COLCHICINE EFFECT ON THE MITOTIC DIVISION OF THE BODY NUCLEUS IN THE POLLEN GRAINS OF SOME *EPHEDRA* SPS.

By P. N. MEHRA, *D.Sc., Botany Department, Panjab University, Lahore.*

(Communicated by Prof. A. C. Joshi, D.Sc., Govt. College, Lahore.)

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Most of the work on artificial induction of polyploidy in plants through colchicine technique is limited to Angiosperms. In Gymnosperms only two cases are on record. Mirol and Stockwell (1939) found polyploid cells in the seedlings obtained from colchicine treated seeds of *Pinus ponderosa*. Later Jenson and Levan (1941) induced polyploidy in *Sequoia gigantea*.

The investigations on the cytological mechanism responsible for the induction of polyploid cells by the use of this drug is again entirely confined to Angiosperms. The present work aims at a study of the effect of colchicine in a Gymnosperm material. The effect of the drug is noted on the mitosis of the body nucleus in the pollen grains of some species of the genus *Ephedra*.

The mature pollen grain in *Ephedra* consists of remnants of two disorganised vegetative cells at one pole (often no trace of these is visible), a large faintly staining tube nucleus on the opposite end, a body cell in the centre enclosing a body nucleus in its middle and a short densely staining stalk nucleus at its periphery towards the end opposite the tube nucleus.

In nature the pollen grains germinate in the mucilage secretion that fills the micropylar tube of receptive ovules and oozes out as a thick mucilage drop at its end. This secretion is utilised for germinating the pollen grains artificially on glass slides. As soon as the pollen is put on the secretion under suitable conditions, its body nucleus is initiated to mitotic division. The details of the technique for germination and preparation of smears is described in a previous paper (Mehra, 1946).

This material is exceedingly suitable for the present study because of the large size of the chromosomes in the genus, the ease with which the division in the body nucleus could be initiated, the short period of 3–8 hrs. (depending on temperature) within which the mitotic cycle is completed, the facility for trying the action of the drug at any particular stage of the mitosis and lastly because the effect could be verified on observations on a large number of cases since a very large number of grains could be smeared on the same slide.

The fixation in all cases was done in Bouin's fixative with Allen's modification P.F.B.$_{15}$ followed by staining with Heidenhain's iron-alum haematoxylin.

Photos 1 and 2 show the metaphase and late anaphase stage respectively in the normal division of the body nucleus of pollen grains germinating in natural mucilage secretion free from the alkaloid and in Photo 3, which represents a later stage, the two daughter nuclei have already been organised.

The problem with respect to the effect of the drug has been tackled from two aspects:

A. The immediate effect of colchicine on the different phases of mitotic division and the changes ensuing thereafter.

B. Alteration in the mitotic cycle when the division starts in the colchicine containing secretion from the very beginning.

A. *The immediate effect of colchicine on the different phases of mitotic division.*

These observations are based on experiments with 0·2% strength of colchicine conducted on grains of four species, viz., *E. foliata* Boiss., *E. intermedia* Schrenk & Meyer, *E. sinica* Stapf and *E. likiagensis* Florin.

The grains are at first germinated on natural mucilage secretion on the slides till the rupture of the exine. The grains or the 'spindle gametophytes' at this time are usually at metaphase. This was verified from controls started simultaneously under similar conditions and given aceto-carmine or acetic-iodine-green stain. In other cases mitosis was allowed to proceed still further till the arrival of succeeding desired stages. 0·4% colchicine solution in distilled water was added in equal quantity to the mucilage secretion in which the grains were germinating at the desired stages and the fluid thoroughly mixed with the help of a needle. The colchicine strength of the medium thus became reduced to 0·2%. The grains were allowed to remain in the medium for 5 or 10 minutes—this short period being allowed for the grains to absorb the drug which could then react with the particular mitotic stage. After this interval fixations were made. Since in many cases a close series of different stages were present in the same slide, results for different phases were obtained even from the same preparation.

In other experiments where the further effect on the mitotic phenomenon after the action of the drug on a particular phase was desired to be investigated, fixations were made at different intervals from cultures started and treated simultaneously and kept under similar conditions.

In the beginning parallel controls were kept in which the same amount of distilled water was added when colchicine solution was added in other sets. Since these did not exhibit any difference from the normal mitotic phenomenon, the necessity for keeping them in later experiments was not felt.

Observations.

The immediate direct reaction of the drug at all stages of nuclear division where a spindle is present (metaphase, anaphase, telophase or when the two male nuclei are organised but the spindle is yet present) is the complete destruction of the spindle. This is observed in slides fixed 5 and 10 minutes after the application of this drug.

At metaphase and anaphase, after the collapse of the spindle, the chromosomes for a while remain in the same position in which they were before the action of the drug, though in a loosened state. If the drug reaction has been at metaphase the diplochromosomes are observed dispersed in the 'equatorial region' with their kinetochores lying almost in the same plane. If it was anaphase, the characteristic anaphasic orientation of the chromosomes with the kinetochores towards the 'poles' and the free arms towards the centre is retained for a brief period. Very soon these arrangements are disturbed and the chromosomes become irregularly scattered and sometimes widely separated. Obviously this is due to movements of the body cell cytoplasm in which the now free chromosomes are embedded.

After the collapse of the spindle at metaphase, if the grains are allowed to continue germination in the colchicine containing medium, these double chromosomes, which have already undergone scattering, ultimately split. The daughter chromosomes then enter into interphasic stage forming restitution nucleus.

If the drug has acted on the anaphasic stage, the chromosomes after the collapse of the spindle undergo irregular scattering within the cytoplasm of the body cell and finally form the restitution nucleus. The details of the process of formation of restitution nucleus are discussed later in the body of the paper.

In cases where colchicine acts upon telophase stage, the immediate effect is the same; i.e., the destruction of the spindle as previously described, but the two daughter nuclei are organised in the normal manner.

Also when the drug acts on the grains in which daughter nuclei have already been organised but the spindle yet not disappeared, the spindle simply collapses leaving the two free nuclei embedded in the cytoplasm of the body cell. The further increase in size of the two male nuclei in the body cell of the grains of *Ephedra* proceeds unhindered by the action of the drug.

Dermen (1940) in reviewing the effect of colchicine on plant cells states, 'Colchicine, like temperature, does not have any effect on resting cells and like temperature its effect is specific to metaphase and leptotene'. This statement needs qualification in that the effect of colchicine is specific at all stages where a spindle is present whether metaphase, anaphase or telophase.

B. *Alteration in mitotic cycle when the nuclear division starts in the colchicine containing medium.*

These experiments were tried on six different species of the genus *Ephedra*. The grains were germinated directly in colchicine containing natural mucilage secretion. *Ephedra intermedia* Schrenk & Meyer, *E. saxatilis* Royle, *E. sinica* Stapf and *E. likiagensis* Florin were all tried on 0·2% strength of the drug while in *E. foliata* Boiss and *E. altissima* Desf 0·1% and 0·2% concentrations were used. For making germinating medium of a particular strength of the drug, the natural secretion was thoroughly mixed with an equal quantity of double strength solution of colchicine in distilled water. Fixations were done at different intervals.

0·1% and 0·2% concentrations of the drug were found to have the same effect in disturbing the divisional mechanism in *E. foliata* and *E. altissima*.

Observations.

Up to the late prophase, the appearance of the nucleus and its structure is the same as in grains germinating in secretion free from the drug. At this stage the chromosomes, each of which possesses two chromatids, are elongated, closely packed and polarised. Their kinetochores point towards the tube nucleus end of the grain while the free arms which are more or less curved in order to accommodate themselves within the boundary of the body nucleus point in the opposite direction. Obviously the arrangement of the chromosomes at the end of the previous telophase is kept up during the interphase. The nuclear wall then disappears and the chromosomes remain for some time in the polarised state. The spindle mechanism is completely inhibited. The double 'metaphase' chromosomes loosen from one another and gradually become scattered within the limit of the body cell. The regular orientation of their kinetochores is lost. The chromosomes gradually become more or less straightened (Fig. 1, Photos 4, 5, 14, 15). This is due to the fact that the spindle being absent they are no longer under any stress which is operative in normal mitotic division when the chromosomes have to orientate variously during attachment to the equatorial region of the spindle. Simultaneously these double chromosomes undergo longitudinal contraction by spiralisation of their chromonemata thereby resulting in an increase in thickness. This contraction is the same as that observed in the normal mitotic cycle during the development of the metaphase.

The scattering of the chromosomes within the body cell is in all possible manners (Photos 4, 5) and must be ascribed to the movements of the cytoplasm in which the now free chromosomes are embedded.

As pointed out above, in the treated material the chromosomes become straightened. Their kinetochore regions become more prominent. The satellites and secondary constrictions remain visible in the same fashion as in untreated preparations.

The double chromosomes undergo splitting, the split starting from the arms gradually proceeds towards the centromere. In many preparations daughter

chromosomes fairly separated all along the arms and still held together at the region of the kinetochore are observed. Finally the split occurs in the region of the cen-

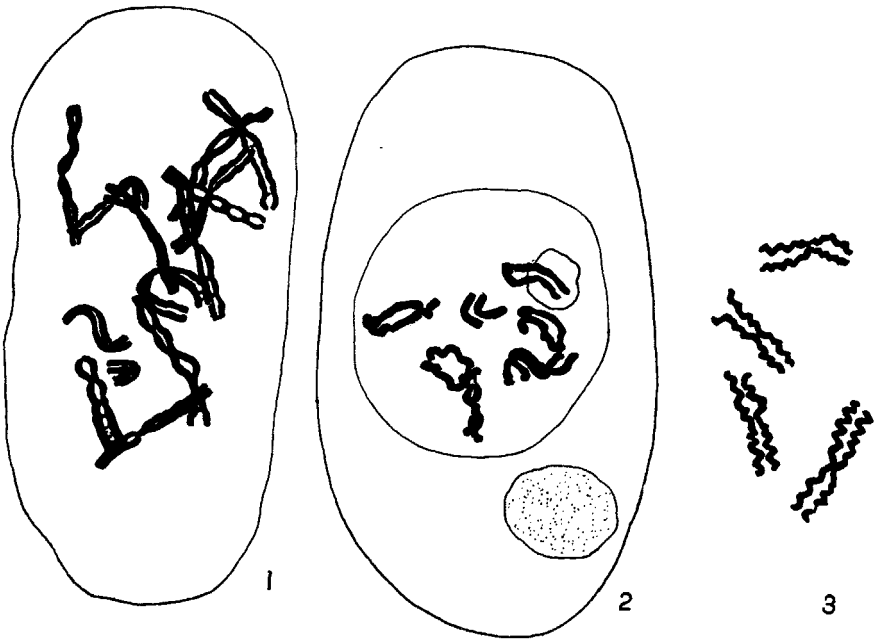

FIG. 1. 0·2% colchicine treated grain of *E. intermedia*, only body cell shown. The diplochromosomes have become widely scattered in the body cell. ×1840.
FIG. 2. 0·2% colchicine treated grain of *E. foliata*. The daughter chromosomes completely separated but still lying parallel. ×1840.
FIG. 3. *E. intermedia*. Diplochromosomes becoming condensed by close spiralisation of the component chromosomes due to the action of the drug. These are drawn from preparation shown in Photos 6, 7. ×1840.

tromere. The two daughter chromosomes may for a time lie parallel to one another (Photo 8, Fig. 2) but soon this arrangement is disturbed and all evidence of their parallel arrangement is lost (Photo 17). As previously pointed out this seems to be due to the excitation movements in the cytoplasm.

The splitting of the chromosomes may occur at any stage during their contraction to attain the size characteristic at the normal metaphase. The splitting for all the members of the complement is simultaneous.

As a rule the daughter chromosomes separate before entering into the interphasic stage. In extremely rare cases the double chromosomes are observed to proceed directly to nucleus formation even before undergoing splitting.

These observations are in strong contrast to those of some other investigators. Thus Levan (1938, 1939) found that colchicine, besides affecting the divisional mechanism, also delays the division of the centromere in the 'metaphase' chromosomes. Nebel and Ruttle (1938) found that the split chromosomes in the stamen hairs of *Tradescantia* do not separate. Eigsti (1940) also reports that in the pollen tube material treated with the drug, the daughter chromosomes remain attached at the region of the kinetochore and enter in the same state in nuclear formation.

After splitting the daughter chromosomes enter into the process of nucleus formation. They never become contracted to the same extent as in a normal telophase prior to the formation of daughter nuclei.

In some preparations in *E. intermedia* and *E. likiagensis* marked condensation of chromosomes is observed in grains germinating in medium with 0·2% colchicine. These condensed chromosomes become much reduced in size and increase considerably in thickness. This high degree of condensation is due to the marked spiralisation of the chromonemata of the double chromosomes. The gyres of the spiral become much compacted (Photos 6, 7, Fig. 3). Even in these condensed chromosomes splitting of the daughter chromosomes occurs invariably before restitution nuclei are organised.

Somewhat similar observations were made by O'Mara (1939) in root tip cells of *Allium cepa* exposed to colchicine. He found in some cells the double chromosomes much contracted, in others relatively elongated in the same root tip and concluded that 'cells which appear to be identical components of meristematic tissue may have significantly different resistance or reactions to the drug'.

The number and form of the nuclei organised from these scattered double number of chromosomes is very variable depending entirely on the position of the chromosomes in the body cell when nuclear formation commences. When chromosomes are close together a single diploid restitution nucleus is organised which may be small, compact and circular in outline and sufficiently dense so as to be sharply demarcated from the cytoplasm in which it is embedded. If the chromosomes are somewhat spread out, a larger nucleus with more or less irregular outline may result. Photo 10 also reproduced in Fig. 4 shows a large oblong restitution nucleus formed in this manner in *E. intermedia* and another similarly formed entering into the short pollen tube given out from the polar end of the grain is seen in Photo 12 and Fig. 5. In *E. likiagensis* a single large restitution nucleus, more or less dumbbell shaped in outline but with a precise wall, is observed in Photo 19 also reproduced in Fig. 7. In case the chromosomes are very widely scattered so that each is separated from the other by a wide space, a diffuse type of nucleus may be organised whose boundary is not clear and whose density is so low that it is not clearly demarcated from the surrounding cytoplasm (Photo 11). When chromosomes happen to lie in groups, two or more nuclei are organised according to the number of groups (Photo 13 reproduced in Fig. 6). These may be of different sizes depending upon the number of chromosomes entering into the constitution of each. They may be circular or amoeboid in form and may be connected with one another by bridge-like processes. In many cases some of these nuclei may not be easily differentiated from the surrounding cytoplasm. Their constitution is obviously very variable being dependent upon just how many chromosomes are present in a particular group when the nuclear formation begins.

It may be pointed out that Eigsti (1940) observed 3-4 occasionally 6 nuclei formed in the pollen tube of *Tradescantia* treated with colchicine but never the formation of a single diploid restitution nucleus. This is due to the fact that because of the narrowness of the pollen tube, the chromosomes cannot lie near one another after the inhibition of the spindle and the separate groups organised separate nuclei. The author reports that the chromatin of these nuclei was not distinguishable from cytoplasm.

The process of formation of the restitution nucleus is on the same lines as the formation of daughter nuclei after a normal mitotic division of the body nucleus except in one respect, i.e., the daughter chromosomes do not undergo contraction to the same degree as in a normal telophase before entering into the organisation of the daughter nuclei. The scattered daughter chromosomes become enveloped by some pale substance which markedly contrasts with the surrounding cytoplasm of the body cell which takes greyish stain. It seems as if some substance is secreted out of the chromosomes. The area of pale substance around each chromosome gradually increases and simultaneously the staining capacity of the chromosomes decreases. Finally all chromosomes lying close to one another become immersed in the pale substance while the chromosomes become gradually faded (Photo 9).

This is followed by the appearance of a wall around this pale area which forms the karyolymph.

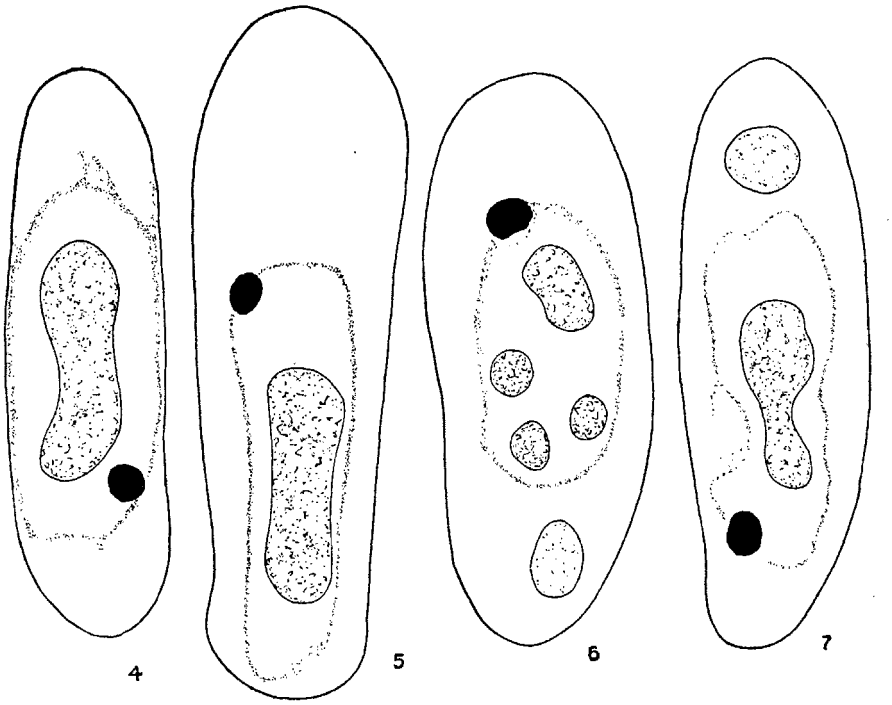

FIG. 4. Formation of large diploid restitution nucleus containing 28 chromosomes in *E. intermedia*. Same as Photo 10. ×870.
FIG. 5. Large diploid restitution nucleus entering into the short pollen tube developed in continuation of the polar end of the grain in *E. intermedia*. Same as Photo 12. ×870.
FIG. 6. Numerous unequal nuclei formed in a colchicine treated grain of *E. intermedia*. Same as Photo 13. ×870.
FIG. 7. A dumb-bell shaped restitution nucleus organised in a colchicine treated grain of *E. likiagensis*. Same as Photo 19. ×870.

Some investigators have held that colchicine retards the chromosome cycle and that is why a number of 'metaphase' stages are observed in colchicine treated material compared to the controls. Levan (1938, 1939) ascribed the delay in the division of centromere as at least one of the causes for the observation of large number of such stages in the colchicine treated material. Eigsti (1940) concluded that colchicine 'inhibits and prevents mitotic cycle but merely retards chromosomal cycle'. On the other hand Dermen (1940) states, 'the elimination of typical metaphase formation and the elimination of anaphases and telophases may account for an apparent increase of metaphase figures (mostly distorted) but actually the metaphases represent the sum total of anaphase and telophase figures which would have been formed normally but are prevented from formation'.

In an experiment to see whether or not colchicine retards the chromosomal cycle, cultures of germinating pollen grains of *E. likiagensis* were started simultaneously. In one of these which served as control the germination was allowed on the natural mucilage secretion to which an equal quantity of distilled water was added while in the other instead of distilled water an equal quantity of 0·4% colchicine solution was added and thoroughly mixed with the help of a needle so that

concentration of alkaloid was reduced to 0·2%. Both cultures were placed side by side under similar conditions and fixation made after 3½ hours. In the controls in many grains the normal mitotic process had been completed during this interval and two male nuclei organised at the two poles. In colchicine treated material on the other hand, many of the grains had formed the restitution nuclei. This is a clear evidence that 'chromosomal cycle' is not retarded by the action of the drug at least in the material under investigation.

The stalk and tube nuclei are not affected in any manner by the action of the drug. In some cases, a certain amount of vacuolation was observed in the cytoplasm of the body cell as well as in the general cytoplasm of the grain outside the body cell in 0·2% colchicine treated material.

The reaction of colchicine on the diploid grains in *E. foliata, E. altissima, E. intermedia* and *E. saxatilis* is the same as on the haploid ones.

The pollen tube growth seems to be slowed down in media with 0·1% or 0·2% colchicine but no detailed study of the effect of the drug on the rate of pollen tube growth in contrast to their normal growth in the unadulterated natural mucilage secretion was carried.

Summary and Conclusions.

The direct immediate effect of colchicine on the metaphase, anaphase and telophase stages of the dividing body nucleus in pollen grains of different species of *Ephedra* results in the complete collapse of the spindle mechanism. At the first two stages the chromosomes become scattered while in the latter stage two daughter nuclei are organised in the usual manner from the groups of telophase chromosomes in the absence of the spindle.

When the mitotic division starts from the very beginning in the presence of the drug the spindle is inhibited.

The chromosomes are sensitive to the action of the drug. In some cases with 0·2% concentration of colchicine, high degree of longitudinal chromosome contraction was observed associated with increase in thickness. This has been found to be due to high degree of spiralisation of chromonemata with consequent compacting of gyres.

The direct effect of the drug in some cases is the vacuolation of the cytoplasm of the body cell and the general cytoplasm of the grain.

The indirect effects of the drug due to the collapse of spindle are (i) the chromosomes become straightened since they are no longer to orientate in various fashions on the spindle, (ii) scattering of chromosomes due to excitation movements in cytoplasm in which the now free chromosomes are embedded.

The satellites, secondary constrictions and centromere are not affected by the drug.

There is no retardation of chromosomal cycle which is completed in about the same time as the normal mitotic cycle. The delay either in the division of centromere or in the splitting of daughter chromosomes does not occur as a rule in this material. On the other hand the splitting of daughters may occur even earlier when the chromosomes have not yet contracted to their normal metaphase size.

In the colchicine treated material the further contraction of the elongated double chromosomes occurs after their liberation from the prophase stage in a manner similar to that occurring in the normal mitotic cycle. Also splitting of daughter chromosomes *definitely* takes place prior to their organisation to form restitution nuclei. Thus both of these processes are independent of spindle mechanism. The chromosomes seem to act like independent units and go through their normal behaviour inherent to them irrespective of the presence or absence of a spindle.

The details in the process of building of restitution nuclei are on the same lines as those of daughter nuclei in the course of normal mitotic cycle.

The number of nuclei formed from the scattered chromosomes within the body cell are variable. In many cases a single circular dense nucleus of diploid nature is formed. Often a large amoeboid rather diffuse nucleus is formed or a number of nuclei of varying sizes and contours, some connected by bridge-like processes, are organised. Evidently when more than one nucleus is formed, these are of variable constitution.

Pollen tube growth seems to be retarded in the presence of 0·1% and 0·2% colchicine containing natural mucilage secretion.

Explanation of Plates.

1–13, *E. intermedia*. 14–19, *E. likiagensis*.

1–3 show normal germination on unadulterated mucilage, in the rest the pollen grains were germinated from the very start in 0·2% colchicine containing mucilage secretion.

1, 2, 3. Germinating grains at Metaphase, late Anaphase and having organised daughter nuclei respectively during the course of normal mitosis.
4, 5. Diplochromosomes widely scattered because of spindle inhibition. Note straightening of chromosomes in contrast to those in Photo 1.
6, 7. Condensation of chromosomes by close spiralisation in response to the direct effect of the drug.
8. Daughter chromosomes completely separated but yet lying parallel.
9. The process of building of a restitution nucleus.
10. Same as Fig. 4.
11. Diffuseness in the formation of restitution nucleus because of wide scattering of chromosomes.
12. Same as Fig. 5.
13. Same as Fig. 6.
14, 15. Parallel to 4, 5 but for *E. likiagensis*.
16. Parallel to 6, 7 but for *E. likiagensis*.
17, 18. After separation the daughter chromosomes have become widely separated.
19. Same as in Fig. 7.

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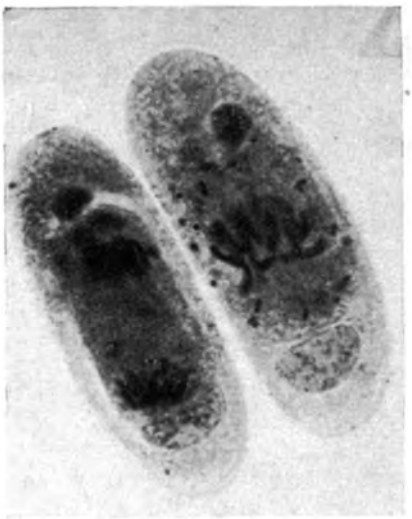

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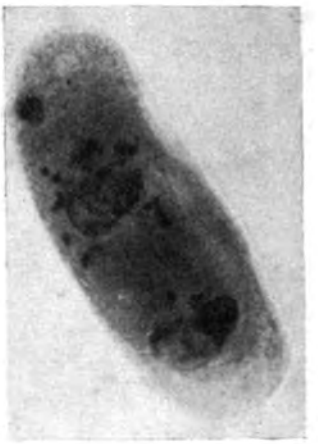

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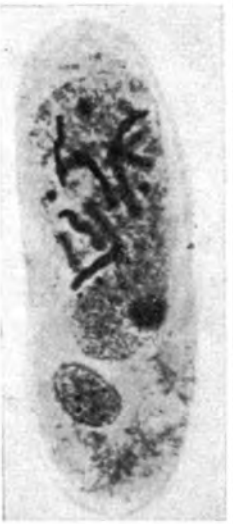

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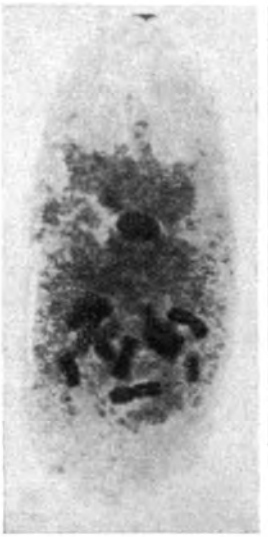

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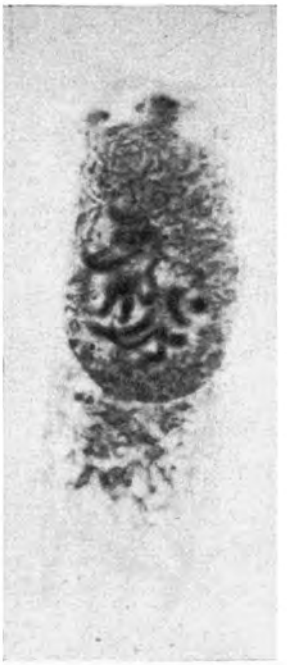

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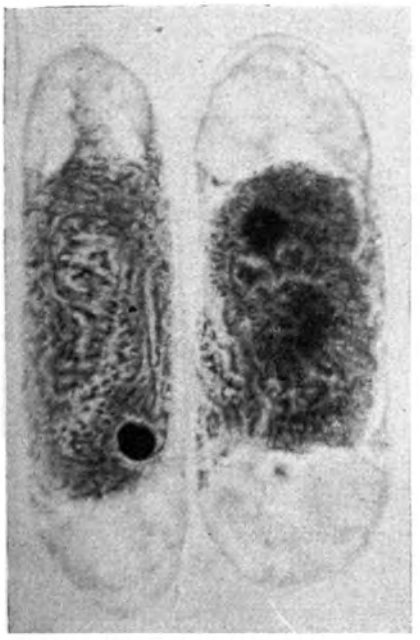

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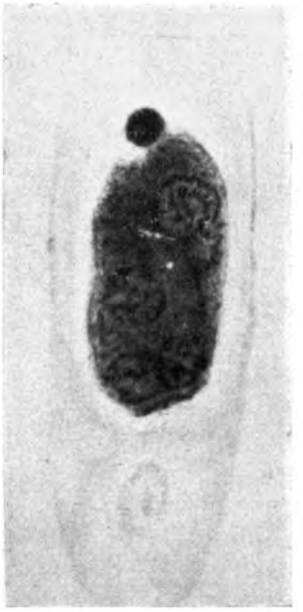

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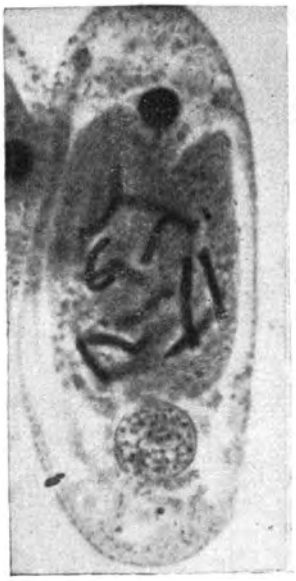

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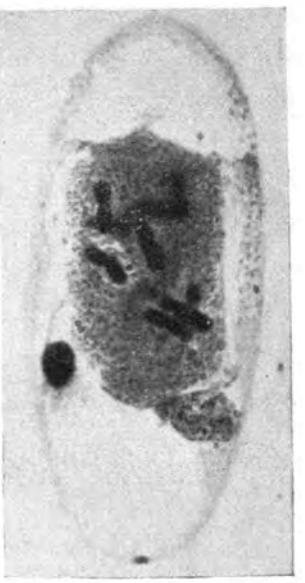

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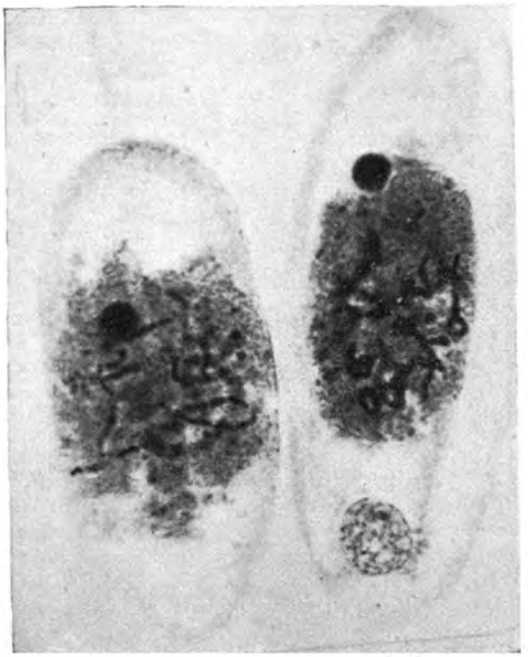

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