THESIS

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A study of spermatogenesis in Podophyllum
including laboratory methods of preparation

Approved

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Preparation of Material.

In the work carried out, using the here-in-described material, it was found that several processes gave good results. But in each case only one process will be described, unless some notable exception is brought to mind. The work, in the main, is based on the methods outlined or suggested in Methods in Plant Histology by Chamberlain. In a few cases it was found that slight modifications in our laboratory work seemed advisable, although there is no great change in results noticeable at any point.

In preparing the material it was taken directly from the plant, in the greenhouse or field, and there transferred to the killing and fixing fluid. The material was always cut into small pieces or freed of superfluous tissue by trimming and in no instance was there any evidence of defect from improper killing. The period allowed for killing, or remaining in the solution, was at least 24 hours; sometimes the buds remained 36 hours without any harmful results. The killing solution used was the Chromo-acetic solution containing a small proportion of osmic acid. The following proportions were found to suit in all cases:

1% Chromic acid .................... 25 cc.
1% Acetic acid ..................... 10 cc.
Water ............................... 55 cc.
1% Osmic acid ...................... 10 cc.
It was not found necessary to bleach for removal of any blackening due to the action of osmic acid.

After being in the killing solution the material was washed under the tap on filter paper in a funnel, allowing the water to fall in a slow drip. The water is allowed to cover the material in the funnel during the process of washing for the whole period of 24 hours.

The hardening process and simultaneous dehydrating was carried out by the use of alcohol. In this process a large excess of the various strengths of alcohol was used. The material was allowed to remain 24 hours in each grade of alcohol, at least beyond the 35%. After much of the work had been done it was found advantageous to pour off the solutions from the material rather than to handle the delicate buds and anthers with forceps. The grades of alcohol used were 15%-35%-50%-70%-85%-95% and absolute.

After hardening and dehydrating, the material was cleared in xylol, using the following gradual transfers:

3 parts 100% alcohol and 1 part xylol, 24 hrs.
2 " 100% " " 2 " " 24 "
1 " 100% " " 3 " " 24 "

From the last the material was transferred to pure xylol and then to xylol containing a small block of paraffine in solution. All these steps were carried out at room temperature.

The infiltration was carried out on the bath, the material being transferred from xylol paraffine to 43° paraffine and allowed to infiltrate 24 hours at a little above the melting point. From here it was transferred to, and imbedded in, 53° paraffine, care being taken to prevent the paraffine crystallizing, as the latter interferes with ribboning.
Sections were fixed to slides by Meyer's albumin fixative, floating the sections in water and warming over moderate heat in order to remove wrinkles from paraffine. The sections were dried from 2 to 24 hrs. on water bath. When sections were dried less than 2 hrs. before placing in turpentine very often the sections washed off before the triple stain was reached.

In order to remove paraffine, the slides were immersed in turpentine in Coplin's staining jars. In case the paraffine was not completely removed, xylol finished this process. The turpentine and xylol were removed by passing the slides through absolute alcohol and then 95% alcohol in Coplin jars. Fleming's triple stain was usually employed and the slides were transferred directly from the 95% alcohol to the saffranin. If haematoxylin or any water stain was used, the alcohol was removed by dipping in water preparatory to placing slides in stain. In dehydrating after the stain, 50%, 95%, and absolute alcohol were used.

After dehydrating, the sections were cleared with clove oil, and in the triple stain excess clove oil, which extracts Gentian violet, was removed by xylol. The latter is especially valuable as the rapidly evaporating xylol allows the Canada balsam to dry so much more rapidly.

The Cell.

As far as structure and origin are concerned the tissue cell is of the same morphological value as the one-celled plant or animal; and in this sense the multicellular plant is equivalent to
a colony or aggregate of one-celled forms. Physiologically the cell tissue can only in a limited sense be regarded as an independent unit; for its anatomy is merged in a greater or less degree into the general life of the organism. Schwann drew the conclusion that the life of the organism is essentially a composite; that each cell has its independent life; and that the whole organism subsists only by means of the reciprocal action of the single elementary parts.

However, it has been demonstrated in growth and development that the cells can be regarded in a limited sense as units. They are rather local centers of a formative power pervading the growing mass as a whole. Many cases are known in which the division of the nucleus was not followed by a division of the cell body, multi-nuclear bodies being formed, consisting of a continuous mass of protoplasm through which the nuclei are scattered. Protoplasmic bridges between germ cells and somatic cells have been demonstrated in plants. (In Volvox by A. Meyer). The latter believes that both animal and plant individual are continuous masses of protoplasm forming a morphological unit whether in a single cell, or multi-nucleated cell, or a system of cells. Basing his conclusions on the observance of cell bridges being formed anew after cell division in Volvox. If this is correct, the nucleus of one cell may control the membrane forming activity of an enucleated fragment of another cell by means of the protoplasmic bridges. A confirmation of this is given by Townsend ('97) who finds in root hairs and in pollen-tubes, that when the protoplasm is broken up, a membrane may be formed by both nucleated
and non-nucleated fragments, by the latter only when they remain
connected with the nucleated masses by protoplasmic threads how-
ever fine. If these threads are broken, the membrane-forming pow-
er is lost.

By cell division the hereditary substance is split
off from the parent body; and by cell division, again, this sub-
stance is handed on by the fertilized egg-cell, or oosperm, to
every part of the body arising from it. The cell has no other
means of origin than by the division of a pre-existing cell. Cell
division proceeds from the center toward the periphery, beginning
with division of the nucleus, continued by constriction and divi-
sion of the nucleus and completed by division of the cell body and
membrane. In some cases the nucleolus seems to disappear entirely
before cell division.

There are two modes of cell division - indirect and
direct. The former is by far the more common and to it has been
given the name of mitosis, or karyokinetic division, , and amitosis
to the latter. It is certain that in all the higher, and in many
of the lower forms of life, mitosis is the typical mode of cell
division. In such cases the process involves three parallel series
of changes, which affect the nucleus,(centrosome in animal tissue)
and cytoplasm of the cell body. These are: (1) Prophases, or pre-
paratory changes; (2) Metaphases, involving the most important
changes in the division of the nucleus; (3) Anaphases, in which the
nuclear material is distributed; (4) Telophases, in which the en-
tire cell divides and the daughter cells are formed.
6.

In the prophase the nuclear substance gives rise to a definite number of intensely staining bodies known as the chromosomes. It is very probable that every species of plant or animal has a fixed and characteristic number of chromosomes, which regularly recurs in the division of all its cells; and in all forms arising by sexual reproduction the number is even. Thus in the lilies, 24, and 16 in the onion. The even number of chromosomes is due to the derivation of one-half the number from each of the parents.

Net-knots, or chromatin-nucleoli contribute to the formation of the chromosomes and in *Spirogyra* where the whole of the chromatin is at one period concentrated into a single mass; the whole chromatic figure appears to arise from a nucleolus. Commonly the nucleolus fades away in situ while the chromosomes and spindle are forming. Strasburger suggests that the true nucleoli are storehouses of kinoplasmic material, which is either used in formation of the spindle, or being cast out of the nucleus becomes available for future mitoses. The chromosomes group themselves in a plane passing through the equator of the spindle, forming the equatorial plate.

The structure resulting from the foregoing changes consists of the (1) chromatic figure and the (2) achromatic figure consisting of spindle and asters which stain but slightly.

The prophases of mitosis are preparatory; metaphase forms the initial phase of actual division. Each chromosome splits lengthwise and sometimes transversely into two exactly similar halves and diverge to opposite poles of the spindle where
each group of daughter-chromosomes finally gives rise to a daughter-nucleus. The chromatic network is converted into a thread which splits throughout its entire length into two exactly equivalent halves and the daughter-nuclei receive precisely equivalent portions of chromatin from the mother nucleus.

In Anaphases, after splitting of the chromosomes, the daughter-chromosomes, arranged in two corresponding groups, diverge to opposite poles of the spindle, where they become closely crowded in a mass. As they diverge, the daughter-chromosomes are connected by a bundle of achromatic fibers known as the connective or interzonal fibers. Hermann regards these fibers as belonging to a central spindle, surrounded by mantlefibers to which the chromosomes are attached and only exposed to view as the chromosomes separate. Almost invariably in division of plant cells the deeply staining thickenings of the equatorial plane forming the cell-plate or mid-body appear at this stage.

In the final stage of mitosis, the entire cell divides in two new cells in a plane passing through the equator of the spindle, each of the daughter cells receiving a group of chromosomes and half the spindle. In many plant cells, the daughter-chromosomes become thickened, contorted and closely crowded to form a daughter spireme similar to that of the mother nucleus. The cell plate finally extends across the entire cell and splits into two layers between which appears the membrane by which the daughter cells are cut apart.

If we trace cell division in anthers of flowering
plants there is found another stage which succeeds the completion of the mitosis above described. Instead of a clearly marked cell wall or equatorial plate being formed at the close of the first mitosis, a second mitosis immediately follows. In this case the second mitosis takes place transversely to the first division, and in the sections prepared the tetrads are found still united by the linin threads.

A resume' of the history of work on tetrad formation is gathered in the following paragraphs.

Henking ('91) first figured a tetrad, or ring, which he interpreted as a chromosome undergoing one transverse and one longitudinal division, thus giving a true reduction division required by Weismann's theory. Osterhout ('97) figured the same as Equisetum. Belajeff ('98) and Atkinson ('99) figured a longitudinal and a transverse division in plant cells. Though different observers found minor variations in the details of tetrad formation, all agreed on the essentials; namely, that there was a longitudinal division of the chromatin thread followed shortly by a transverse division, thereby forming a number of segments equal to one-half the number of somatic chromosomes. These segments were looked upon as each composed of two somatic chromosomes united end to end. In the ensuing divisions these segments were divided once longitudinally and once transversely, thus giving a qualitative reduction in accordance with Weismann's hypothesis.

On the other hand, the researches of Carnoy ('86), Boreri ('87), Hertwig ('90), and especially Brauer ('93) on Ascaris led them to conclude that both divisions in tetrad formation were longitudinal; that is, the tetrads arose by a double longitudinal
division instead of by a longitudinal and a transverse division. The conditions in Ascaris, however, are complicated from the fact that the number of chromosomes in the somatic cells is very much larger than in the germ cells; therefore, it may be questioned whether the chromatin body dealt with in the germ cells is a true chromosome in the sense in which that term is used for other organisms. Most of the investigators of this period, however, believe that, as a general thing, tetrads, in the strict sense, are not formed. Miss Sargent ('95) concluded that both divisions were longitudinal in Lilium. This was soon concurred in by Strasburger ('95), Farmer and Moore ('95), Dixon ('96), and by a number of other workers. Two years later Strasburger and Mottler ('97) figured a longitudinal and a transverse division in Lilium and several other angiosperms. Practically similar results were obtained by Ishikauva ('97) in Allium, and by Belajeff ('98) in Iris. Schaffner ('97) also working with Lilium, figured a transverse division in the first mitosis and a longitudinal in the second. Atkinson ('99) in Arisaema represents a tetrad formation which he interprets as a transverse and longitudinal division, the transverse appearing in the first mitosis.

Allen ('04) in a preliminary note on the microspore formation in Lilium, finds that after the formation of the mother cell there is a long growth period in which the chromatin is in a reticulate condition. During the latter part of the growth period the chromatin changes from irregular reticulate masses into threads which become arranged in pairs, the moieties of each pair parallel, as they pass into synapsis. In synapsis the chromatin is massed
in a tight knot at one side of the nucleus, often pressed against the nuclear wall with the nucleolus. As these parallel threads enter synopsis they move closer together and finally fuse to form a single thread which shows no evidence of its bivalent character for some time after its formation. Just previous to the union of the two parallel threads their substance is differentiated into linin and chromatin, the latter aggregated into small granules, chromosomes. With the fusion of the threads the chromosomes fuse in pairs. The chromatin is in synopsis for several days. The threads emerge from synopsis and become distributed throughout the nucleus, forming twelve loops which segment transversely at or near the point where they are in contact with the nuclear wall to form the reduced number of chromosomes. Previous to this transverse segmentation there is a longitudinal fission of the thread, the chromosomes, which are still apparent, first dividing. Allen believes that this longitudinal fission represents a separation of the paired threads which fused in the presynaptic stages. He finds a second contraction stage when the chromatin threads are drawn away from the nuclear wall. This contraction, however, is not nearly so marked as in the case of synopsis. The first mitosis is longitudinal and probably separates the two threads which fused in synopsis, thus giving a true reduction. The heterotypic chromosomes are not, as a general thing, V-shaped, but rod-shaped, as they pass to the poles, though often remaining attached at their equatorial end, thus forming a V-shaped body. The second division is also longitudinal and divides the daughter-chromosomes of the first mitosis.

That the synaptic knot is a region of great activity
is indicated by the way in which it resists the extraction of stains (safranin and iron-haematoxylin). That this is due not to the mass effect alone is shown by the fact that a small section cut from one side of the knot behaves in the same way. The contraction of the chromatin threads into the synaptic knot invariably occurs at one side of the nuclear cavity and in close contact with the nucleolus, the latter being almost surrounded by the threads at times. Often loops of thread or threads extend outward some distance from the knot, but these loops are always few in number, much less than the number of chromosomes — and show no such regularity in arrangement and number as those figured by Farmer and Moore ('05). Montgomery ('05) reports that he finds the synaptic knot always on the side of the nucleus bordering upon the greater bulk of cytoplasm in the cell.

After synapsis is reached the nucleus ceases to increase in size.

From the results obtained it seems highly probable that with the fusion of the gametes in fertilization there is a nuclear but not a chromatin fusion and that the maternal and paternal chromatin retain their identity throughout the sporophytic existence of the plant, finally fusing, in so far as it fuses at all, in synapsis. That is, the sporophyte is a sort of double-celled phase of the organism. Thus Cook and Swingle ('05) in an interesting article, argue that the sporophyte is not an asexual but a highly sexual generation or phase in that it is produced "during the actual process of conjugation". These writers hold that "it was not the reduction to fewer chromosomes, but the retention of the double number that constituted the important step in sexual reproduction
and made possible the evolution of complex higher organisms." If, as is generally admitted, the chromatin controls the metabolic activities of the cell, it would seem that the above theory is not without considerable foundation. The familiar fact, that an offspring more often possesses certain characters of the one parent to the exclusion of the other, would indicate that it is the chromatin of the latter that is controlling the physiological processes of the organism. Nor are we without evidence that the maternal and paternal chromatin remains distinct during the sporophyte phase.

If the above is true, the explanation of synapsis is that it is the end-result of fertilization. Thus the two phenomena of fertilization - stimulus to growth and mingling of ancestral characters - are quite widely separated, the former coming at once with the union of the gametes, and the latter with synapsis. The idea - not new - that the offspring is not the offspring of the parents, but of the grandparents, would find support in the results obtained.

Mitosis of Pollen Mother-cells and Formation of Pollen Grains.

In studying mitosis in the Podophyllum it is clearly seen that four cells result from the two reduction mitoses.

This is clearly demonstrated because at the beginning of the mitoses each mother-cell becomes encased in a tough-walled sac or cell wall (Fig. 1) and remains in this same envelope until its four descendants become fully grown pollen grains.

The pollen sacs are early marked out in the stamen and are four in number. The cells within them become the primitive pollen forming cells. The cells are all alike until warm weather
when the pollen sacs begin to grow, at which time the central cells begin to increase much faster than the two outer layers, (Fig 2) where the central cells in a transverse section of a pollen-sac are larger and have proportionately larger nuclei and nucleoli than the peripheral cells. The latter become differentiated as nurse cells, Fig. 3, forming a double row of cells whose nuclei divide by amitosis without a subsequent division of the cytoplasmic body. Meanwhile, the central pollen mother-cells grow to the proportional size shown in figure 4. The ultimate fate of the nurse cells is that of a shrunken state (Fig. 5) by the time each pollen mother cell has divided into four young pollen cells. Evidently the nurse cells have been nourishing the reproductive cells as the former eventually disappear.

In the pollen mother-cell the nucleolus is large and perfect in outline showing no vacuoles. The skein is made up of a chromatic and an achromatic material. It is smooth and wiry with darker masses at the intersections of the strands of the skein. Fig. 6.

A more developed cell is shown in Fig. 7. Here the spireme is shown much broken up and its remains have been gathered against the nuclear membrane.

The remains have acquired the power of staining more deeply, or have had other material added to them to take the stain as the nucleolus has been undergoing a disintegration. The chromatin matter gathers in granules around the periphery and there the granules increase in size while the nucleolus decreases in bulk. The whole nucleus enlarges as this proceeds.
At the time at which the nucleolus is dissolved, or shortly before, a series of achromatic fibrils appear in loose formation around the edge of the nucleus, whose wall becomes indistinct and disappears (Fig. 8). The chromatin material has mostly left the nucleus and been added to the chromatin particles which are evidently the future chromosomes of the first reduction divisions. They appear in their regular shape, size and arrangement, (Fig. 9), the fibrils showing the spindle. The cytoplasmic material is increased to form a complete shell about the whole figure which is beginning to divide its chromatin.

Fig. 10 shows the stage in which, with a higher power, the equatorial plate might begin showing. Fig. 11 shows an intermediate stage just prior to Fig. 10.

Fig. 12, taken from a Tradescantia, shows the two nuclei reformed, and very distinct separations on the spindle fibers clearly indicate the future cell divisions. Before the cytoplasmic division takes place, the second reduction division has started. (Fig. 13 & 14). The two figures of this process occur at the same time and usually at right angles to one another.

The dark cytoplasmic zone is reformed around each of the two new figures. Figures 14 to 20 show the changes occurring in this second mitosis which results in, or has for its object, the formation of the tetrads (Fig. 19-20). These, in turn, become invested in cell walls and constitute the fully developed pollen grains. The nuclei of the four cells rarely appear in any one given section, owing to the position of the spindles by which they were formed, being at right angles to each other. The completed pollen grain of the
Tulip is shown by Fig. 21, which shows both generative and tube, or somatic, nuclei.

The young cells now develop a cell wall of their own of a very peculiar pattern, (with external spikes or knobs as developed in the Mallow (Fig. 22)) and having broken out of the original cell wall, which still encloses the four, they lie massed in the sac to await its ripening and rupture. The drying up of the scant remains of the former nurse cells sets them entirely free from any connection with the pollen sac.

Literature.

Atkinson - College Botany.
Campbell's University Botany.
Cardiff - A Study of Synapsis and Reduction.
Chamberlain - Methods in Plant Histology.
Dahlgren & Kepner - Principles of Animal Histology.
Duran - Development of the Sexual Organs and Sporegonium of Marchantia Polymorphia.
Strasburger - Concerning Reduction.
Problem of Karyokinesis.
Wilson - The Cell.
Fig. 1 - Podophyllum peltatum - Mother cell showing formation of chromatin threads.

Fig. 2 - Layer of tapetal cells and mother cells showing relation in regard to size.

Fig. 3 - Tapetal, or nurse cells, wall about nuclei plainly showing.

Fig. 4 - Full grown mother cells showing relation in size to nurse cells.

Fig. 5 - Shrunken nurse cells.

Fig. 6 - Mother cell in spireme stage.

Fig. 7 - Open spireme.

Fig. 8 - Chromatin gathered at equatorial plate.

Fig. 9 - Metaphase showing separation of chromosomes.

Fig. 10 - Telophase of 1st division.

Fig. 11 - Anaphase of 1st division.

Fig. 12 - Tradescantia showing nuclei formed.

Fig. 13 - Showing diad stage complete.

Fig. 14 - Metaphase of 2nd division giving a longitudinal and equatorial view.

Fig. 15 - Three groups of chromosomes, the fourth not showing, in anaphase, 2nd division.

Fig. 16 - Anaphase of 2nd division.

Fig. 17 & 18 - Anaphase passing into telophase.

Fig. 19 & 20 - Telophase.

Fig. 21 - Completed pollen grain of Tulip.

Fig. 22 - Partial pollen grain of Mallow showing the projections.

Fig. 23 - Two nuclei showing on changing focus of microscope.

Fig. 24 - Completed tetrad.