

Cytological studies in *Ascobolus immersus*

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INTRODUCTION

Ascobolus immersus is a heterothallic, coprophilic fungus belonging to *Ascomycetes* (*Euscomycetes*), order *Pezizales*. Mature asci of *A. immersus* contain eight large, dark pigmented, unordered ascospores. The spontaneous mutations concerning spore pigmentation are very frequent and were used intensively (Lissouba, Mosseau, Rizet and Rossignol, 1962; Gajewski, Kruszewska, Makarewicz, Paszewski, Surzycki and Bielawska, 1963; Makarewicz, 1964; Surzycki and Paszewski, 1964) to study intragenic recombination. As in spite of great progress in genetical analysis of *A. immersus* nothing is known about its cytology, we had to provide some observations concerning ascus development and meiotic divisions.

MATERIAL AND METHODS

Observations were made on *Ascobolus immersus* wild strain from collection of Department of General Genetics (Warsaw). To obtain material suitable for cytological study + and — strains were crossed on sterilized horse dung in Petri dishes. After 10 — 15 days in temp. +22°C perithecia were formed with young asci in different stages of development. Perithecia were fixed together with thin layer of dung. For staining perithecia were removed from the dung using dissecting microscope. For spore germination study the mature spores were germinated on agar with NaOH (0.15%) in Petri dishes in temp. 39°C for 18—24 hrs. After germination spores were transferred together with thin layer of agar on the slide with egg albumen and then stained.

Fixation. Material for smears was fixed in Carnoy fixative (3:1) for 30 min. to five days. Fixed material could be stored in the refrigerator for several weeks. Material for permanent preparations was fixed in Carnoy, Helly and mercuric chloride-formalin fixative. Small pieces of dung didn't interfere with embedding, cutting and staining.

Special methods. Some types of pretreatment were useful e.g. 1% NaOH; 1 KOH; 1N HCl; mixture of 1N HCl with 2% aluminum alum, 2% chromic alum and 2% iodic acid. The best results were obtained with material hydrolysed in 1N HCl for 8 — 10 min. at temp. 60°C. Such

kind of pretreatment caused asci to separate, the cytoplasm to stain very lightly as opposed to the rather heavy staining of untreated asci, also spindles were better visible.

Staining. Several methods of staining were tried: 1) Orcein in 45% acetic acid, 2) Orcein in 45% propionic acid, 3) Orcein in 45% acetic + propionic acid, 4) Carmine in 45% acetic acid, 5) Haematoxylin Regaud, 6) Acid fuchsin — aurantia — toluidine blue, 7) Azur B, 8) Feulgen, 9) Feulgen — carmine technic (McIntosh, 1953), 10) Giemsa HCl (Hrushovetz, 1956), 11) Aceto — iron haematoxylin HCl (Lowry, 1963). The best results were obtained with the two last methods.

Giemsa HCl. Material was placed in Carnoy's type fixative (6 parts absolute alcohol, 1 part glacial acetic acid, 1 part lactic acid) for 10 min., rinsed in 95% ethanol, and then transferred to 70% ethanol. Perithecia free from the dung were placed in cold 1N HCl for 10 min., hydrolysed in 1N HCl at 60°C for 7 min., washed several times in distilled water, and finally placed in phosphate buffer solution of pH 6.9. Material from the buffer was transferred to Giemsa stain (1:1 with buffer solution) and stained overnight. After washing in buffer solution material was placed on microscope slide in the drop of buffer.

Aceto-iron-haematoxylin HCl. Perithecia were fixed in Carnoy (3:1). After fixation they were rinsed several times in distilled water. Material was hydrolysed in 1N HCl and then rinsed thoroughly several times in distilled water but it was kept in water not longer than several hours. Dissected perithecia were put on a slide in the drop of the stain. Material was covered with the cover slip and heated just below the boiling point of the stain. Pressure was applied under several thickness of blotting paper. The slide was passed quickly over a spirit flame 5 — 6 times.

Microscopy. A Zeiss microscope was used with Planchromatic HI 100/1,25 objective and PK $\times 16$ ocular. A green Kodak Wratten Filter in "B" glass and projective 4:1 ocular were employed during photography. Camera lucida was used for drawings.

RESULTS

The development of asci and meiosis. The ascus development in *Ascobolus immersus* is very much as in *Ascobolus magnificus* (Wood, 1953), *Neurospora* (Singleton, 1953), and *Sordaria* (Carr and Olive, 1958). The earliest stage observed in preparations of ascogenous hyphae was crozier formation (Fig. 1). The two haploid nuclei of the crozier undergo conjugate division. At metaphase of conjugate division in the crozier two plates are laying closely to each other (Fig. 2). Cell with four haploid nuclei is formed after conjugate division. Each of the four nuclei has distinctly visible nucleolus.

Cross walls cut off the uninucleate ultimate and basal cells from binucleate penultimate cell from which the ascus develops (Fig. 3). Proliferation of the crozier occurs through the fusion of the ultimate and basal cells. In each of the two nuclei of the young ascus just prior to its fusion a prominent nucleolus and chromatin strands are visible. Shortly after fusion the chromosomes undergo contraction (Fig. 4) and synapsis begins (Fig. 5) like in *Sordaria* and *Neurospora*. Beginning from this stage it was possible to see the centriole placed near the nucleolus (Fig. 6).

Before meiosis numerous bodies were observed in the cytoplasm of young asci (Fig. 7, 8). They are probably mitochondria because they stain with Regaud haematoxylin after Helly and mercuric chloride fixative and stain red with acid fuchsin — aurantia — toluidine blue after the same fixatives. But on the other hand they stain also with aceto-haematoxylin after acetoalcohol fixative (fixation 2—4 days) which usually destroys mitochondria.

The young ascus grows quickly and the nucleus occupies the central position. In the pachytene the chromosomes and the nucleus are much bigger (Fig. 9). Approximate dimensions of asci and nucleoli at various stages of development as seen in squash preparations are as follows:

Stage	Ascus lenght in μ	Nucleolus diameter in μ
Synapsis — late pachytene	25 — 100	4 — 7
After I Division	80 — 100	3 — 4
After II Division	85 — 100	2 — 3
After III Division	80 — 100	1,5 — 2

Fig. 10 shows the nucleus with distinct membrane and big nucleolus in late pachytene. In the nucleus pairing between homologous chromosomes is almost complete and the centriole is visible. By full pachytene the ascus length reaches about 100 μ , the nucleolar diameter increases from about 2 μ to about 5 μ and the nucleolar chromosome elongates from about 2 μ in the contracted presynaptic condition to more than 10 μ . The bivalents clearly differ in lenght but it was never possible to analyse the whole complement.

The chromosomes through diplotene to metaphase I undergo contraction and condensation. Diplotene nucleus is rather difficult stage for detailed observations. It is scarcely possible to distinguish individual bivalents. The chromosomes become thicker and more fuzzy in appearance (Fig. 11). During early diakinesis they look more distinct in outline (Fig. 12). In later diakinesis the chromosomes are much thicker and contracted, reaching the size about 1,0—1,5 μ . Figs 13, 14 and accompanying drawings (13a, 14a) show metaphase with about eight bivalents but sometimes also one small fragment laying apart is visible.

Plate I

Fig. 1—8. Fig. 1. Ascogenous hyphae (note different stages of crozier formation). Fig. 2. Conjugate divisions in the crozier. Fig. 3. Cross walls formation in the crozier (arrow indicates binucleate penultimate cell). Fig. 4. Young ascus with the fusion nucleus (note contracted chromosomes). Fig. 5. Synapsis of the chromosomes. Fig. 6. The nucleus of young ascus (arrows indicate big nucleolus and centriole). Figs 7, 8. Asci with numerous cytoplasmic bodies (mitochondria?). All magnifications $\times 1100$. Staining — Figs. 1—6. Acetohaematoxylin, smears; Fig. 7. Acid Fuchsin — Aurantia — Toluidin Blue, permanent preparation; Fig. 8. Regaud Haematoxylin, permanent preparation.

Plate II

Fig. 9—14. Fig. 9. Early pachytene (note big nucleolus). Fig. 10. Late pachytene (arrows indicate big nucleolus and centriole). Fig. 11. Early diplotene (note fuzzy appearance of chromosomes). Fig. 12. Early diakinesis. Figs 13, 13a. Middle diakinesis. Figs 14, 14a. Late diakinesis.

All magnifications $\times 1100$ except 13a, 14a; Figs 13a, 14a drawings $\times 2800$. Staining — Acetohaematoxylin, smears.

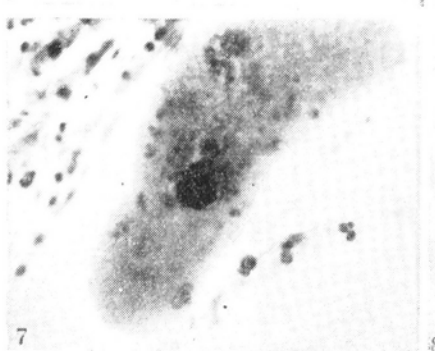
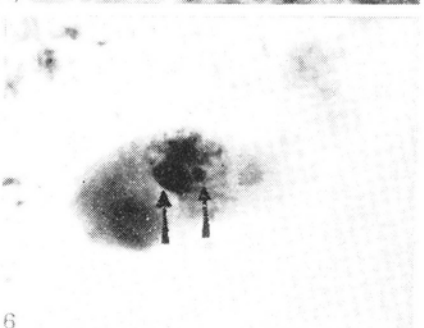
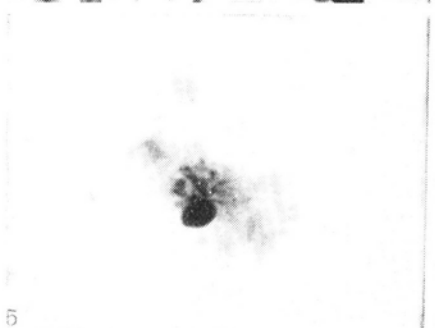
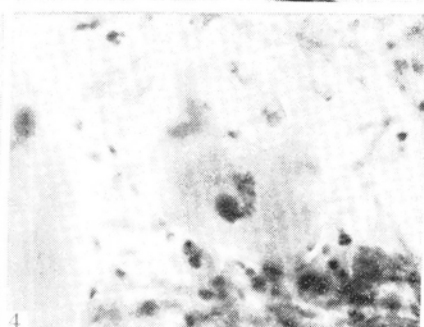
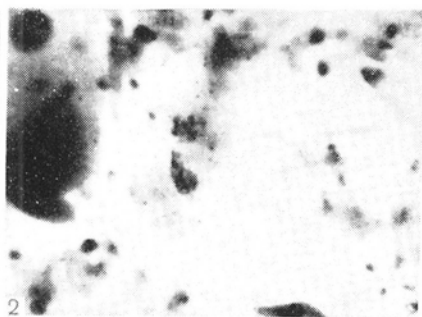
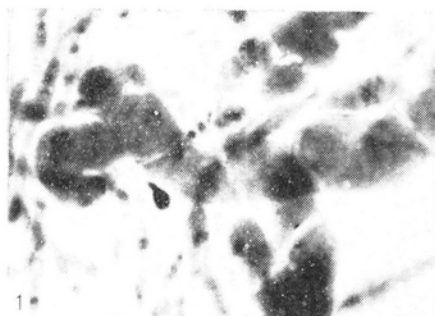
Plate III

Fig. 15—22. Fig. 15. Metaphase I (arrow indicates spindle). Fig. 16. Early anaphase I (note asynchronous disjunction). Fig. 17, 18. Telophase I (the same ascus in two focal levels), dark body between two chromosome groups is nucleolus passing out into the cytoplasm. Fig. 19. Two sister nuclei after I division. Fig. 20. Prophase of II division (arrow indicates old nucleolus). Fig. 21, 22. Metaphase II, two focal levels in the same ascus. All magnifications $\times 1100$. Staining — Acetohaematoxylin, smears.

Plate IV

Fig. 23—30. Fig. 23, 24. Four sister nuclei after II division (note different arrangements of the nuclei). Fig. 25—27. Different stages of III division, Fig. 25, 27 two focal levels and Fig. 26 camera lucida drawing of the same ascus. Fig. 28. Beaks formed by the centrioles after III division. Fig. 29. Ascus with eight nuclei before wall formation of the spores. Fig. 30. Typical ascus with eight spores (to the left) and abnormal ascus with nine spores (to the right). All magnifications $\times 1100$ except Fig. 30. $\times 450$. Staining — Acetohaematoxylin, smears.

Plate I



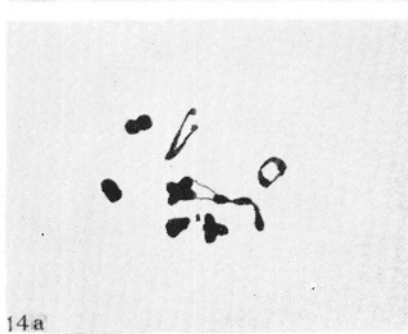
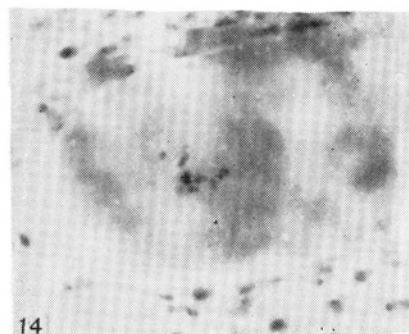
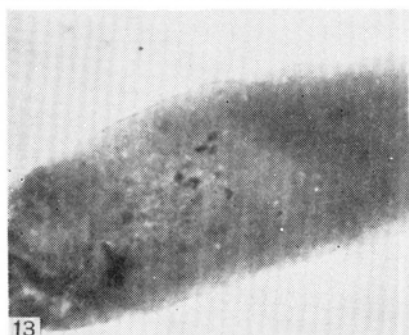
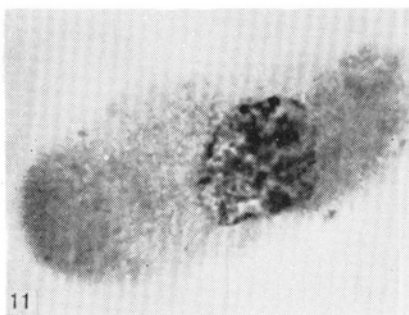
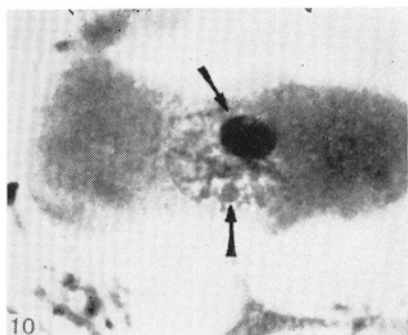
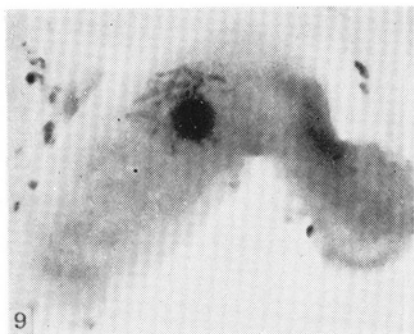


Plate III

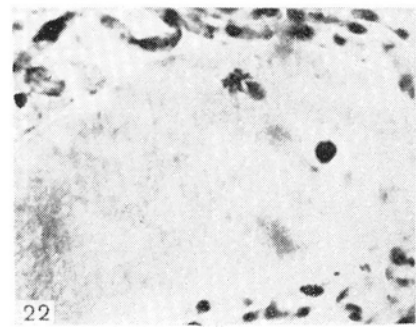
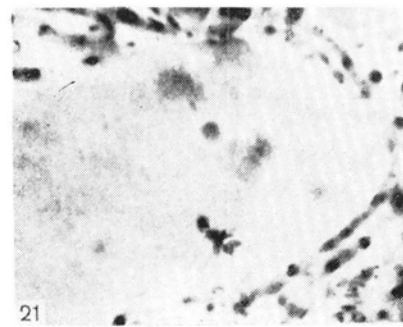
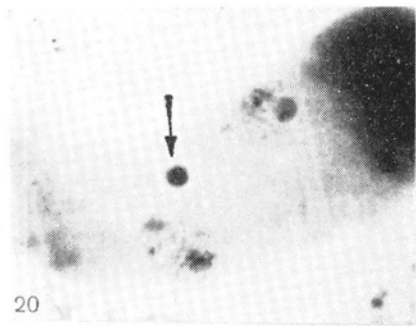
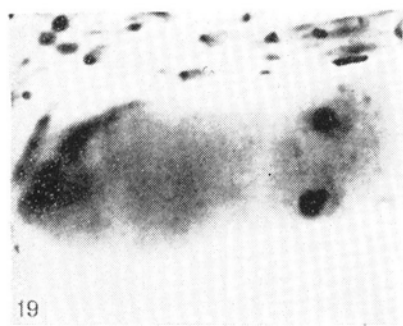
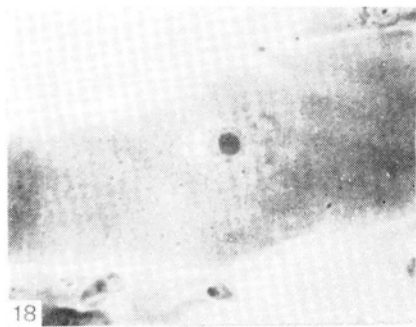
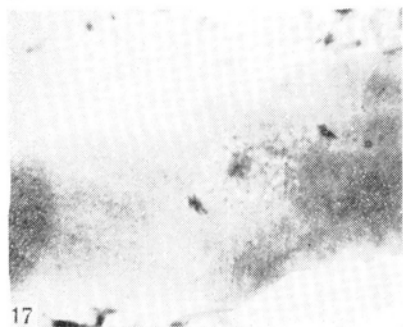
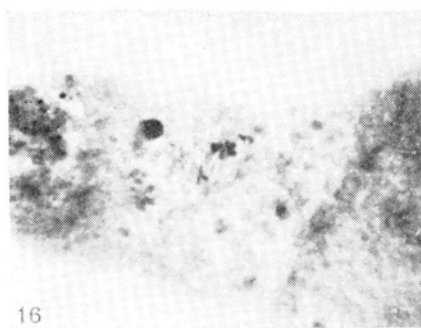
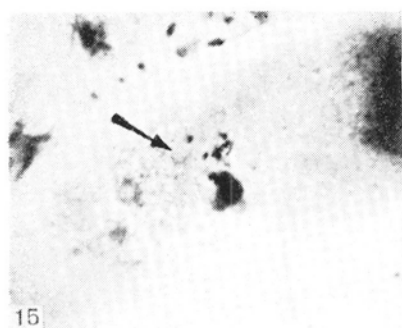
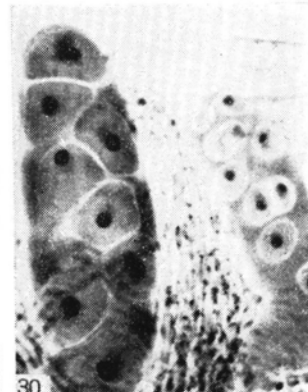
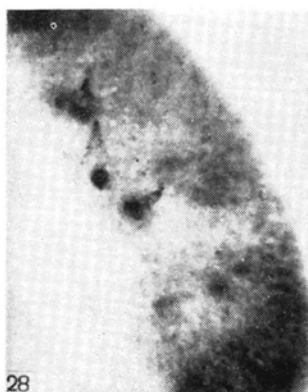
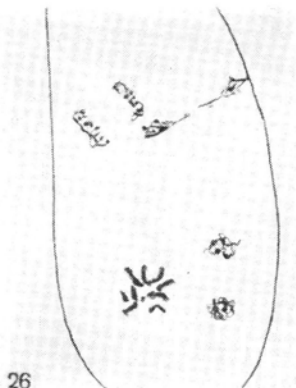
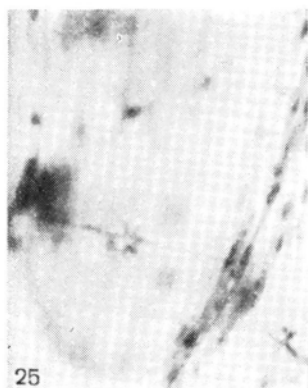
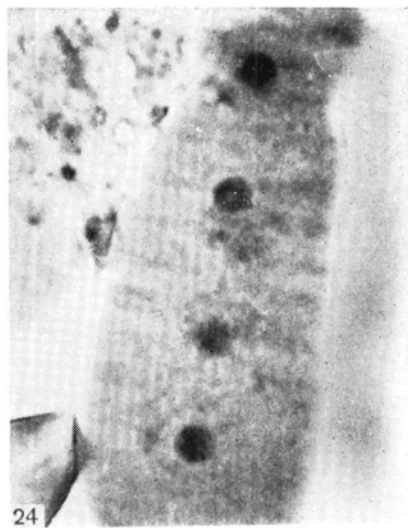


Plate IV



During metaphase I an intranuclear spindle is formed (Fig. 15) usually more or less parallel to the long axis of the ascus but transversal position of the spindle was also observed. In anaphase I chromosomes pass to the poles. Frequently early anaphase figures show asynchronous disjunction of daughter chromosomes (Fig. 16). Figs 17, 18 show two early telophase groups at the opposite poles of the spindle and the old nucleolus in the middle of the spindle.

After telophase I during short interphase a new nucleolus is organised in each of the two nuclei. The chromosomes elongate. Fig. 19 shows two sister nuclei after first division, the new nucleoli are prominent. In Fig. 20 there are two sister nuclei in early prophase II and an old nucleolus between them. During prophase II the chromosomes contract so that by metaphase II they are very short (Figs 21, 22). The axes of second divisions are usually perpendicular (Fig. 23) or rarely parallel (Fig. 24). During second division the old nucleolus of the old diploid nucleus is still present in the cytoplasm.

After interphase which follows second division the chromosomes contract and the third division begins. The divisions of the nuclei in one ascus are not fully synchronised and their axes are not regularly orientated (Figs. 25—27). Camera lucida drawing shows the four nuclei in different stages of third division (Fig. 27). Centrioles are sometimes clearly visible in this division (Fig. 28). They are big and prominent because they play probably additional role in ascospore delimitation.

The eight nuclei resulting from third division are surrounded by the walls and spores are formed (Fig. 29). Each ascus usually contains eight uninucleate spores (Fig. 30 to the left). But occasionally asci are formed with abnormal ascospore numbers from 1 to 11 and even with 15 spores (Figs 31, 37). Fig. 31 shows an ascus in which six spores are degenerating and Fig. 32 ascus with 15 spores resulting from additional mitoses. In Fig. 38 there is a fragment of an ascus containing seven ascospores instead of eight but one is binucleate. It is obvious that two nuclei from third division are surrounded by a common wall.

Spore maturation and germination. After third division and spore walls formation the ascus and spores grow quickly. Enlargement of the nuclei of spores is also prominent: the nucleus reaches 11 μ and nucleoli about 7 μ in diameter. Fig. 39 shows a typical spore in this stage with big nucleus, prominent nucleolus, chromatin threads and centriole. Big nucleolus indicates that nucleus of the young growing spore is in very active metabolic condition. In older spores the structure of nucleus is different — it is composed of two spheres, internal more and external less darkly stained. It was also observed that in some spores nucleus occupies a position near the spore wall (Fig. 40). The cytoplasm stained uniformly in young spores becomes granular and many vacuoles are formed (Fig. 41).

Prior to germination spores became bigger and swollen. At the same time many consecutive divisions of spore nucleus take place (Fig. 42). Germinating spore contains numerous nuclei which pass to the young hyphae proliferating in many points through the spore wall (Fig. 43).

Somatic mitosis. Vegetative cells of *A. immersus* are typically multinucleate (Fig. 44). The nuclei are randomly distributed within the cell. The number of nuclei per cell varies widely, in normal hyphae from 3 to 10, six on the average. In vegetative hyphae of "colonial" mutants the number of nuclei reaches 20—30 per cell (Fig. 45). Near the cross walls separating two cells of mycelium small granules were observed, two on each side of the wall. Like in other fungi they probably play some role during migrations of the nuclei from cell to cell. Resting nuclei in older hyphae appear as intensely stained small sphaerical bodies (Fig. 44, 45). In the resting nuclei it is possible to distinguish one, two or three Feulgen positive "chromocenters". The nuclei showed less densely stained central parts. The appearance of resting nuclei was the same after staining with haematoxylin, propiocarmine and Feulgen.

In young actively growing hyphae of germinating spore the nuclei are distinctly bigger than in old ones, and adopt various shapes (Fig. 43). They stain less intensely and appear to have a granular or fibrous structure. In such hyphae the nuclei are intensively dividing and this is the

Plate V

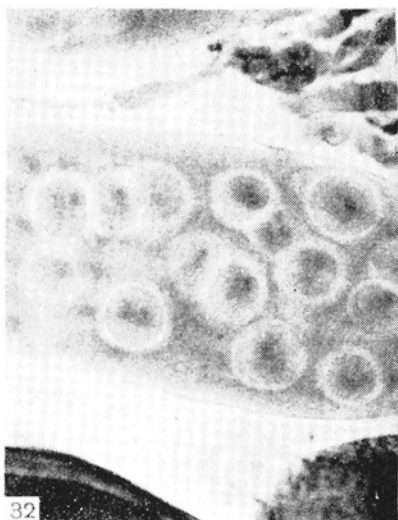
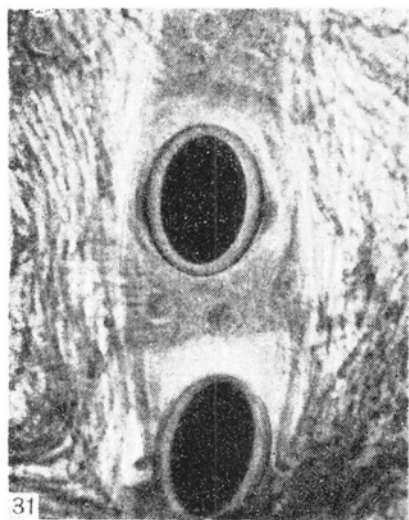
Fig. 31—38. Abnormal types of asci with different ascospore numbers. Fig. 31. Ascus with two normal and six degenerating ascospores. Fig. 32. Ascus with 15 ascospores resulting from additional mitosis. Fig. 38. Abnormal binucleate ascospore.

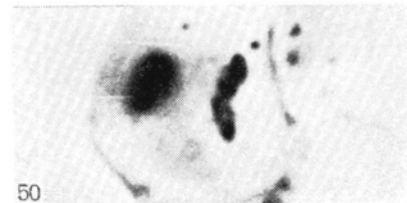
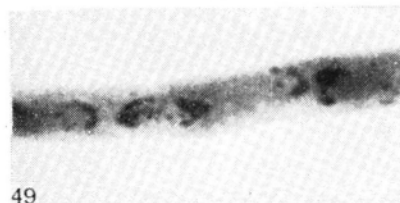
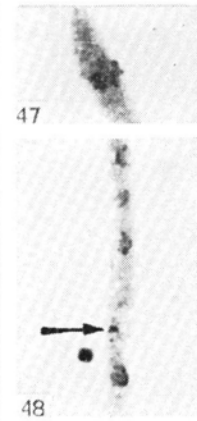
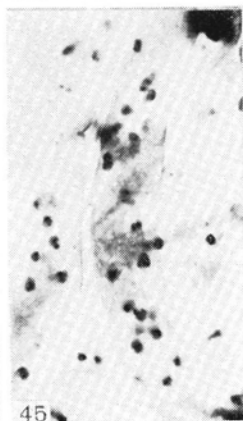
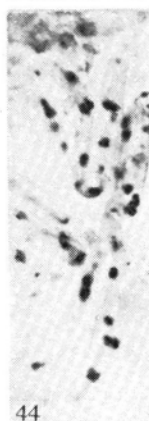
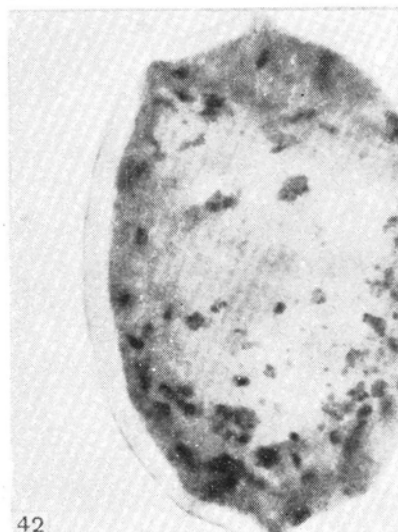
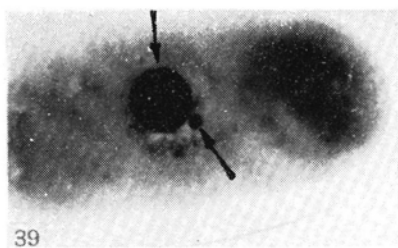
All magnifications $\times 450$ except Fig. 32, 38. $\times 1100$. Staining — Acetohaematoxylin, smears.

Plate VI

Fig. 39—50. Fig. 39. Typical uninucleate spore (arrows indicate nucleolus and centriole). Fig. 40. Mature spores with nuclei near spore wall. Fig. 41. Mature spore with numerous vacuoles. Fig. 42. Germinating spore (note numerous dividing nuclei). Fig. 43. Germinating spore (note numerous young hyphae with dividing nuclei). Fig. 44, 45. Resting nuclei in old hyphae (Fig. 45 — colonial mutant). Fig. 46—48. Nuclei in different stages of divisions in young hyphae; Fig. 46 — prophase, Fig. 47 — metaphase, Fig. 48 — prophase and metaphase in one hyphae (arrow indicates metaphase in side view). Fig. 49. Anaphase of mitotic division in young hyphae. Fig. 50. Figures of mitotic division overstained with Acetohaematoxylin.

All magnifications $\times 1100$ except Fig. 40, 41 $\times 450$ and Fig. 49, 50 $\times 2100$. Staining — Fig. 39—45 and Fig. 50 Acetohaematoxylin, smears; Fig. 46—49, Giemsa HCl, smears.





best stage to study nuclear divisions. A typical prophase nucleus can be recognised by its conspicuous nucleolus (Fig. 46). In later stages nucleolus was not observed, chromosomes became contracted and organised as typical metaphase plate (Fig. 47 polar view and Fig. 48 lateral view). In this stage the number of chromosomes is about eight or nine rods of various sizes (eight bigger and one very small). Anaphase configurations were also recognised (Fig. 49). The divisions of the nuclei in a cell of young hyphae appear to be synchronised to some extent, usually only one stage of division being represented by the nuclei of one cell. Some figures different from normal mitotic divisions were also observed (Fig. 50).

DISCUSSION

The subject of this investigation was to provide cytological informations to complete the genetic studies on *Ascobolus immersus* which are in progress in this laboratory. In this the finding that spores are uninucleate and that asci with number of spores other than eight are not so rare is of interest.

The haploid chromosome number is eight or nine. Eight or nine bivalents can be counted in meiosis. This results are confirmed by examination of mitotic division in vegetative mycelium. Similar results were also obtained in *Ascobolus magnificus* (Wood, 1953). Chromosome number established by Wood for *A. magnificus* was approximately eight. Chromosomes of *A. immersus* seem to be small in comparison with other *Ascomycetes*. For example the longest chromosome in pachytene nucleus of *Neurospora crassa* is 18.9μ (Singleton, 1953), in *Sordaria fimicola* 21μ (Carr and Olive, 1958) and in *Ascobolus* one of the longest distinctly visible chromosome is only about 10μ long.

The occurrence of precocious synapsis appears to be similar to that reported for *Neurospora crassa* (Singleton, 1953), for *Cochliobolus sativus* (Hrushovetz, 1956) and for *Sordaria fimicola* (Carr and Olive, 1958). This investigation confirms Carr's conclusion that precocious synapsis is an integral part of nuclear cycle in *Ascomycetes*. Recently Lu has demonstrated that in *Basidiomycete* *Cyathus stercoreus* synapsis also takes place very early (Lu, 1964). So it seems that precocious synapsis is even more general phenomenon in fungi.

In *Ascobolus* like in *Neurospora* and *Sordaria* in diplotene a fuzzy appearance of nuclei was observed. The chromosomes which are easily distinguishable in pachytene, in diplotene are like a mass of thickened rather fuzzy strands. Moens (1964) presented evidence that diffuse stage exists in *Lycopersicon esculentum* and in some other plants as well. He also suggested that synapsis in *Lycopersicon* is complete in interphase, possibly prior to chromosome duplication.

The observations concerning nuclear divisions in vegetative mycelium provide evidence that somatic nuclei of *A. immersus* divide by mitosis in essentially the same way as the vegetative nuclei of other fungi. These findings are in agreement with those of Ward and Ciurysek (1961, 1962) for *Neurospora crassa* and for one of the *Basidiomycetes*. Bakerspigel (1959) have attempted to provide evidence for another mechanism of somatic nuclei division in fungi. The figures similar to those given by Bakerspigel were also found in *Ascobolus* (Fig. 50) but it seems that they were caused by overstaining and are not sufficient evidence to postulate amitotic division of somatic nuclei in fungi. The claim that somatic nuclei do not divide by mitosis also fails to find support on theoretical ground as it was thoroughly discussed by Ward and Ciurysek (1962).

SUMMARY

Conjugate divisions occur in the binucleate crozier of *A. immersus*. Two cross walls are laid down in four nucleate crozier in such a way that the penultimate cell is binucleate. The binucleate cell grows quickly and young ascus is formed. The two haploid nuclei of young ascus fuse and this is accompanied by a fusion of their nucleoli. In early stage of first meiotic division pairing between highly contracted chromosomes takes place. After pachytene chromosomes become fuzzy in appearance and diffuse stage of the nucleus is observed. A third division (mitotic) follows and results in the formation of eight haploid nuclei. Ascospores delimitation begins at this stage. Each spore is uninucleate and no more divisions in spores have been observed. The presence of centrioles and spindles in nuclear division is demonstrated. The haploid chromosome complement as determined from diakinesis and metaphase stages of meiosis and mitosis in vegetative nuclei is eight or nine chromosomes. Spore germination is described. The process of nuclear division in vegetative mycelium of *A. immersus* follows the conventional mitotic sequence.

We wish to thank Prof. Wacław Gajewski, Head of the Department of General Genetics, Polish Academy of Sciences, for his stimulating criticism during this work.

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(Entered: 15.X.1964.)

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Badania cytologiczne nad Ascobolus immersus

Streszczenie

Opisano podział jąder sprzężonych, powstawanie worków oraz przebieg mejozy u *Ascobolus immersus*. W wyniku pierwszego i drugiego podziału mejotycznego powstają w workach cztery haploidalne jądra. Po mejozie następuje podział mitotyczny, który daje osiem jąder. Zarodniki, które powstają w worku w liczbie ośmiu, są jednojądrowe. Liczbę chromosomów określono na podstawie diakinezy i metafazy podziału mejotycznego oraz na płytkach metafazowych w kiełkującej grzybni na 8 lub 9.