toluene treatment; the minced material is placed in a well-stoppered bottle, toluene added, and the bottle immersed in a freezing mixture of ice and salt for 10-12 hours. This modification has been found to yield comparable and concordant results.[37] The fluid obtained at 1 ton pressure to the sq. inch is centrifuged at 3000 R.P.M. for 15 mins. to free it from all debris and the clear centrifugate used for subsequent analysis.

Artificial Culture of the Male Gametophyte of *Ephedra foliata* Boiss and *Ephedra Gerardiana* Wall. and a Study of the Number and Morphology of their Chromosomes.

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EPHEDRA FOLIATA Boiss (*E. peduncularis* Boiss) is a native of the Punjab plain. Plants of *E. Gerardiana* Wall. (*E. vulgaris* Hook. f.) were raised in the Government College Botanic Garden, Lahore, from seeds brought by Prof. Kashyap from Zanskar (about 12,000 ft. above the sea) and sown in October 1928. The plants produced flowers for the first time in 1933. In its natural habitat at high altitudes the flowers of *E. Gerardiana* appear during June and July and the seeds are set in the later half of August. These ripen by the end of September before the onset of the severe winter which brings the period of vegetative activity to a close and the plants enter the dormant phase of life to resume their activity during the next summer. The conditions are different in the Punjab plain. The period of vegetative activity is during the spring months of March and April. The plants of *E. Gerardiana* bear flowers during this period —from the middle of March to the middle of April—this period coinciding with the flowering period of the native *E. foliata*. After this the seeds are set which ripen about the middle of May. The plants then enter the resting period because of the strong heat of the plains.

The spindle-shaped pollen grain at maturity possesses a sculptured exine with ridges running longitudinally from pole to pole. Remains of two evanescent vegetative cells on one side, a stalk nucleus embedded in the peripheral part of the cytoplasm of the naked body cell in the centre and a rather large tube nucleus at the other end completes the structure of the pollen grain at the time when it is shed.

The pollen grains were germinated on the mucilaginous secretion that oozes out of the ripe ovules, placed on glass slides kept in a moist chamber. It is possible to germinate the pollen grain of one species on the mucilage secretion of the other. The secretion absorbs water from the surrounding atmosphere of the glass chamber and gets diluted. The grains gradually absorb the nutritive medium, swell in size, and at the same time prophasic changes start in the body nucleus. On the pollen grain becoming highly turgid the exine ruptures by two splits starting on opposite sides from the tube nucleus end of the grain and extending to about the middle of its length. This throws out the grain bounded by the intine with a jerk from the inside of the outer coat which immediately undergoes torsion. Thus liberated the grain increases to about double its former length. By this time the body nucleus is in the mid-prophase or early metaphase stage. All the further changes take place outside the exine in the medium. The total time for the complete division of the body nucleus and the organisation of the two male nuclei on the opposite poles is about five hours from the time the pollen grains are put into the secretion.

The pollen tubes are given out after about six to eight hours. From one to as many as four or more tubes may be given out from different sides of a grain, sometimes in a most irregular manner. More commonly only a single tube is given out usually laterally from just near the tube nucleus end of the grain but it may grow out from the mid-lateral position, or as a direct continuation of its tube nucleus end. When a number of tubes are given out, only one develops further, and the others remain

37 Narasimhacharya and Sastri, *J. Indian Inst. Sci.*, 1931, 14A, 2.

small and empty except for a thin lining of cytoplasm along the wall. Tubes growing in the medium for a period of 48 hours become quite elongated (attaining a length of 600-700μ) hyaline, and vacuolated and if kept for a longer period show signs of the degeneration of the male nuclei. The latter by the time they enter the pollen tubes from pollen grains have already increased considerably in size and the further growth takes place in the tubes.

The germination of pollen grains has also been tried on different strengths of sachharose solution in water. There is practically no germination in 10% and 20% solutions. The germination in 40% solution is better than in 30% and the former strength is perhaps the best for securing germination, but by far the best results are obtained in the natural mucilage secretion described above.

A new method has been evolved for making cytological investigations of the number and morphology of the chromosome of the species by making smear preparations of the germinating pollen grains at the time when the body nucleus is undergoing division.

The pollen grains are germinated on glass slides in suitable media—mucilage secretion from the ripe ovules being utilised in the present case. After the exine is ruptured the body nucleus in the grain is to be found at the different stages of mitotic division from mid-prophase onwards, the early prophasic changes having occurred before the rupture of the exine. The grains are now fixed by putting two or three drops of the desired fixative on the slide. Out of a number of fixatives tried Bouin's fixative with Allen's modification P.F.B$_{15}$ gave decidedly the best results, the second best being Flemming's for Gasteria and smear method (W.R.T.) and Flemming's weak solution with only a few drops of osmic acid (not so much as is described in the original formula) which are excellent for prophasic stages. The fixative is allowed to act for two to four hours but a prolonged action of the fixative upto twelve hours does no harm. The fixative on the slide should not be allowed to evaporate and the slide is therefore kept in a moist chamber. The most delicate part of the process now is the handling of these microscopic objects.

A clean slide is smeared with Meyer's albumen as in ordinary paraffin technique and the drop of the fixative with the grains suspended is poured on to it. This is now spread uniformly over the slide by gently tilting the slide in various directions. If the fluid is not enough a drop or two of water may be added. This is done to ensure the uniform distribution of the grains over the slide. A long cover-slip is next placed upon the slide just as in making balsam mounts. The liquid is then carefully sucked out by gently placing a piece of blotting paper on one side of the cover-slip. Some of the grains may be sucked out by the current but mostly they remain in position. This suction is continued to such an extent that any further withdrawal of water introduces air bubbles under the cover-slip. The pressure exerted upon the grains by the weight of the cover-slip reinforced by the adhesive force of the disappearing water on the cover-slip is sufficient to fix the tiny objects to the albumen coating on the glass slide. The cover-slip is now removed by flooding the slide with water and gradually and carefully sliding away the cover-slip preferably under water when the force of buoyancy facilitates the process a great deal. Some of the grains are sure to be washed away during this process but a large number of these stick to the slide.

The next process is washing. If the fixation is done in chrome-acetic mixture the slides are washed in ordinary Stender dishes in flowing water at least overnight. If Bouin's fixative is used, the process takes a longer time but gives excellent results. The slides are first washed in water for about five minutes to remove the superficial fixative and then passed through the various grades of alcohol to remove all traces of picric acid. In 30%, 40%, 50% they are kept at least overnight in each. They are then passed back in the reverse order through the successive lower grades keeping in each for about four hours till they are brought back into water. The washing is further completed in running water for about four to six hours. This process must be strictly followed to ensure the complete removal of the fixative before proper brilliancy of stain in the preparation can be obtained.

The slides are now ready for staining in the usual way. Two to three hours mordanting in iron alum and 2-3 hours staining in $\frac{1}{2}$% Haematoxylin give excellent results. The chromosomes and chondriosomes take

a jet black stain against the greyish white background of the cytoplasm.

An important point in mounting the preparations in balsam is that the balsam should be thin, otherwise the grains are liable to undergo plasmolysis.

This process described above possesses several distinct advantages over those usually in vogue for the study of the chromosomes in plants. The tediousness of cutting microtome sections is avoided. As in the " Pollen Mother Cell Smear Method " the greatest advantage that is secured is the immediate fixation of the living material by the direct contact of the fixative used—a feature which is most essential in delicate cytological works where it is highly desirable to get accurate results with least possible distortion of the original features present in the living condition. A number of fixatives can thus be tried within a relatively short period for testing their efficiency. A distinct advantage over the "Pollen Mother Cell Smear Method" is that on account of the reduced number of chromosomes in the gametophyte, the number of chromosomes and the form of the individual chromosome are ascertained with greater ease and accuracy, since the congestion due to the double number of chromosome is avoided and this is particularly advantageous in cases where the chromosome number is large. Another advantage is that with a little skill different stages in the mitotic division of the body nucleus from the prophase onwards to the formation of distinct male nuclei (and even the later stages when these increase in sizes) can be secured on the same slide. This is most essential in following the alteration in the chemical nature of the chromosomes as they pass through the various phases, so far as these are exhibited by their reaction to the stains, since in the same slide, the period of mordanting, staining and destaining remains constant for the chromosomes at the different phases of nuclear division.

So far as the writer is aware, no such method of working out the number and morphology of the chromosomes in plants from a study of their male gametophytes grown under artificial cultural conditions, has been described previously by any other author.

The technique above described for handling microscopic objects can be utilised with great advantage for making detailed cytologic investigations of such minute objects as Desmids, Protococcus, etc., which are otherwise difficult to handle. The previous method of making cytologic studies by microtomic sections of such minute organisms involves a great wastage of the material during the passage through various grades and is not capable of easy handling. This method can be utilised with greater ease and without unnecessary wastage of the material.

It can be stated definitely from the author's observations that the haploid number of chromosomes in *E. foliata* is seven. Three of these possess median fibre attachment constriction and are V-shaped. One of these three probably possesses a secondary constriction in one of the arms. The other two possess submedian fibre attachment constriction and the length of the longer arm is about $1\frac{1}{2}$ times that of the shorter. They are further distinguished from one another by the larger size of one of these as compared to the other. The remaining two chromosomes have sub-terminal fibre attachment constriction. One of these again is smaller than the other and possesses a satellite at the end of the longer arm.

Similarly, the writer is in a position to state definitely that the haploid number of chromosomes for *E. Gerardiana* is undoubtedly 14—thus showing diploidy over *E. foliata* which possesses the basic number 7. The diploidy, however, is limited only to the number of chromosomes and not with respect to the type of chromosomes or in other words the chromosome compliment of *E. Gerardiana* does not show an exact duplication of the type of chromosomes met with in *E. foliata*. In *E. Gerardiana* 4 chromosomes possess median fibre attachment constriction and one at least of these possesses a trabant or satellite at the end of one arm and a secondary constriction at the end of the other. Six possess sub-median fibre attachment constriction, three possess attachment constriction which might be regarded as intermediate in position between sub-median and sub-terminal and one possesses sub-terminal fibre attachment constriction.

That the constant position of the fibre attachment constriction in chromosomes is a feature of great importance in the identification of different chromosomes in a complement is clearly realised in the present study. The attachment constriction becomes apparent in each chromosome as early
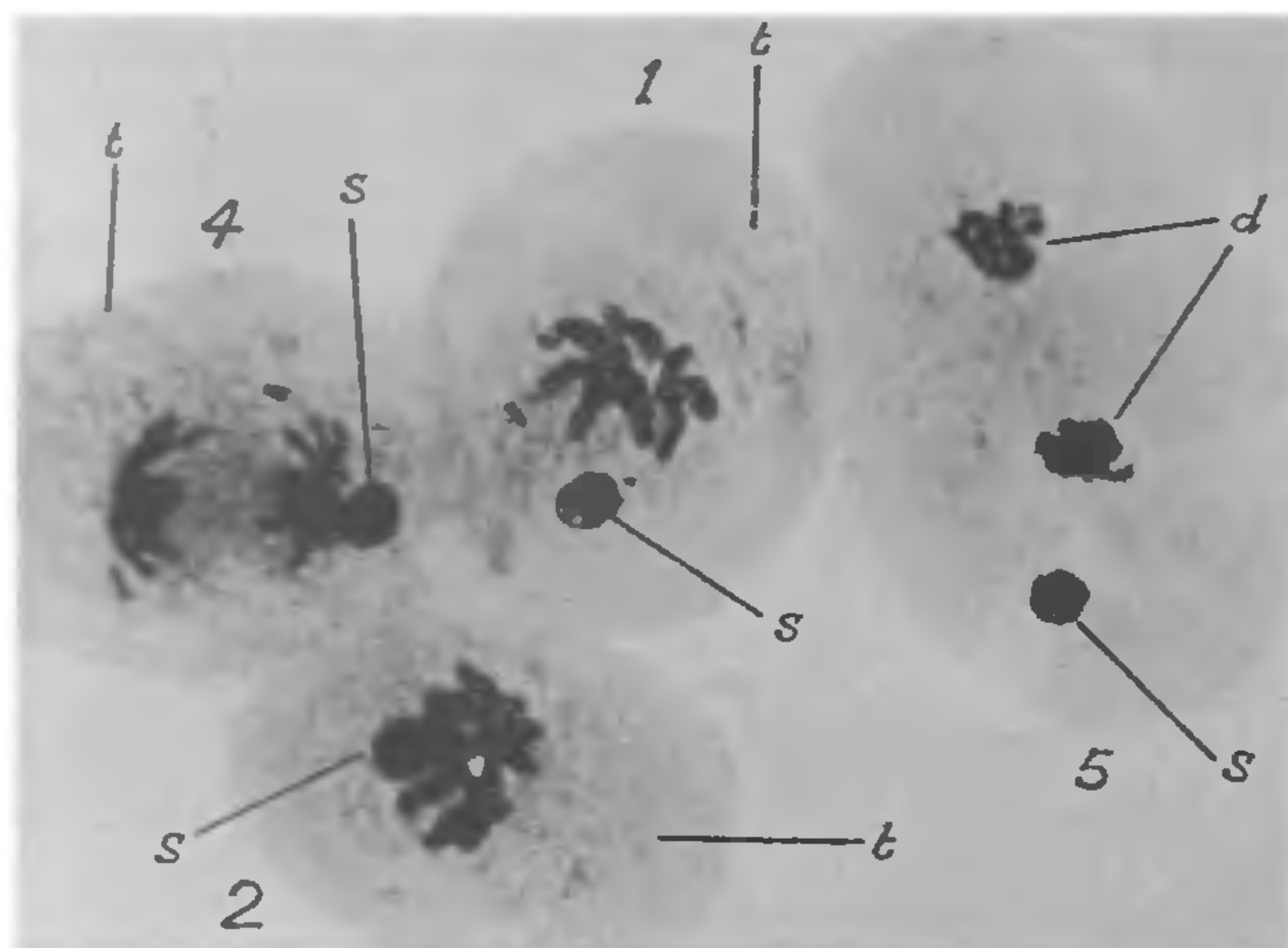
as the mid-prophase and most clearly in the late prophase while the wall of the nucleus is still intact and long before the appearance of any spindle fibres.

An important difference from the observations of other investigators is that the form of the chromosomes does not remain constant during the different phases of mitosis and therefore cannot be relied upon in the identification of the chromosomes.

Chondriosomes are found irregularly scattered or regularly arranged in the cytoplasm of the body cell in preparations fixed in chrome-acetic acid mixtures. Each is found to possess what looks like a vacuole in the centre and these chondriosomes stain jet black with iron-alum hæmatoxylin. In preparations fixed in Bouin's fluid, the chondriosomes are either completely absent or present a faded appearance. It seems they are in some way chemically altered by the action of the fixative. In living specimens the chondriosomes appear as refractile granules in the cytoplasm of the body cell.

I express my deep sense of gratitude to Dr. S. R. Kashyap under whose guidance this work was carried on for very kindly allowing me to use the material from the Government College Botanic Garden and for critical and other valuable suggestions made during the course of the present investigation.
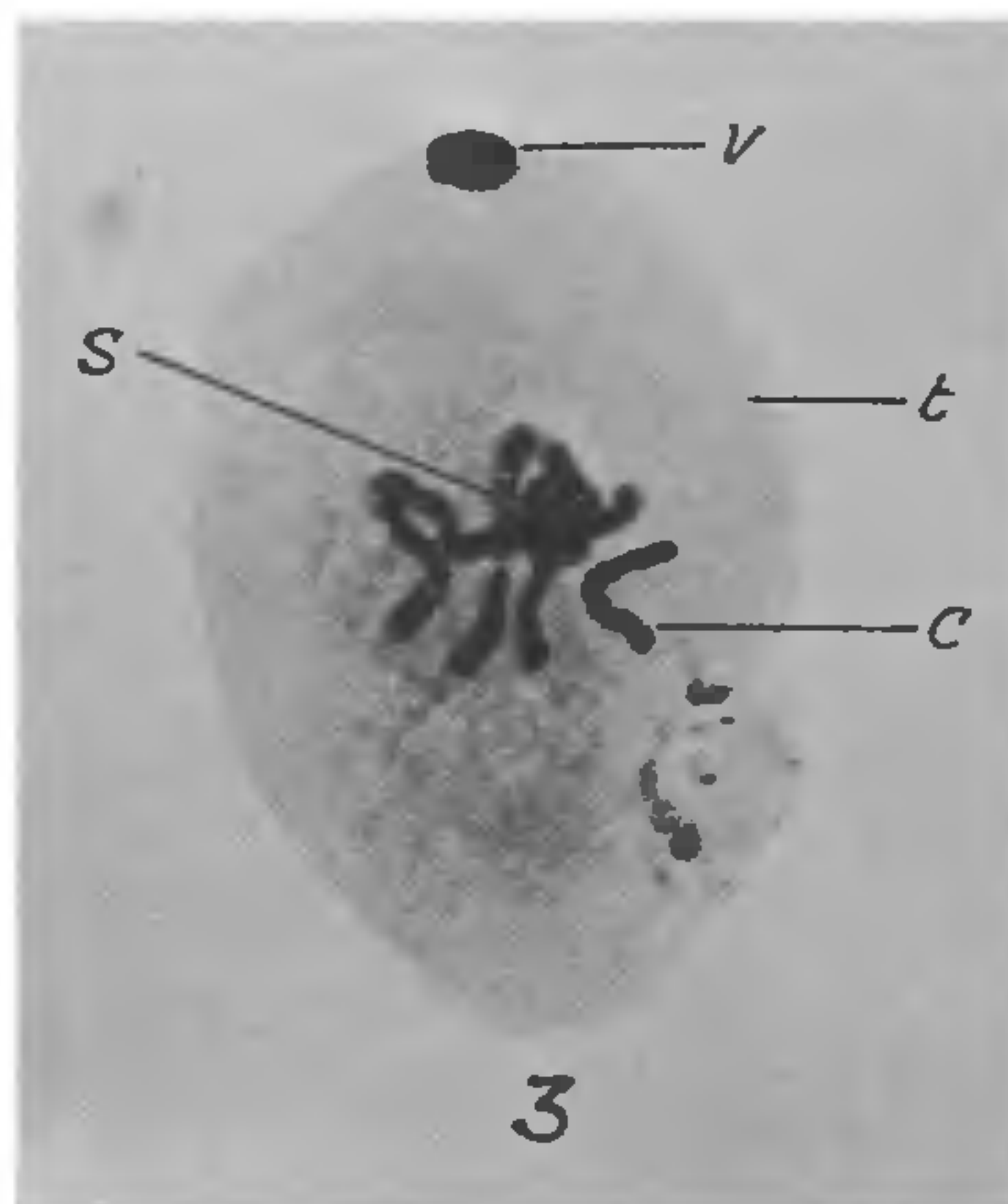
The photographs are of the germinating pollen grains of *Ephedra foliata* Boiss, showing the body nucleus at various stages of mitotic division.

Figs. 1, 2 & 3.—Body nucleus at metaphase ; in Fig. 3, seven chromosomes can be clearly made out. c—chromosome.

Fig. 4.—Late anaphase.

Fig. 5.—Beginning of telophase. d—daughter nuclei being organised.

v—remains of the vegetative cells, s—stalk nucleus, t—tube nucleus.