Studies on Giant Amoeboid Organisms

2. Nuclear Division and Cyst Formation in *Leptomyxa reticulata* Goodey with Remarks on the Systematic Position of the Organism

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SUMMARY: Excellent cytological preparations may be made from growth of *Leptomyxa reticulata* on cover-slips on a film of agar.

The organism is multinucleate. Each resting nucleus contains a deeply staining mass, the nucleolus, which is surrounded by a clear zone in which chromatin material is scattered. There is always a definite nuclear membrane. The nuclei divide simultaneously by intranuclear mitosis and the whole process is completed in a short time. At prophase the dispersed chromatin granules aggregate and later assume a thread-like structure and enter upon the metaphase. The formation of the spindle can be seen at the beginning of the metaphase. No centrosomes are seen at any stage. During anaphase the chromosomes begin to separate and it is difficult to count their numbers. The nuclear membrane disappears at late anaphase. At telophase the daughter chromosomes fuse together, the connecting threads break and the daughter nuclei are formed.

Under suitable cultural conditions and on certain strains of bacterial food supply, multinucleate cysts are produced in clusters. The process of cyst formation is described.

Leptomyxa reticulata Goodey, 1914 is widely distributed in soils of Great Britain (Singh, 1948). The study of the selection of bacterial food by *L. reticulata* has shown that under suitable cultural conditions and with certain bacterial associates these multinucleate organisms produce multinucleate cysts in clusters (Singh, 1948).

McLennan (1930) found *L. reticulata* associated with hop disease in Tasmania, but after a careful study concluded that it was a secondary invader and had no significance as a causative agent of the disease. These organisms were both in the root and on its surface, and in the lower part of the aerial bine. She thought that *L. reticulata* could penetrate the hop root either through the root hairs or by direct penetration through the epidermis, and after entering the root hairs, travel down into the cortical tissue and pass readily from one cell to the other.

For cultivating *L. reticulata*, McLennan (1930) used Erlenmeyer flasks with 100 ml. of tap water containing boiled wheat grains. In a synthetic food solution not containing carbon (Knop) and in soil infusion *L. reticulata* did not grow well. This she thought due to the insufficient development of bacterial food supply. It may be pointed out that nutrient media are often unsuitable either for isolation or growth of holozoic organisms because they may encourage the growth of inedible organisms and possibly of organisms producing toxic substances (Singh, 1945, 1946*a*, *b*, 1947*a*, *b*, *c*, 1948).

McLennan (1930, p. 36) says: 'When the organism found on the hop is

compared with *Leptomyxa reticulata* the two forms are found essentially similar, and there is no doubt that the hop organism is correctly placed in this genus.' She concluded that the hop organism differed from *L. reticulata* Goodey in the following respects. (1) The cysts formed by the hop organism were much more irregular in shape and on the average much larger than those described by Goodey (1915). (2) The ectocyst in the hop organisms never approached the thickness attained in some cysts of *L. reticulata* figured by Goodey. On the basis of these differences she named the hop organism *L. reticulata* Goodey var. *humuli* (n.var.). The creation of this variety is probably not justified because the cultural conditions were such that mature cysts were unlikely to be formed. Judging from McLennan's diagrams, it seems certain that she was dealing with immature cysts or with those that were in the process of formation.

Goodey (1915) and McLennan (1930) have given a good general account of *L. reticulata*, although they could neither find the nuclear division in these multinucleate forms nor were they able to determine the mode of cyst formation. From their accounts it seems that one large individual gives rise to one multinucleate cyst.

The mode of nuclear division and cyst formation described in this paper may be of interest to systematists of the *Proteomyxa*, a group in which the great majority of forms have been very imperfectly studied.

MATERIAL AND METHODS

Leptomyxa reticulata was isolated from soil on non-nutrient agar (1·5 % agar in 0·5 % NaCl; pH 6·8–7·0) with *Aerobacter* strain 1912 (Singh, 1941, 1948). A pure line culture started from a single cyst was used throughout the work. *L. reticulata* forms cysts in large numbers only with certain bacterial associates.

L. reticulata did not grow very well on cover-slips covered with a suspension of a 2–4-day culture of a suitable bacterium in 0·8 % NaCl. In the presence of a thin layer of non-nutrient agar *L. reticulata* grew luxuriantly and produced cysts. Thin films of plain agar were made as follows. One or two drops of hot melted agar were put on a $2 \times \frac{7}{8}$ in. cover-slip and spread into a thin film by quickly covering with a second slip. When the agar had solidified the upper cover-slip was gently removed, and one or two drops of a thick suspension of a suitable bacterium in 0·8 % NaCl were gently smeared on the agar surface. A small piece of agar containing *L. reticulata* was cut from an actively growing culture and put face downwards in the centre of the film. The cover-slips were kept in moist chambers in Petri dishes in order to prevent the drying of the agar and were incubated at 20–21°. Every precaution was taken to prevent contamination of the agar films. After 2–3 days, when some of the *L. reticulata* had moved on to the film, the piece of agar used for inoculation was removed.

Carnoy fixative was extensively used to make permanent preparations and gave better results than Bouin and Schaudinn. *L. reticulata* was fixed for 20–40 min. and then put into 95 % ethanol for 24 hr. The cover-slips were then

brought through 70, 50 and 30 % ethanol to water, and at this stage the film of agar was gently removed from the cover-slip. As *L. reticulata* are large organisms, they sink into the agar and stick to the glass when the film of agar is removed after fixation. About 60–90 % of them remained on the cover-slips in a beautifully extended position as seen during life. The preparations were stained for *c.* 6 hr. in iron alum, overnight in haematoxylin and mounted in the usual way.

LEPTOMYXA RETICULATA

A general account of *L. reticulata*, its movements and division by plasmotomy has been given by Goodey (1915) and McLennan (1930). According to Goodey (1915) on nutrient-bouillon-agar *L. reticulata* has a waxy translucent appearance, the protoplasm being very compact and often disposed in an irregularly branched dendritic manner. When transplanted from agar into a liquid medium it assumes its normal shape and condition by stretching out into a thin sheet of almost transparent protoplasm. The abnormal shape observed by Goodey on nutrient agar was most probably due to the uncontrolled bacterial food which may have produced unfavourable metabolic substances.

When *L. reticulata* was grown on non-nutrient agar with a suitable bacterial food the organisms always stretched out into a very thin sheet of almost transparent protoplasm so that at times it was difficult to see them. Under suitable cultural conditions the size of *L. reticulata* was found to be much larger than those recorded by Goodey (1915) and McLennan (1930). When fully stretched an individual may attain a length of 3 mm. or more. As pointed out by Goodey, it assumes all kinds of fantastic shapes (Pl. 1, figs. 1–9), so that it may appear as though several species are present in the same culture. When the food supply is exhausted, and with drying of the agar, some of the individuals shrink so as to appear mycelial. In this form they remain alive for a month or more without forming cysts, and fresh cultures can be obtained from them without difficulty. The mycelial appearance was also sometimes observed in actively growing cultures, but under these conditions the protoplasm was nicely stretched out, and very branched. A single large *L. reticulata* looks like a small plasmodium of an endosporous Myxomycete, but without the regular reversible streaming movement which is found in the latter. The number of nuclei varies a great deal depending on the size of the individual; up to several hundred nuclei have been found in one large *L. reticulata*. In an actively growing culture a few very small individuals may be present (Pl. 1, figs. 4–8) containing 8–20 nuclei. They look like small true soil amoebae, and are the results of division by simple plasmotomy in which the daughter individuals produced are often of very unequal size (Pl. 1, fig. 10). As was noted by Goodey (1915) no nuclear division in the dividing individuals was observed either during plasmotomy or just afterwards. It is interesting to watch under a low-power microscope the process of division of the individuals by plasmotomy and the fusion of two or more of them into one, processes already recorded by Goodey.

Goodey (1915) says (p. 84): 'Stained examples show that the nucleus consists

of a central deeply staining mass, the karyosome, surrounded by a clear zone which is often somewhat oval or spindle-shaped and does not seem to possess a definitely stainable membrane. Some sort of membrane is, however, present, for the nuclear sap is quite distinguishable from the surrounding cytoplasm. The process of nuclear division is quite obscure at present, but from the examination of a large number of living and stained specimens it seems that some kind of fragmentation of the karyosome takes place.' As noted below, the karyosome, which I call the nucleolus, does not seem to take any direct part in the intranuclear mitosis of *L. reticulata*. McLennan (1930) showed that each resting nucleus has a definite nuclear membrane, and the chromatic material is scattered evenly between the karyosome and the nuclear membrane. This observation of McLennan is in accord with my findings.

Nuclear division

Mitosis in endosporous Myxomycete plasmodium was so rarely observed by the earlier workers that they concluded that nuclei divide both by amitosis as well as by mitosis. With the development of better cultural methods, a few workers definitely proved that all the nuclei divide by mitosis simultaneously. The whole process is completed in a short time.

A large number of observations was made to find whether the nuclei in *L. reticulata* divide by mitosis, amitosis or both. At first no nuclear divisions were found. A few individuals were found containing nuclei very much smaller than are usually met with in individuals containing resting nuclei. It was thought that the nuclear division might either be completed within a short time or the nuclei might be dividing at some particular time of the day or at night. Accordingly, large numbers of cover-slip preparations were made at hourly intervals from 10 a.m. to 6 p.m. and from 8 to 12 p.m. Nuclear division was ultimately found in the material fixed during both the day and night. Out of the many thousands of large and small individuals examined only three large ones and one small one were found in the stage of nuclear division. All the nuclei in the small individual, numbering 20, were in the telophase of mitosis. All the stages of mitosis were found in each of the three large individuals containing several hundred nuclei. It seems that all the nuclei divide more or less at the same time and the process is completed in a very short time. Many *L. reticulata* were found in which the nuclei were only half the normal size. They apparently had just completed mitosis.

Details of mitosis

The resting nuclei in *L. reticulata* are either spherical or spindle-shaped (Pl. 2, figs. 20–22); and both kinds may be present in the same individual. The mitosis in some respects is like that found in vascular plants, the achromatic figure being devoid of centrosomes and asters. The nuclear membrane is a definite structure which can be seen up to anaphase (Pl. 2, figs. 23–32) and the nuclear division is intranuclear.

In each resting nucleus there is only one nucleolus. The first stage in the nuclear division is the aggregation of dispersed chromatin granules in prophase. The granules stain deeply with iron alum haematoxylin (Pl. 2, figs. 23, 24). The nucleus at this stage generally attains its greatest diameter. The deeply staining granules fuse together and begin to assume a thread-like structure. Soon afterwards, at metaphase, the chromosomes are compact and irregular and the spindle forms (Pl. 2, fig. 25). Later, the spindle is completely formed without any centrosomes (Pl. 2, figs. 26, 27).

During the anaphase the chromosomes begin to separate (Pl. 2, figs. 28–34). It is very difficult to count them accurately. In some nuclei it appeared that there were four chromosomes at each side of the spindle and in others in the same individual only two could be distinguished. In the late anaphase the nuclear membrane is not distinctly seen.

At telophase the spindle shrinks and appears as dark wrinkled lines (Pl. 2, figs. 35–38). The daughter chromosomes fuse together into a darkly staining mass. The connecting threads break and the daughter nuclei begin to form (Pl. 2, figs. 38, 39). The chromatin beings to fragment inside the nucleus, which at this stage again has a definite nuclear membrane. The nucleolus persists throughout anaphase and does not seem to take part in cell division. In telophase it either gradually fades away or is left out when the daughter nuclei begin to form. Sometimes more than one darkly staining mass can be seen inside each nucleus. The mode of formation of the new nucleolus could not be ascertained. It first appears as a small body which grows as the nucleus begin to grow.

The formation of cysts

Under suitable cultural conditions *L. reticulata* produces multinucleate cysts in clusters (Singh, 1948) resembling the sclerotium of an endosporous Myxomycete. The process of cyst formation is usually very slow, and mature cysts are produced only after several days. Parts of branching protoplasm are slowly drawn into dense and irregular masses which gradually become rounded (Pl. 1, figs. 12, 13). These rounded parts secrete an outer cyst membrane, or ectocyst, of varying thickness. The contents of the cyst continue to contract and secrete another wall, the endocyst, which is usually rounded. The ectocyst may be rounded or irregular (Pl. 1, figs. 15, 16). Usually the cysts break off from the parent individual when they are mature. Several cysts were seen in the process of formation attached to the parent individual in fixed preparations (Pl. 1, figs. 12, 13); these individuals showed no sign of nuclear division. The nuclei in the cyst-forming area were spherical and packed together, whereas the nuclei in the rest of the individual were either spindle-shaped or spherical and typical of resting nuclei. After the cysts are broken the parent individual continues a trophic existence. Sometimes rounded or irregular bits of protoplasm are cut off from a large individual, and give rise to double-walled cysts. As shown in Pl. 1, figs. 17 and 18, one ectocyst may contain up to ten endocysts, each of which is multinucleate (Pl. 1, fig. 19). Under good cultural conditions a single large multinucleate organism is completely broken into a number of

cysts, the number depending on the size of the individual (Pl. 1, fig. 14). Up to 40 or more cysts may be produced from a single individual, which then resembles a sporangium. This formation of multinucleate cysts in clusters is a very interesting feature in the life cycle of *L. reticulata*. When the cysts are fully mature they can be easily separated from each other.

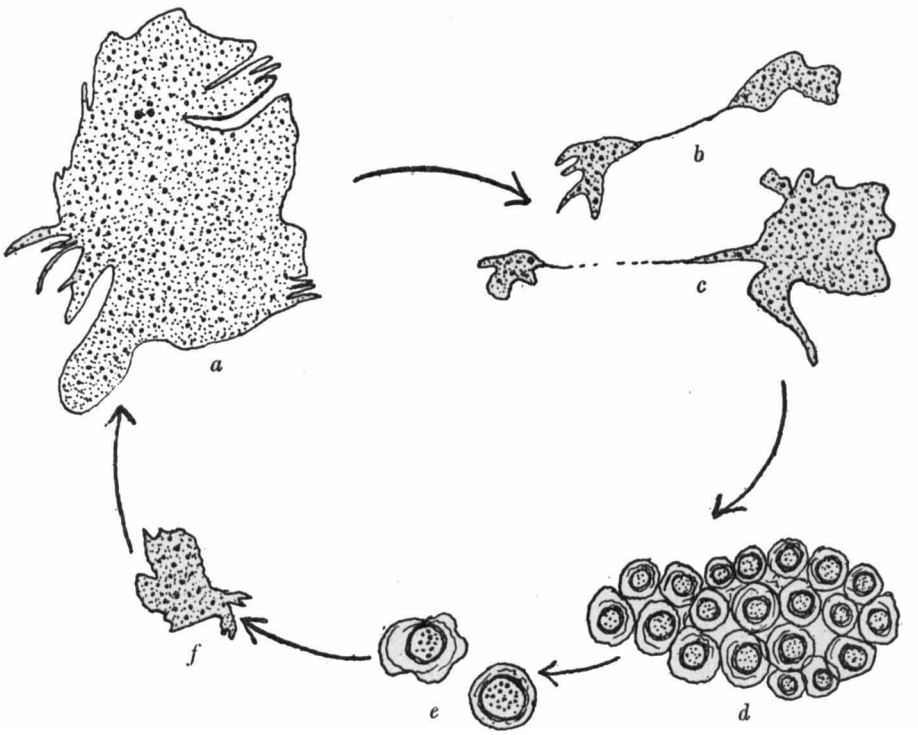

Fig. 1. Diagrammatic representation of the life cycle of *Leptomyxa reticulata*; *a*=fully grown multinucleate individual; *b* and *c*=division by simple plasmotomy; *d*=a cluster of multinucleate cysts formed from one individual; *e*=two multinucleate cysts; *f*=a small multinucleate individual after emerging out of a multinucleate cyst.

Excystation

When cysts are transferred to non-nutrient agar or to a few drops of 0·8 % NaCl on cover-slips supplied with suitable bacterial food they excyst readily. The process of excystation has been described by Goodey (1915). Multinucleate individuals have always been found to grow out of the cysts. Many of the cysts formed under good cultural conditions do not excyst and are probably dead.

THE SYSTEMATIC POSITION OF *LEPTOMYXA RETICULATA*

A number of mostly imperfectly studied forms which did not seem to fit in with the rest of the Rhizopods have been put into the group *Proteomyxa*. Since the discovery that *Leptomyxa reticulata* produces multinucleate cysts in clusters resembling the sclerotium in endosporous Myxomycetes, a considerable

time has been spent in finding whether a flagellate stage occurs in its life cycle. As observed by Goodey (1915), a single cyst gives rise to one multinucleate individual which feeds on bacteria and grows into a giant form. The fusion of two or more individuals may result in the formation of one larger individual. The life cycle of *L. reticulata* (Fig. 1) seems to justify its inclusion in Rhizopoda.

It is curious that such a remarkable form as *L. reticulata* has been ignored by the writers of text-books on Protozoology. The mode of cyst formation and nuclear division in *L. reticulata* surely justifies the creation of a new genus as suggested by Goodey (1915). Goodey (1915), Sandon (1927) and McLennan (1930) have put this organism in the order Amoebaea. Schaeffer (1926) excludes rhizopods with reticulate pseudopodia from this order. In the present state of our knowledge, it is my opinion that the genus *Leptomyxa* should be put in the family Vampyrellidae (Doflein) belonging to the order Proteomyxa (Lankester), and ranking with the few well-recognized genera like *Arachnula* and *Vampyrella*, etc., thus bringing it nearer to the Amoebaea.

Leptomyxa flabellata (Goodey, 1915) has been isolated from a number of arable soils in Great Britain, but I have not yet studied the life cycle of this interesting form.

The observations of McLennan (1930) on the occurrence of *L. reticulata* in hop tissues is very interesting. It is possible that some of the Plasmodiophorales may have evolved from free living forms like *L. reticulata*.

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EXPLANATION OF PLATES

PLATE 1

Leptomyxa reticulata. Figs. 1–18 are drawn from the same magnification. The magnification of fig. 19 is indicated below it. Cover-slip preparations fixed in Carnoy and stained with iron alum haematoxylin.

Figs. 1–9. Individuals of different sizes and shapes.
Figs. 10, 11. Division by simple plasmotomy.
Figs. 12–14. The formation of multinucleate cysts.
Figs. 15, 16. Typical double-walled mature cysts.
Figs. 17, 18. Cysts containing several endocysts.
Fig. 19. A part of the endocyst highly magnified to show the nuclei.

PLATE 2

Intranuclear mitosis in *Leptomyxa reticulata.* Cover-slip preparations fixed in Carnoy and stained in iron alum haematoxylin.

Figs. 20–22. Typical resting nuclei.
Figs. 23, 24. Prophase stages in mitosis showing the aggregation of chromatin in one place.
Figs. 25–27. Metaphase stages and the formation of spindle.
Figs. 28–34. Anaphase stages and the separation of chromosome to two poles of the spindle. The nuclear membrane has disappeared in the late stages.
Figs. 35–38. Telophase stages and the formation of two daughter nuclei.
Fig. 39. Four small nuclei just after mitosis.

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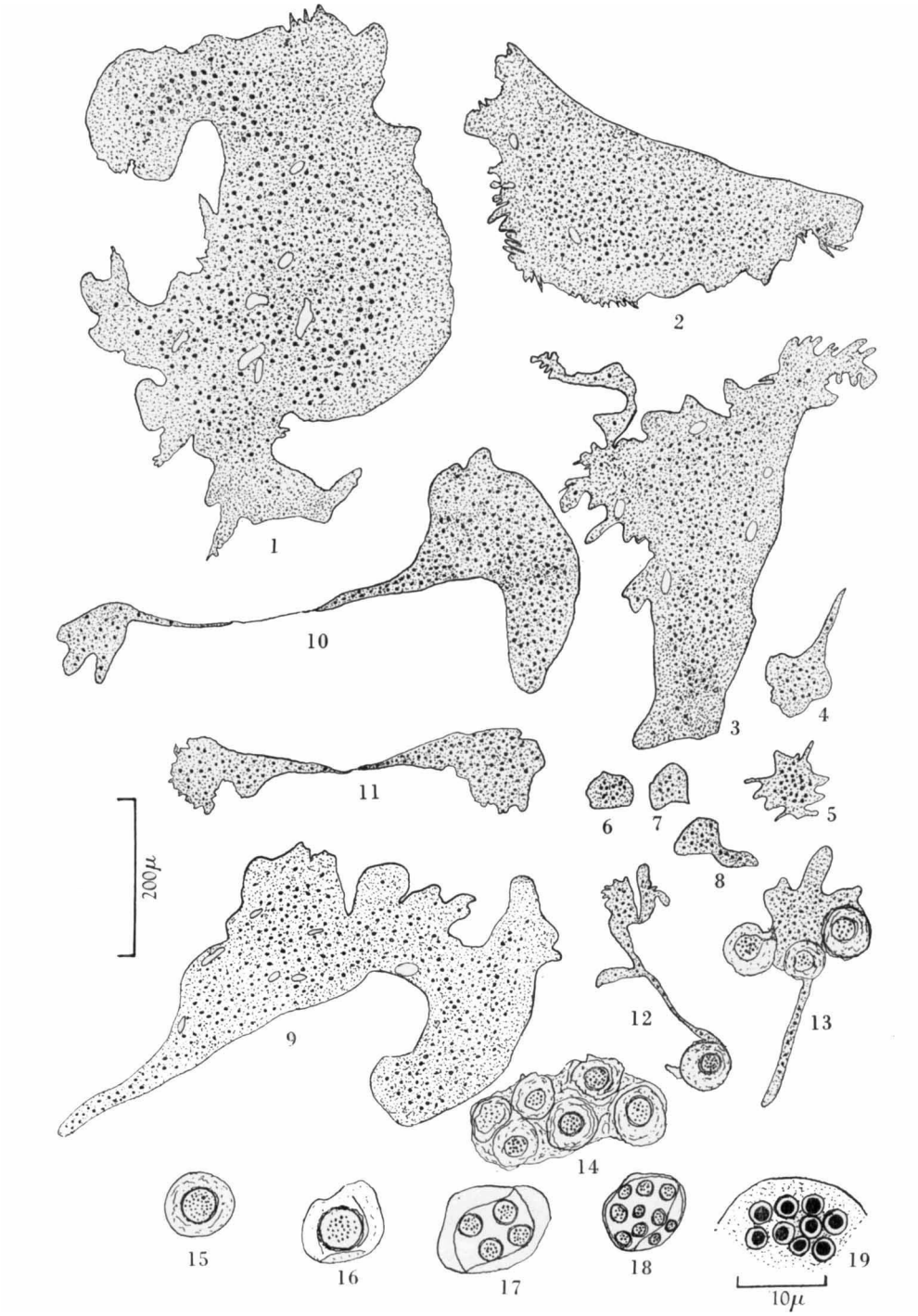
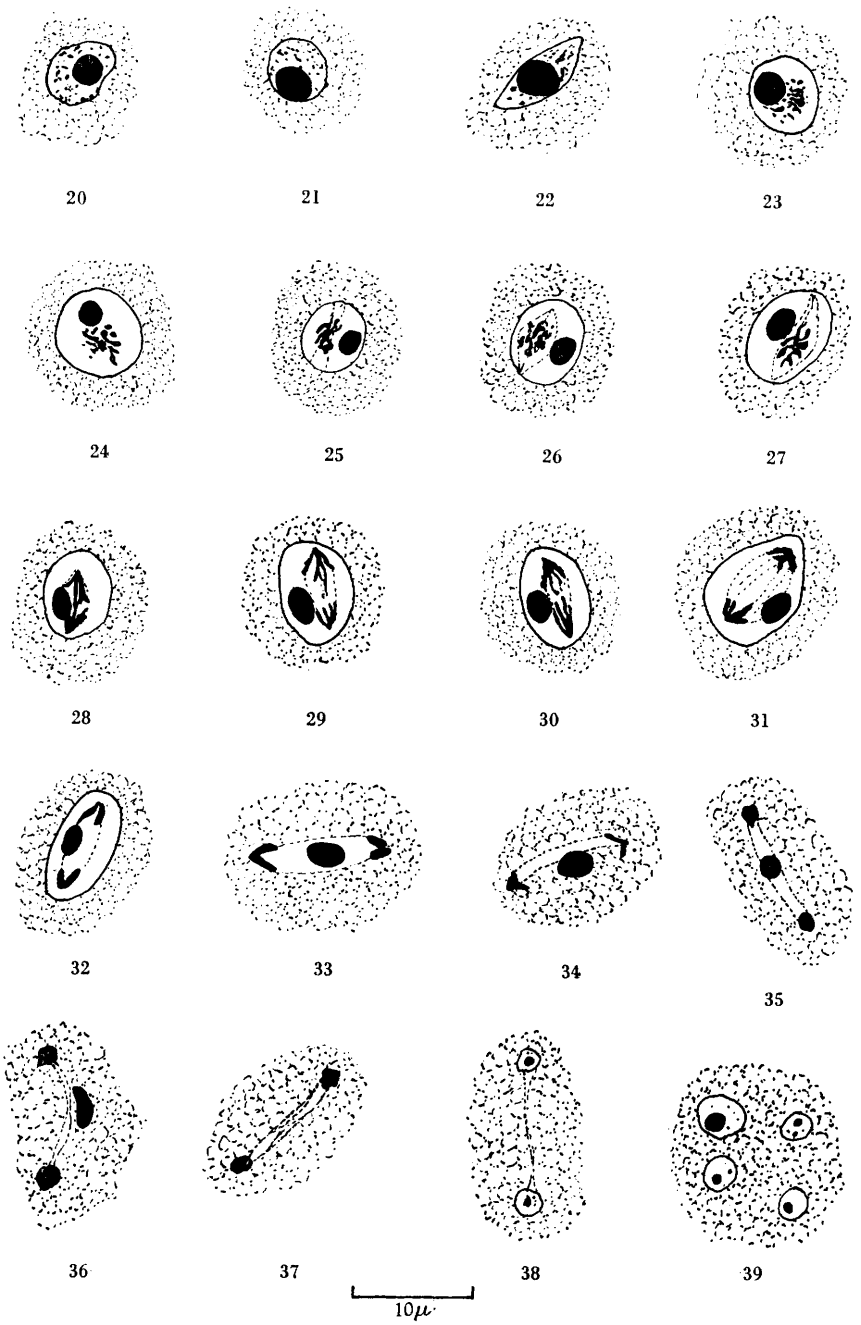


Figs. 1–19

B. N. Singh—Division and cyst formation in *L. reticulata*. Plate 1



Figs. 20–39