not preserved after fixation in CrAF. There remain, thus, in the cell only dissociated phosphate groups of nucleic acids to react with fuchsin cations at this pH. As nearly all RNA is removed during hydrolysis, DNA is, therefore, the only substance in the cellular structure, with abundant anionic groups at pH 2.6 after fixation in CrAF and Feulgen type hydrolysis. This suffices to give an almost specific staining of chromatin.

There are no free aldehyde groups in cellular structures of most plant cells after fixation, the lignified cell walls excepted. The main source of aldehyde groups after Feulgen-type hydrolysis is the DNA in chromatin. The liberated aldehyde groups of DNA interact with the undissociated amino-groups of fuchsin (Schiff-base reaction). This

reinforces the chromatin staining. There is some unspecific binding of fuchsin but the latter may be removed during washing in buffer after staining.

The method outlined gives a more intense staining of chromatin than does the Feulgen method and it is almost as specific as the latter. The cytoplasm and the nucleoli in almost all cell types are unstained. The nucleoli of the secondary nuclei and of the egg cells may be, however, weakly stained. They contain a large amounts of RNA, not wholly removed during hydrolysis. The cytoplasm of the pollen tube discharged into the embryo sac is also weakly stained. The same staining effects may be obtained with thionine or other related basic dyes with unsubstituted amino groups.

Other staining methods used are: Feulgen, PAS, iron-alum haematoxylin and crystal violet. The Feulgen reaction is weak in *Helianthus*, and haematoxylin and crystal violet overstain the cytoplasm of the pollen tube discharged into the embryo sac.

## OBSERVATIONS

### Embryo sac

The ovule of *Helianthus annuus* is anatropous, unitegmic, and tenuinucellar. After disintegration of the nucellus, endothelium differentiates early in the embryo sac development from integumental and funicular innermost layers.

The mature embryo sac (Fig. 1) is surrounded on all sides with a single layer of endothelium. Its shape is fusiform, greatly narrowed in the micropylar part. It consists of a three-celled egg apparatus, a central cell and two antipodals.

The egg apparatus is composed of two twin synergids and an egg cell affixed on them (Figs 2—4). It exhibits bilateral dorso-ventral symmetry. The symmetry plane of the egg apparatus is independent of the symmetry plane of the ovule.

The micropylar part of the egg apparatus is formed solely of the laterally expanded micropylar parts of the two synergids directly adjacent to the endothelium (Fig. 4). This micropylar part of the synergid is separated from its remaining part by an indentation. Owing to this, on the longitudinal section it has the shape of a hook. This part of the synergid may be called the synergid cap, or better, following the suggestion of McLean and Ivimey-Cook (1956), the synergid hood. The term synergid cap was introduced by Strasburger (1878) to denote the cellulose or other materials accumulation at the tip of the synergid, it is, however, now very seldom used in this original sense. Steffen (1951) recommends, however, its use in this sense as a general

term, leaving the term filiform apparatus for the distinctly differentiated caps.

In the indentation separating the synergid hoods, the micropylar foot of the egg cell is fixed. The remaining part of the egg cell adheres closely to both synergids. Beginning from this indentation the whole egg apparatus is enclosed in the central cell. The egg cell and the central cell are thus separated from the micropylar canal by the joined micropylar hoods of the synergids (Fig. 4).

All the three cells of the egg apparatus are elongated, and pear shaped. The synergids have, in the expanded chalazal part a large central vacuole, and dense cytoplasm in the micropylar hoods. The filiform apparatus is absent. The synergid nuclei lie in the outer part of the cytoplasm surrounding the central vacuole at mid height of the cell. They are ovoid or elongated in shape, and they give a distinct Feulgen reaction.

In the egg cell the main part of the cytoplasm with the nucleus lies in the chalazal part (Fig. 4). The pear-like widened central part of the cell is highly vacuolized, whereas the narrow micropylar part of the egg cell is filled with dense cytoplasm. The nucleus of the egg cell is sometimes so close to the chalazal border of the cell that it may be flattened on that side (Fig. 3). Usually, however, it is spherical.

The egg cell nucleus has a poorly visible chromatin which hardly stains in the Feulgen reaction. After fuchsin it is a little stronger stained, with the chromocentres outlined. The nucleolus of the egg cell nucleus is rather large and usually highly vacuolized.

The egg apparatus is enclosed in the cytoplasm of the central cell. The cytoplasm of the latter reaches on one side the synergid hoods, and on the other the antipodal cell. The chalazal part of the cytoplasm is highly vacuolized, frequently with one central vacuole, whereas smaller ones are scattered in the entire cytoplasm (Figs 2, 3). On many slides, owing to the shrinking of cytoplasm during fixation, a rather large cleft is visible between the cytoplasm of the egg cell, and that of the central cell. There are no cell wall materials in his cleft. The secondary nucleus formed by fusion of the two polar nuclei lies in the middle of the central cell in a mass of cytoplasm right under the egg cell. The cytoplasm surrounding the secondary nucleus is usually connected by transvacuolar strands with the parietal cytoplasm of the central cell. The shape of the secondary nucleus varies: it is usually ellipsoid, irregular, with a slight depression on the side facing the egg cell (Fig. 2).

The nucleus contains one enormous nucleolus. A vacuole formed by the fusion of numerous minute vacuoles frequently fills most of the nucleolus in the mature embryo sac. The secondary nucleus may contain 1—3 additional nucleoli.

The chromatin of the secondary nucleus is poorly visible, it gives no

Feulgen reaction and stains very weakly with fuchsin at pH 2.6. It is, however, evident that the entire interior of the nucleus is filled with a delicate reticular structure. The nuclear mebrane is well pronounced and rather thick (Fig. 2).

In the chalazal part of the embryo sac there are two antipodal cells, one lying above the other (Fig. 1). The chalazal antipodal is somewhat wedge-like, the central one is elongated and quadrangular. Both cells are highly vacuolized in the mature embryo sac. Numerous nuclei are present in the centre of each of these cells; they lie close to one another, and are partly fused forming a mass difficult to examine. These nuclei contain an increased amount of DNA as indicated by the very strong Feulgen reaction and have large nucleoli. The antipodals do not degenerate after fertilization. They were found to be fully active even at the heart stage of the embryo.

The description of the cell boundaries is a separate problem. There is no megaspore wall around the whole cell complex constituting the mature embryo sac. On the basis of the PAS reaction and the presence of stained cell walls not adhering to the cytoplasm shrunken during fixation, it may be concluded that the megaspore primary wall constitutes the peripheral parts of the synergid, central cell and antipodal cell walls.

It appears that each synergid is surrounded by its own cell wall on its entire surface. This wall is thickest and stains most intensively in the peripheral part of the hoods. The contiguous walls of the two synergid hoods are also intensively stained, but on the sections they are often apart. It would result from this that they are not joined by any solid intercellular material. The inner walls of the synergid hoods are not smooth but folded and fit into one another. In the chalazal part of the synergids the wall is very thin. Between the egg cell and the synergids, the common cell wall is most distinct in the micropylar part.

The egg cell in its micropylar and peripheral parts has a well outlined but thin and poorly staining cell wall, whereas in the chalazal part there is only the plasma membrane without the cell wall.

The lateral wall of the central cell is thick. It is a part of the primary megaspore wall. The central cell is separated from the antipodal one by a common cell wall. At the border with the egg apparatus, however, the central cell seems to have no cell wall.The latter is certainly absent between the central cell and the egg cell. Thus, between the central and the egg cells there are only two plasma membranes adhering to one another. On thin sections it is easy to see that the plasma membranes of these cells frequently separate under the influence of the fixative forming a cleft. No trace of any cell wall materials can be found in this cleft.

The antipodals have distinct, thick, intensely staining cell walls.

## Entry of pollen tube into embryo sac

The pollen tube of *Helianthus annuus*, similarly as in other *Compositae*, passes to the embryo sac through the style and the micropylar canal closed by the synergid hoods, and in the end part of this canal it swells club-like. It has a delicate, poorly staining wall and a strongly staining basophilic cytoplasm which makes it visible on sections stained with basic dyes. The very moment of the pollen tube entry into the embryo sac have been not observed. There is no doubt, however, that it always passes through one of the synergids. This must occur very quickly, since on sections either pollen tubes contacting the synergid hoods or pollen tube contents inside the already destroyed synergid are seen. It is difficult to notice the wall of the emptied pollen tube. It would seem, therefore, that the pollen tube almost instantly penetrates into the synergid through the hood and then bursts at once ejecting violently, fountain-like, its contents into the synergid. The explosive discharge of the pollen tube contents causes the immediate bursting of the synergid at a predetermined site, that is in its chalazal part next to the egg cell, where the synergid wall is thinnest, and the discharge of the contents with the sperms between the central cell and the egg cell. After bursting of the pollen tube the synergid is destroyed and its entire contents stain very intensely with basic dyes (Fig. 5). Sperm transport through the synergid is very rapid, since the sperms of the first pollen tube has never been observed within the synergid.

Supernumerary pollen tubes have been found in 1/3 of the fertilized embryo sacs examined. Even three additional pollen tubes may enter one embryo sac. The sperms of additional pollen tube which has penetrated into the already destroyed synergid migrate much slower than those of the first pollen tube, and they can be observed in the successive stages of their path. A detailed description of penetration of the supernumerary pollen tubes into the embryo sac and their fate there is given in the chapter on fertilization anomalies.

The cytoplasm of the pollen tube with the two sperms flows out from the synergid to the widening space between the central cell and the egg cell, surrounding the latter partly. The vegetative nucleus of the pollen tube remains in the synergid. Exceptionally, it flows out with pollen tube cytoplasm as far as the outlet of the destroyed synergid. The synergid nucleus remains usually in its place with the surrounding cytoplasm, it does not disappear immediately and is well visible in the destroyed synergid into which even the contents of two pollen tubes have been discharged. These two nuclei, the one of the synergid and the vegetative nucleus of the pollen tube, degenerating slowly within the destroyed synergid and giving a strong Feulgen reaction, correspond to the X-bodies described for the first time by L a n d (1900) in a fertilized embryo sac of *Compositae*.

Sperm nuclei, their displacement and transformation before fusion

The sperms of *Helianthus annuus*, as well of the other *Compositae* investigated, seem to be devoid of their own cytoplasm, in mature pollen grains and in pollen tubes. In the light microspore only naked sperm nuclei are observed.

In the pollen grains and pollen tubes the sperm nuclei are very long, filiform or rather band-like. They appear double with spiral structure as shown by aceto-carmine smears. In the pollen grains they are usually straight. In the pollen tubes their shape varies, and they are often undulated. They give a strong Feulgen reaction.

After entrance of the pollen tube into the synergid, the sperm nuclei become spirally coiled. They are maximally condensed and tightly coiled in ball-like bodies when lying in the lowest position between the egg and the central cell. They then begin to uncoil gradually and penetrate into the female cells (Figs 5, 6). The sperm nuclei enter usually both female cells simultaneously (55—75 min after pollination, Table 1), sometimes, however, one enters the egg cell earlier. Penetration of the sperm nuclei into the central and the egg cell must occur very quickly, since their passage through the plasma membrane has never been observed. Therefore it is not known whether some of the pollen tube cytoplasm enters together with the male nuclei. This problem has so far not been investigated in detail. Immediately after penetration of the sperm nuclei into the female cells no traces of the male cytoplasm have been noted either in the egg cell or in the central cell. It would seem therefore, that the pollen tube cytoplasm filling the space between the egg cell and the central cell remains separate and does not fuse with that of these two cells. It is possible that it is later digested in situ or it may fuse with the cytoplasm of the central cell.

After penetration of the sperm nuclei into the female cells, uncoiled sperm filaments were observed in the egg cell cytoplasm close to its nucleus or on the surface of the secondary nucleus membrane (Figs 7—10). Just before penetration into the female nuclei, the sperm filaments are already uncoiled and loosely rolled up, bending and changing direction at several points (Fig. 11). During despiralization, in many places the duplicity of the sperm filament becomes visible giving it the appearance of a chain of eights (Fig. 12).

The changes in the shape and structure of both the sperm nuclei taking part in fertilization are the same and occur usually simultaneously until they penetrate into the female nuclei. They are very rapid (Table 1).

The sperm nuclei of the additional pollen tubes only undergo despiralization and condensation. These changes last generally longer than in the first sperm nuclei and lead to a greater condensation of chromatin.

## Nuclear fusion and postfertilization events

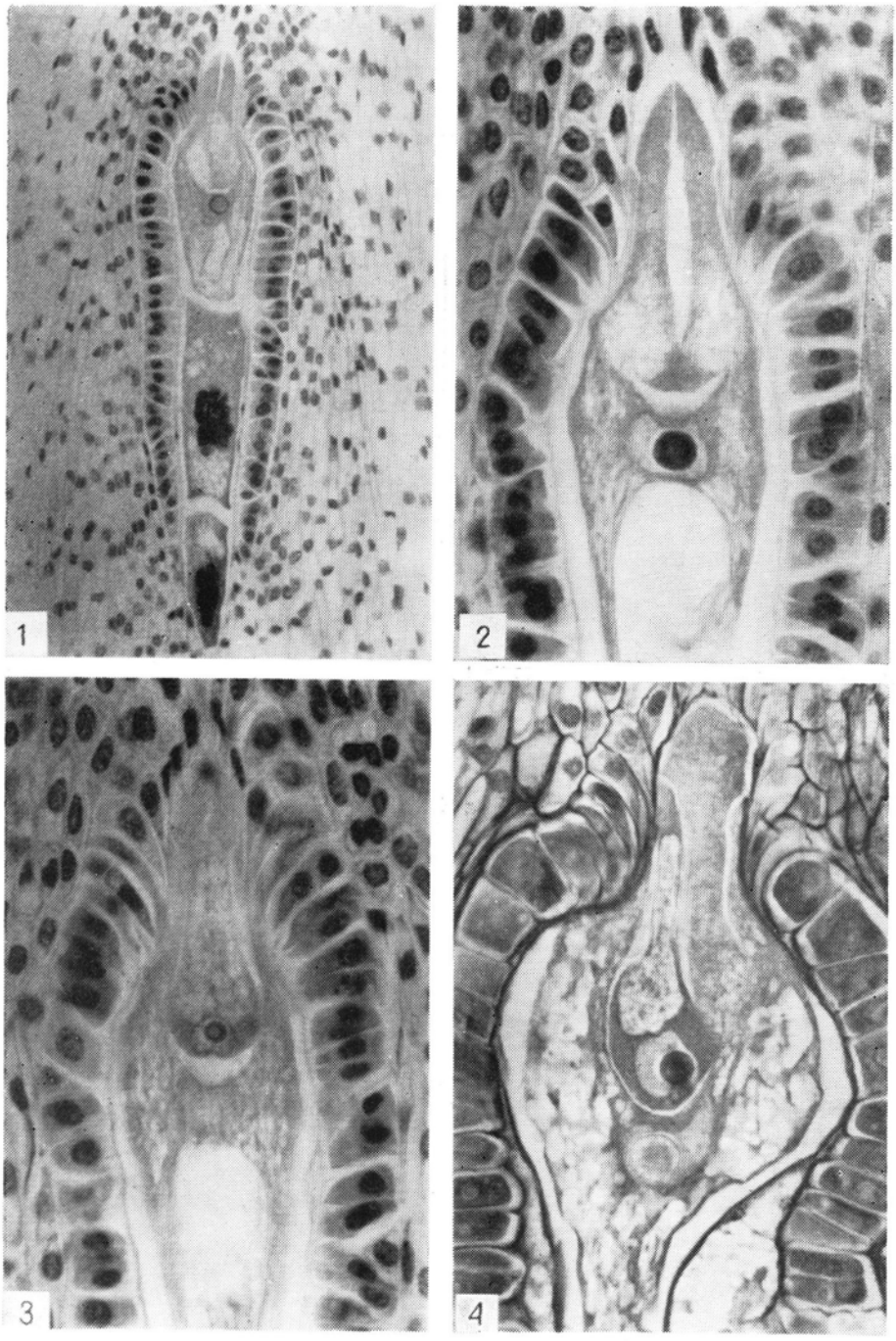
Penetration of sperm nuclei into both the female nuclei of the embryo sac can be followed on the same section. They enter into the both nuclei almost simultaneously. The later transformations of the sperm nuclei however, in both the female nuclei, observed on the same section differ in their stages although their course is similar: in the egg cell the changes are much slower and easier to follow. The sperm nucleus which in the form of a coiled thread penetrated into the egg cell stops at the nuclear membrane (Figs 13—14). The thread uncoils and elongates, it stains weaker at this stage, and then gradually sinks into the egg nucleus. Figures 15—18 show the sperm nuclei at the moment of their penetration into the female nuclei. The part of the sperm filament which is already immersed in the egg nucleus has a loosened structure.

The sperm nuclei then sink gradually into the female nuclei, and not simultaneously along their entire length. The sperm nucleus filament at this moment is loosely coiled and shows a varying configuration.

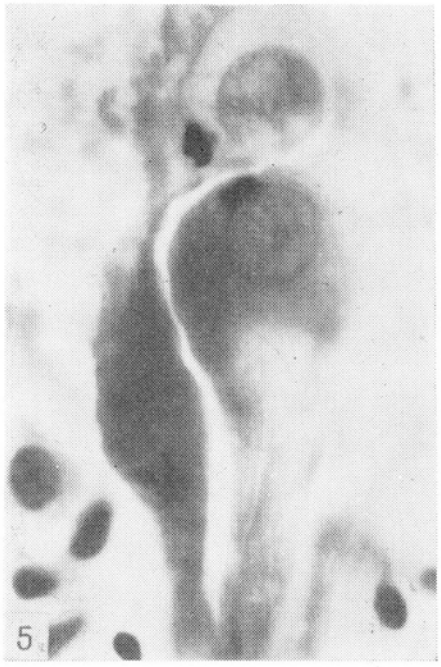
The part of the sperm nucleus filament immersed in the female nucleus stains weaker and has a looser structure than the part which is still outside. The uncoiling and loosening of the sperm nucleus structure thus starts at once after its penetration into the egg cell, and may be the direct response to the contact with the nuclear sap. The ribbon-like sperm nucleus assumes within the female nucleus a spireme-like configuration and falls apart into chromosomes (Figs 19, 20). These chromosomes exhibit at first a distinct spiral structure which gradually uncoils, and stain more and more weakly. Finally within the male chromatin area a nucleolus is formed (Figs 21, 22). At first visible in the form of a small granule, it gradually increases in size, but always remains much smaller than the female nucleolus. At this time the male chromatin, at first well visible and limited to the site of entrance, is further dispersed. The chromatin of the zygote nucleus has at this time the appearance of a delicate reticulum with numerous small chromocentres. The largest number of chromocentres is visible on the side where the male nucleus entered. Minute chromocentres may also be seen on the periphery of the female nuclei. The process of karyogamy thus ends by the dispersion of male chromatin in the egg nucleus, and eventually by fusion of the nucleoli (Fig. 23). This process, from the moment of penetration of the sperm nuclei into the female ones up to complete chromatin dispersion, lasts a much shorter time in the primary endosperm nucleus (ca. 30 min) than in the zygote nucleus (ca. 3 h, Table 1).

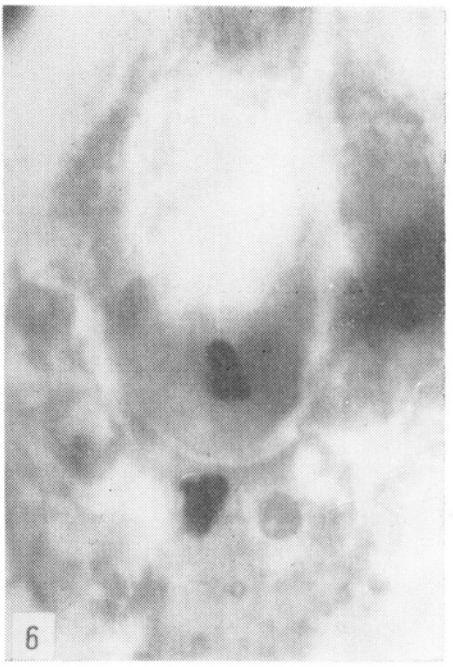
The processes preceding mitosis in the primary endosperm nucleus occur very rapidly and mitosis may start as early as one half hour after

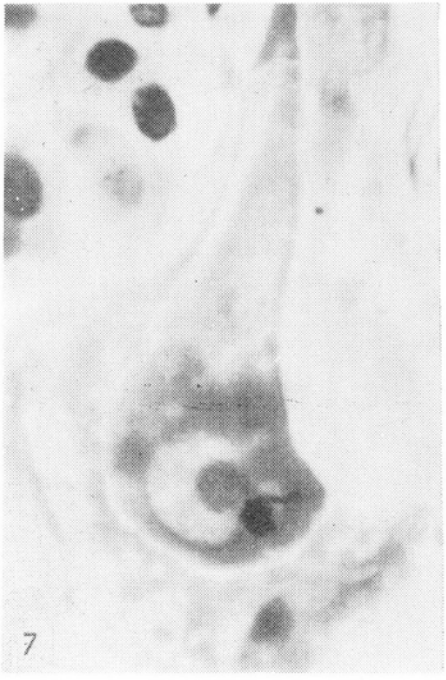
Plate I

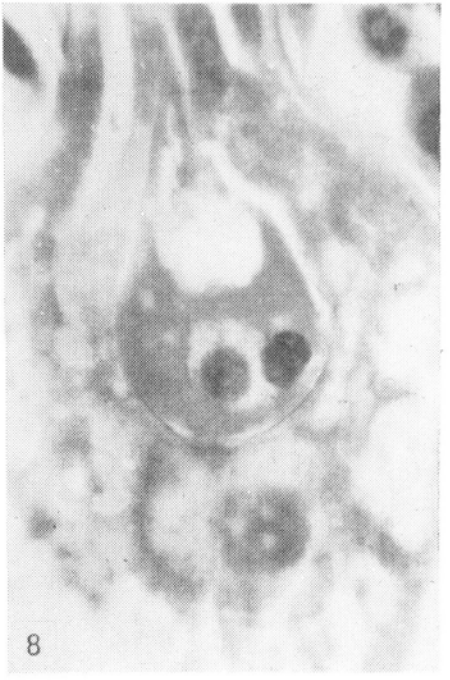



Figs 1—4. Structure of the embryo sac: fig. 1 — mature embryo sac, × 250; figs 2 and 3 — egg apparatus, front view, × 750; fig. 4 — egg apparatus, side view, × 750.

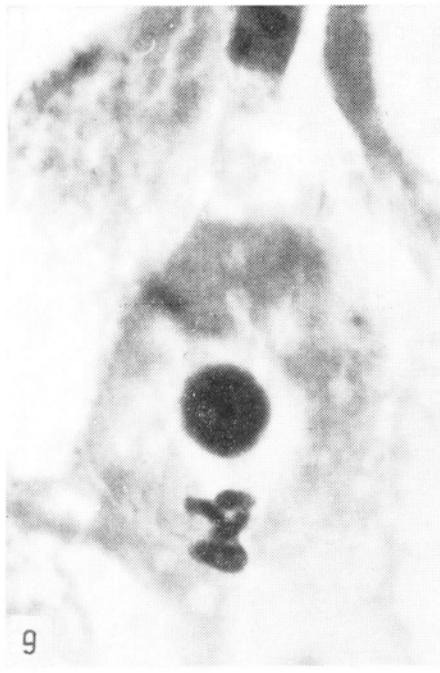


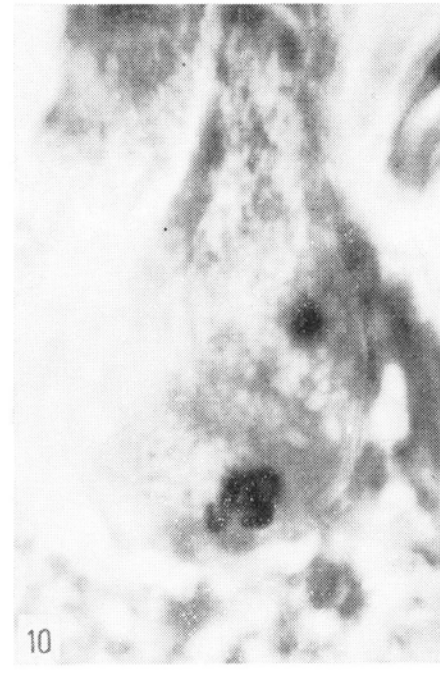



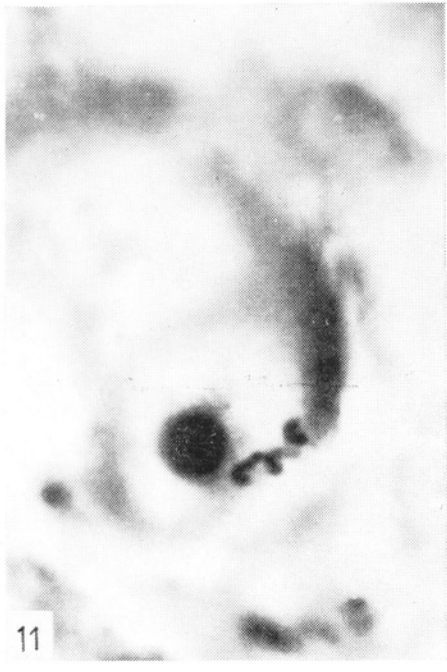



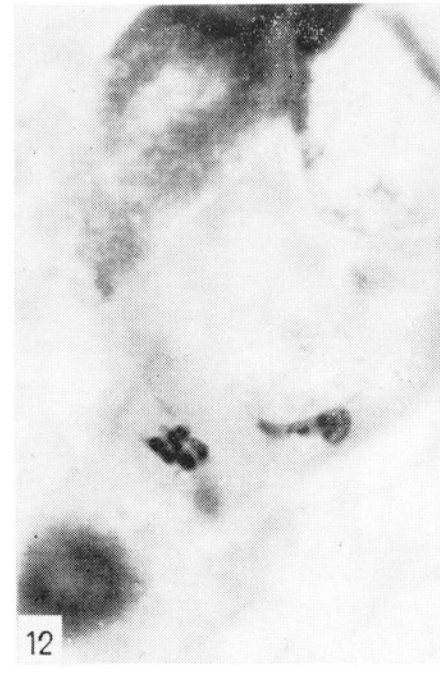




Figs 5—8. Sperm nuclei whithin female cells, × 1000

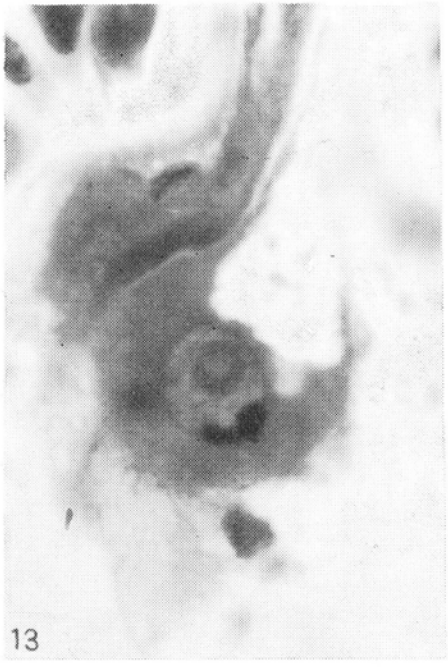


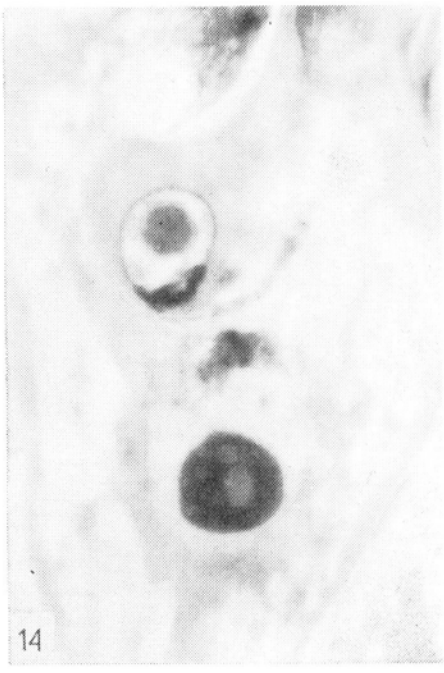



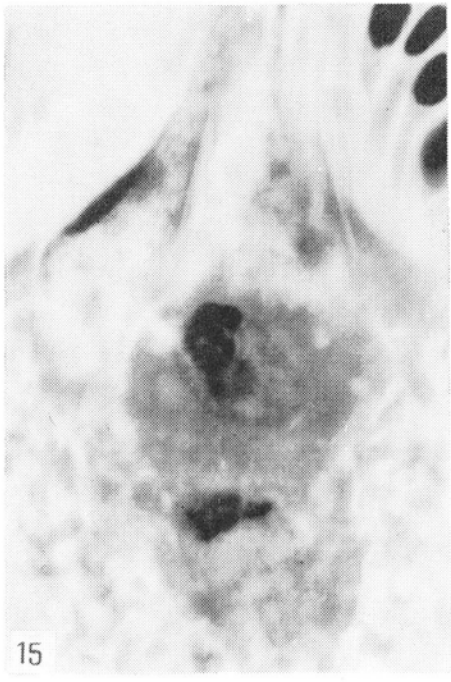



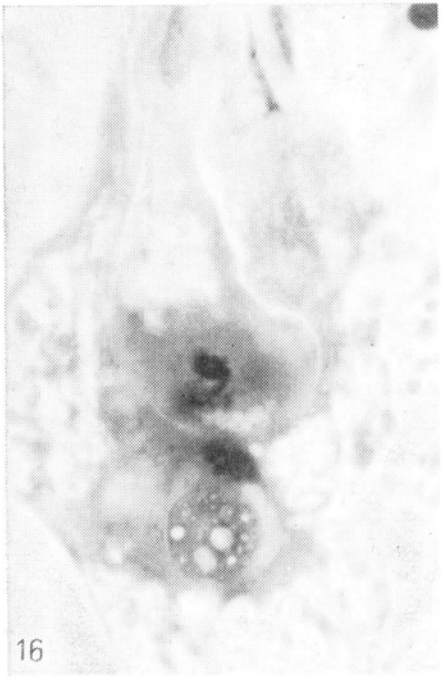




Figs 9—12. Unfolded, partly uncoiled sperm nuclei in contact with female nuclei, × 1000.




Figs 13—16. Sperm nuclei partly embeded in female nuclei, × 1000.

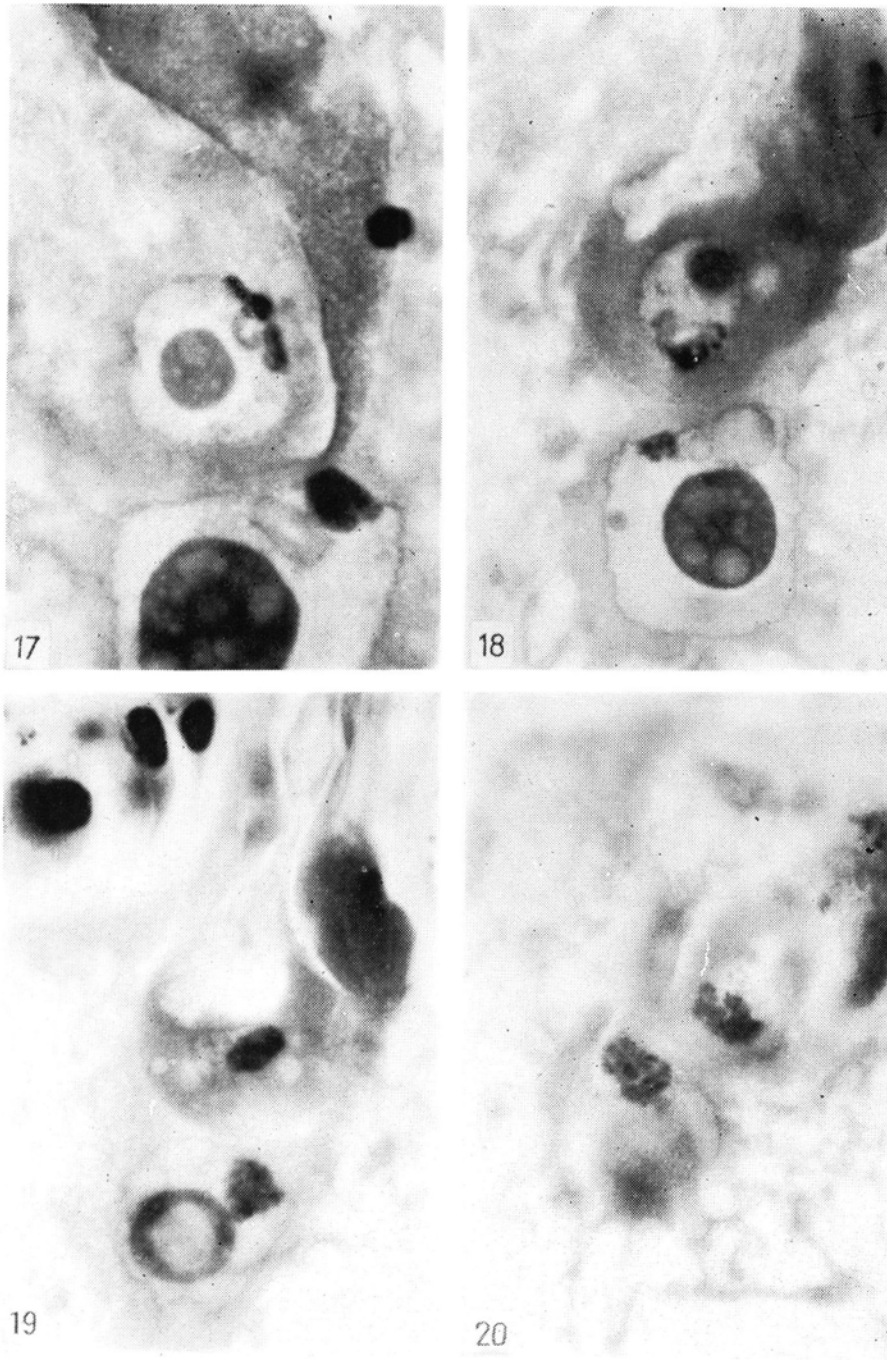



Fig. 17. Penetration of a part of a sperm nucleus into an egg nucleus and its despiralization, × 1500. Figs 18—20. Successive stages of sperm nuclei despiralization in female nuclei, × 1000.

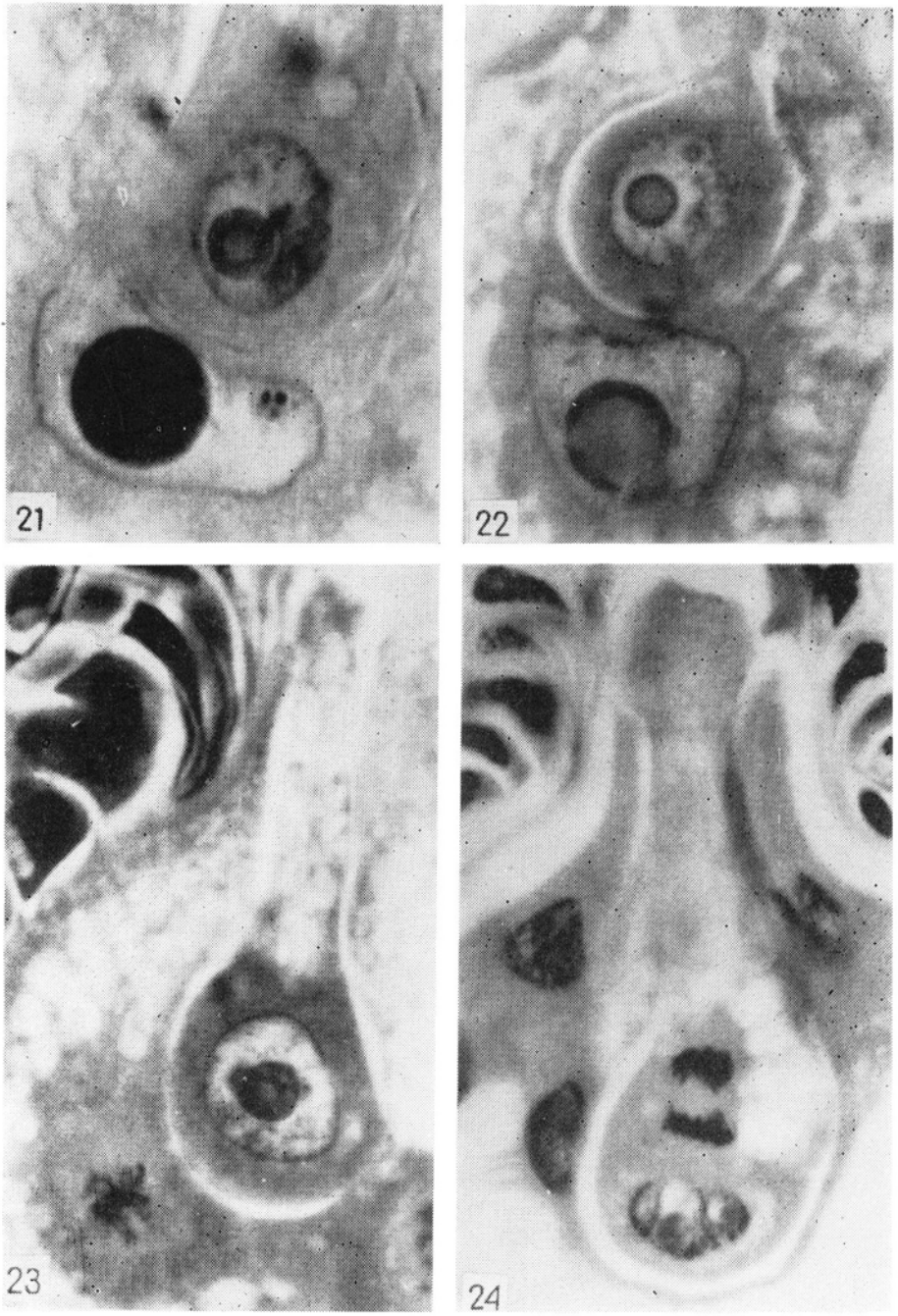



Fig. 21. Further uncoiling of male chromatin in female nuclei, male nucleolus with three vacuoles in primary endosperm nucleus, × 1500. Fig. 22. Male nucleolus just formed in zygote nucleus, × 1000. Fig. 23. Fusion of nucleoli in zygote nucleus, prometaphase in 2-nuclear endosperm, × 1000. Fig. 24. Two-celled proembryo, sixten-nuclear endosperm, × 1000.

penetration of the sperm nucleus into the secondary nucleus of the central cell. Three hours after fertilization, that is 4 h after pollination, 8-nucleate endosperm was found in 3 embryos, and 16-nucleate in one. Transition of nuclear endosperm into cellular endosperm takes place after formation of 32 nuclei, and coincides with the stage of the 8-celled embryo.

The first division of the zygote nucleus has not been observed. The two-celled embryo was not noted earlier than 3 h after penetration of the sperm nucleus into the egg nucleus, that is 4 h after pollination. Six hours after pollination all the ovules examined contained a two-celled embryo and 8- or 16-nucleate endosperm. Eight hours after pollination the embryo has 8 cells arranged in 4 tiers and the endosperm consists of 32 cells.

### Supernumerary pollen tubes in the embryo sac. Embryo sac and fertilization anomalies

Supernumerary sperm nuclei in the embryo sac have frequently been found. On 53 slides, that is in 1/3 of the fertilized embryo sacs examined, additional sperm nuclei were noted. Two pairs were found in 40, 3 pairs in 8, 4 pairs in 5 embryo sacs. These supernumerary pairs of sperm nuclei were brought by additional pollen tubes after abundant pollination.

The second pollen tube enters the embryo sac always after penetration of the first one, through the second synergid. The sperm nuclei of the second pollen tube are ejected from the bursting synergid together with the surrounding cytoplasm between the egg cell and the central cell, but they generally do not reach the cytoplasm of these cells. Only the sperm nuclei of the first pollen tube take part in fertilization of the female nuclei.

The third and fourth pollen tubes penetrate into the already previously damaged synergid. The sperm nuclei brought by these pollen tubes migrate much slower that those of the first and second pollen tubes and usually remain within the degenerating synergid. The supernumerary sperm nuclei remain rather long in the embryo sac. Degenerating sperm nuclei were observed in the destroyed synergid at the 8-celled embryo stage.

Abnormal embryo sacs are very rare; among several hundreds ovules examined only one abnormal embryo sac was found. In this ovule the embryo sac contained no egg cell. The synergids and the central cell were normally developed. One pollen tube had penetrated into this embryo sac, and both the sperm nuclei were near to the secondary nucleus of the central cell. In another ovule two embryo sacs were found. The upper one was normally developed (Fig. 4), and the additional incomplete embryo sac was situated between the chalazal part of the first one, next to its antipodals, and the endothelium.

Symptoms of anomaly in the fertilization process were also very rare. One of them was the beginning of embryo development without the development of endosperm, and another — beginning of endosperm development without fertilization of the egg cell.

## DISCUSSION

### The egg apparatus: spatial relationships and the boundaries of cells

In all angiosperms the egg apparatus occupies the apical, that is the micropylar part of the embryo sac. It thus lies on the pathway of the pollen tube to the embryo sac. The pollen tube — with the exception of rare cases of aporogamy — penetrates directly into the egg apparatus (Schnarf, 1929). Therefore, in order to answer the question by which way the sperms brought by the pollen tube enter into the egg cell and into the central cell, the structural organization of the egg apparatus has to be known in detail. However, detailed descriptions of the structural relationships between the synergids, the egg cell and the central cell are very scarce. The main cause is the difficulty in obtaining adequately spatially oriented sections and faulty fixation causing excessive shrinking of the cells. This is the main source of the discrepanciens in the descriptions of pollen tube entry into the embryo sac.

Members of the *Compositae* are convenient for studying the structural organization of the egg apparatus. The axis of the embryo sac coincides with that of the elongated ovule and the ovary axis, it is, therefore, easy to obtain appropriately oriented sections.

The present investigation demonstrated that the micropylar part of the egg apparatus in *Helianthus* is composed solely of synergid hoods, and that the micropylar part of the egg cell is fixed in the indentation separating the synergid hoods from the main part of the synergid. Thus, there is no doubt that the micropylar part of the central cell reaches only to synergid hoods, surrounding the egg cell and chalazal parts of the synergids. These spatial relationships between the synergids, the egg cell and the central cell are the result of their individualization during cytokinesis and subsequent growth (Gerassimova-Navashina, 1954).

These relationships are probably a characteristic feature of the organization of the embryo sac in all angiosperms, since they have been described both in dicotyledons (*Helianthus* and *Crepis*, and *Gossypium* — Jensen, 1965) and monocotyledons (*Scilla* — Gerassimova-Navashina and Batygina, 1958; *Triticum* — Batygina, 1962).

Thus, the pollen tube can penetrate into the embryo sac through one of the synergids or between them. Normally it passes through the synergid (Nawashin, 1909; Ishikawa, 1918; Steffen, 1951, Vazart, 1955; Van der Pluijm, 1964; Jensen and Fisher, 1967; Diboll, 1968; Cass and Jensen, 1970, and many others). In exceptional cases the pollen tube may penetrate deep between the synergids, but then it does not open (Schnarf, 1929).

The boundaries delimiting the cells of the egg apparatus in the *Helianthus annuus* embryo sac are typical for angiosperms. The synergids and the upper, micropylar part of the egg cell are surrounded by cell walls. But in the lower, chalazal part of the egg cell there is only a plasma membrane without any cell wall materials between this membrane and the central cell plasma membrane. This was demonstrated first in the *Oenothera* embryo sac by Ishikawa (1918). The expanded lower part of the synergid may have a thin cell wall, as seems to be the case in *Helianthus*, and as it was found in *Capsella* by Schultz and Jensen (1968) and in *Hordeum* by Cass and Jensen (1970), or it may be without cell wall materials as described by Van der Pluijm (1964) in *Torenia*, Jensen (1965) in *Gossypium*, J. Vazart (1969) in *Linum* and Godineau (1966 and 1969) in *Crepis* and *Picris*. This part of the synergid is the predetermined site for release of the pollen tube contents from the synergid between the egg cell and the central cell plasma membranes.

## The pollen tube, its entrance into the embryo sac and opening

The pollen tube of *Helianthus annuus* swells while approaching the synergid hood. In this dilated end most of the cytoplasm, sperms and the vegetative nucleus are accumulated.

It is probable that the pollen tube entering the synergid dissolves the synergid wall by excreting enzymes without damaging its own wall, but this point is not fully understood.

There are no data on the chemical components of the pollen tube tip wall at this very moment, except that it is PAS-positive.

Dashek and Rosen (1966) have shown on *Lilium* pollen tubes, growing in vitro, that their tip wall contains pectin and an acid mucopolysaccharide. In crassinucellate ovules, e.g. in *Oenothera* (Ishikawa 1918, Kaienburg 1950) the pollen tubes penetrate between the nucellar cells dissolving the pectinous middle lamella. They must excrete pectinase without damaging their own walls.

Kroh (1964) states that in the cruciferous stigma "the pollen tubes dissolve the pectic constituents of the cellulose-pectic layer without

attacking the cellulose lamellae". It is possible that the cell wall lysing enzymes diffuse from the pollen tube in inactive state and are then activated by substances excreted by the female tissue as in the cross--compatibility reaction in *Brassica* (Röbbelen 1960, Linskens 1969).

The pollen tube, immediately after entering into the synergid, probably bursts, and its contents are violently ejected fountain-like, disrupting the synergid wall in the predetermined site, and flowing out between the egg cell and the central cell into the artificially widened space between their plasma membranes. This is the simplest interpretation of the facts observed.

The penetration of the pollen tube contents from the synergid into the "cleft" between the egg and the central cell was described first by Nawashin (1909) in *Helianthus* and *Lilium* and then by many others, e.g. in the light microscope: Gerassimova in *Crepis* (1933), Steffen (1951) in *Impatiens*, Vazart (1955) in *Linum* and other species, Gerassimova-Navashina and Korobova (1959) in *Scilla*, and in the electron microscope: Fisher and Jensen (1969) in *Gossypium* and Godineau (1969) in *Picris* and other *Compositae*. Godineau states that the pollen tube contents penetrate into the intercellular space delimited by plasma membranes.

The main function of synergids is no doubt to bring the pollen tube contents with the sperm between the egg cell and the central cell without damage to these cells, as was postulated first by Gerassimova-Navashina and Korobova (1959) and Van der Pluijm (1964).

The bursting of one of the synergids bringing the discharged pollen tube cytoplasm with the sperms to the right position between the female cells, is probably due to the explosive bursting of the pollen tube immediately after its entrance into the synergid.

In culture media of too low osmotic values the pollen tubes burst explosively discharging fountain-like their contents into the medium. The sudden bursting of cells may be caused, too, by various other factors diminishing the turgor pressure resistance of the cell wall. All this is known as plasmoptysis (Fischer 1906, Küster 1956). Plasmoptysis is widespread among lower plants as a mechanism releasing generative cells, zoospores, ascospores and sperms.

Plasmoptysis is probably the mechanism of pollen tube opening. This was taken into account by Steffen (1951) and suggested by Van der Pluijm (1964) as the underlying cause of pollen tube opening, as well of the bursting of the synergid and bringing the sperms between the egg and the central cell.

## The sperms, the sperm nuclei and their spiralization-despiralization cycle

In *Helianthus*, as in other *Compositae*, the sperms are of an extreme, peculiar type.

The mature sperms of *Compositae* are apparently free of cytoplasm (Schnarf 1941), although the generative cells do have their own cytoplasm as shown by many authors in the light microscope (e.g. Davis 1961) and in the electron microscope by Lombardo and Gerola (1968). The sperm nuclei lose their cytoplasm during mitosis in the generative cell, as in *Crepis* (Gerassimova-Navashina 1951), or immediately after this mitosis, as in *Echinops* (Poddubnaja 1927).

Extremely elongated, thread-like sperm nuclei in the pollen grains and the pollen tubes of *Helianthus*, *Crepis* and other *Compositae*, examined by us on acetocarmine smears, have marked duality, forming twisted double bands. In most condensed state, when lying between the egg and the central cell after discharge of the pollen tube contents, they are half-folded and tightly coiled (Gerassimova 1933, Fig. 7). Thereafter, in the egg or central cell cytoplasm they uncoil and unfold, elongating many times, as was observed by Gerassimova (1933) in *Crepis* and by us in *Helianthus*. They evidently undergo spiralization and despiralization. In partially despiralized state the sperm nuclei bands of *Helianthus* seem to be double and twisted on all their length, i.e. their longitudinal halves are relatively coiled. If this doubleness corresponds to half chromatids, as it seems to do, then in the ribbon-like sperm nuclei the chromosomes must be united end to end. It is proven to be so in *Crepis* (Gerassimova 1933, Fig. 21) and *Helianthus* when band-like sperm nuclei fall apart into despiralized chromosomes after entrance into the female nuclei.

The mechanism of sperm nuclei transformations during fertilization in *Compositae* seems to be, therefore, the very mechanism underlying chromosome spiralization and despiralization. This mechanism is environment-dependent, as has been experimentally demonstrated.

## Postfertilization events, the cell cycle time in the proembryo and in the nuclear endosperm

Fertilization in *Compositae* is of an extremely premitotic type (*Helianthus* type). The newly formed sperm nuclei assume after early telophase a singular thread-like form (Gerassimova-Navashina 1951) with condensed chromosomes, described and discussed in the preceding paragraph, and undergo post-telophasic transformation not

earlier than within the female nuclei after karyogamy (Gerassimova-Navashina, 1969, and this paper). In the nuclear sap of female nuclei they fall apart into chromosomes. The chromosomes despiralize, and form a small nucleolus which eventually fuses with the female nucleolus. Afterwards, the zygote and the primary endosperm nuclei pass to the rest period. It is during this rest period that the male chromosomes may be reduplicated.

In the primary endosperm nucleus of *Helianthus* the rest period after fertilization is surprisingly short. The minimal interval between the entrance of the sperm nucleus into the secondary nucleus and the first endosperm metaphase is not longer than 30 min.

Evidently all the molecular machinery required for the extremely rapid structural chromosome transformations, and triggering of their reduplication is at hand in the nuclear sap of the secondary nucleus. The 16 nucleate endosperm may be formed within 3 hours, i.e. the shortest nuclear cycle time observed in the *Helianthus* endosperm is not longer than 45 min. The interval between the 2-nucleate stage and the 32-celled stage equals 4 h. The average nuclear cycle time is thus 60 min.

The rest period in the zygote nucleus is of longer duration. The first 2-celled proembryo was observed 3 h after karyogamy, and the 8-celled proembryo after subsequent 4 h. The shortest cell cycle time in the early proembryo of *Helianthus annuus* is therefore not more than 2 h, whereas in the apical meristem of growing seedling root of this plant the average cell cycle lasts 8 h (Van't Hof and Sparrow 1963). Taken into account the differences of temperature (27°C in controlled fertilization experiments and not mentioned, laboratory temperatures during growth of seedling roots), and the fact that the cell divisions in the proembryo are partition divisions i.e. segmentations halving the cell size, the difference in the cell cycle time is not so striking as it seems to be.

The difference between the nuclear cycle time in the endosperm and the cell cycle time in the proembryo is worth considering, the mean nuclear cycle being two times shorter. This was to be expected. But the cycle time itself is very short.

## Mechanisms accelerating the succession of nuclear divisions in the endosperm

Very rapid succession of mitoses occurs at the cleavage of amphibian eggs. In *Xenopus laevis* the average cell cycle time lasts about 15 min. DNA synthesis occurs in every cell cycle, and it begins in late telophase before mitosis is complete. There is thus no $G_1$ phase, and $G_2$ phase is very short or absent (Graham 1966, Graham and Morgan 1966). The interphase is therefore atypical, no nucleoli are formed, and all

the new proteins required for the cleavage divisions are synthesized on long-lived ribosomes and m-RNA formed during maturation of the egg (Davidson 1968).

This is the mechanism accelerating blastomere divisions in amphibia and sea urchins (Duspiva 1971). But the mechanism accelerating the endosperm nuclei divisions in *Helianthus* is different.

Before fertilization, the central cell nucleolus is enormous, indicating active synthesis of ribosome precursors. The ribosomes are dispersed all over the central cell, as was manifested by the RNA-staining reaction (unpublished cytochemical investigations on RNA distribution in the *Helianthus* embryo sac before and after fertilization). They do not suffice, however, to maintain the rapid sequence of mitoses. In the nuclear endosperm there is a typical interphase with large nucleoli. The nucleoli are very active forming ribosome precursors. The presence of numerous ribosomes around new formed nuclei is indicated by the RNA-staining reaction. (The basophilic cytoplasm around the dividing endosperm nuclei of *Silphium laciniatum* is shown on fig. 10 of Land's (1900) paper). It is therefore probable that most, if not all, the proteins required for reduplication of chromosomes and for normal course of the nuclear endosperm mitoses are formed on the new ribosomes. The rapid succession of mitoses in the nuclear endosperm must, thus, depend upon the accelerated cyclic production of new proteins synthesized on the newly formed ribosomes as well as upon the accelerated cyclic nucleic acids syntheses. All this in turn must be dependent on the preformed pools of proteins and nucleic acids precursors as well as on the energy sources stored in the central cell during its long maturation period before fertilization.

The successive splitting of polynemic chromosomes is suggested as another mechanism accelerating nuclear divisions (Darlington 1955). This, in the modified form of endoreduplicated chromosomes, may be the case in the proembryo and the nuclear endosperm in *Hordeum* and related genera.

The fertilization process in *Hordeum, Triticum* and other related species was examined recently by Vazart (1955, 1958), Batygina (1962) and Cass and Jensen (1970). It follows from these studies that the fertilization of the egg cell is of premitotic type, the zygote nucleus entering after karyogamy into a long rest period (18 h in *Triticum* — Batygina 1962). On the other hand, the fertilization of the central cell is of a modified postmitotic type. The sperm nucleus fuses with one of the polar nuclei. The fertilized polar nucleus enters into a very short rest period. Then, in both polar nuclei prophase begins, and hereupon a fusion of both prophase nuclei is accomplished, i.e., there is a modified tripple fusion (Vazart 1955 for *Hordeum*).

According to D'Amato et al. (1965) sperm nuclei in pollen grains of *Hordeum* have a duplicated 2 C amount of DNA. Microspectrography measurements made by the two wave method (Mericle and Mericle 1970) revealed that the DNA content in the zygote nucleus amounts to 16 C, and in the two-celled proembryo to 8 C, falling in the later stages of proembryo development to the typical diploid level 2 C—4 C.

The DNA content in the endosperm nuclei was not determined. If the DNA content in each of the polar nuclei and in the egg cell nucleus before fertilization would be the same as in the sperm, and this is to be expected, it would amount to 2 C. There seems to be no time or opportunity for further endoreduplications before the first endosperm mitosis in *Hordeum*. It follows that repartition of previously endoreduplicated chromosomes may occur only during the first endosperm mitosis. On the contrary, there is ample time for two endoreduplication cycles of chromosomes during the long rest period of the zygote nucleus.

It may be that two different mechanisms exist in angiosperms accelerating the rate of nuclear divisions in the proembryo and endosperm development: the one described in *Hordeum* based on successive splitting of preformed, endomitotically duplicated chromosomes (Mericle and Mericle 1970), and the second suggested in this paper, based on accelerated cyclic desoxyribonucleic and ribonucleic acids syntheses as well as on rapid proteins synthesis on the newly formed ribosomes, dependent upon the preformed precursors pool.

If, however, as it seems to be, all the cyclic, endogenous biological rythms are controlled by some elementary feed-back mechanisms and their interactions (cf. Hess and Boiteux 1971), then it is the acceleration of these mechanisms that is the very cause of the surprisingly short nuclear cycle in the *Helianthus* endosperm.

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## *Podwójne zapłodnienie u Helianthus*

Streszczenie

Opisano przebieg podwójnego zapłodnienia u słonecznika śledząc częstość występowania kolejnych jego etapów po różnym czasie od momentu sztucznego zapylenia (tabela 1).

Szczególną uwagę zwrócono na:

— przestrzenną organizację strukturalną aparatu jajowego i sposób wyodrębnienia komórek,

— przebieg wnikania łagiewki do mikropylarnego kołpaka synergidy i przelanie się zawartości łagiewki z synergidy między komórkę jajową i centralną komórkę woreczka zalążkowego,

— przekształcenia struktury jąder plemnikowych przed wniknięciem ich i po wniknięciu do cytoplazmy obu żeńskich komórek oraz wewnątrz jąder żeńskich po kariogamii.

Stwierdzono, że średni cykl komórkowy w pierwszym etapie rozwoju prazarodka do osiągnięcia 8-komórkowego stadium trwa 2 godziny, a średni cykl jądrowy w bielmie — 1 godzinę. Przekształcenie bielma jądrowego w bielmo komórkowe następuje w stadium 32-jądrowym.

W dyskusji podkreślono rolę strukturalnej organizacji aparatu jajowego w procesie zapłodnienia.

Wskazano na to, że wspólną przyczyną otwarcia łagiewki i przedostania się jej zawartości wraz z obu jądrami plemnikowymi między komórkę jajową a komórkę centralną jest prawdopodobnie plazmoptyza, tj. nagłe pęknięcie szczytowej części łagiewki i wybuchowe wyrzucenie jej zawartości, pociągające za sobą pęknięcie ściany synergidy w predeterminowanym miejscu.

Nitkowatą strukturę jąder plemnikowych *Compositae* zinterpretowano jako szeregowe złączenie chromosomów sugerując, że zmiany ukształtowania jąder plemnikowych w czasie zapłodnienia są następstwem spiralizacji i despiralizacji chromosomów.

Rozważając szybkie następstwo mitoz w bielmie jądrowym powołano się na obserwacje cytochemiczne, wskazujące na syntezę RNA i wytwarzanie nowych rybosomów w każdym cyklu jądrowym w bielmie oraz na argumenty przemawiające za każdorazową reduplikacją chromosomów. W bielmie jądrowym jest zatem normalna, pełna interfaza.

Niezwykłe skrócenie cyklów mitotycznych w bielmie jądrowym *Helianthus* jest prawdopodobnie uzależnione od procesów przygotowawczych zachodzących w centralnej komórce w okresie dojrzewania woreczka zalążkowego i bezpośrednio wywołane przez przyśpieszenie procesów regulujących przebieg normalnego cyklu mitotycznego.