Hillhousia mirabilis, *a Giant Sulphur Bacterium.*

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[Plate 9.]

The organism which forms the subject of this paper has been under the observation of one of us for a number of years, but its true nature was not immediately recognised. It has been found in various parts of England and Ireland since 1892, always occurring in very stagnant pools and marshy bogs.

Until quite recently it was only found very sparingly amongst Algæ, Infusoria, etc., generally in situations where there was much decomposing organic matter. During the past winter, however, the organism has been found in abundance in the mud of the shallower part of an old pool in Worcestershire. This has enabled us to make cultures of it, and to make a study of its general biology and structure. We have named it *Hillhousia mirabilis.**

The cells are solitary but gregarious, occurring freely among other organisms and numerous small particles of decaying organic matter. They secrete very little or no mucus, even in a culture, so that colonies are not built up.

They are for the most part shortly cylindrical, with hemispherical ends, and are usually straight. Some individuals exhibit a very slight curvature, and others may be observed which are slightly attenuated towards each extremity. The cells are from one and three-quarters to three and a-quarter times as long as broad.

For a unicellular bacterium the dimensions of this organism are phenomenally large. *The diameter of the cell* varies from 20 to 33 μ, and *averages about* 26 μ. *The length* varies from 42 to 86 μ, and *averages about* 60 μ.

In its normal healthy condition the cell is packed with globules of oily amorphous sulphur of various sizes, the largest of which have a diameter of about 10 μ (Plate 9, figs. 1 to 3). These globules are very crowded, and, when isolated, are seen to be somewhat irregular, although rounded (fig. 17). Their refractive nature gives the organism almost a black appearance under

* The generic name "*Hillhousia*" has been given in honour of Prof. W. Hillhouse, the retiring Professor of Botany in the University of Birmingham, to whom both authors owe much in the way of kindly advice and criticism.

the microscope, but a pale yellow or yellow-green colour is quite obvious on careful inspection by transmitted daylight.

Owing to the large quantity of sulphur they contain, the cells are very heavy, sinking in the water almost like sand-grains.

The cells are motile, exhibiting slow oscillatory and rolling movements. *The organism is a peritrichous bacterium with several hundred short cilia* disposed all over the exterior of the cell-wall. The cilia can be seen immediately on fixation either with a 5-per-cent. carbolic acid solution or with a 40-per-cent. formalin solution. The action of these reagents results in a cessation of the movements of the cilia in from 10 to 20 seconds, during which period many of them are thrown off and become disintegrated. Some of these cilia exhibit a contractile or wriggling movement after having been thrown off, indicating that the contractility of the cilium is not dependent upon attachment at the base. The cilia are plainly observed by the use of the above reagents, but only within a few seconds of the moment of fixation (figs. 15 and 16).

The cilia can also be clearly seen in active movement by examining the living organism by means of the Zeiss or Leitz dark-ground illuminator. It is likewise possible to demonstrate the presence of cilia by immersing the living organisms in a drop of indian ink. The cilia appear as a halo around the cell, and the minute particles of ink can be seen in rapid motion on the edge of the halo. This movement is of a different character from the Brownian movement of the minute particles in the water. The tiny particles of ink can be seen to be lashed about and driven into small eddies.

Cell-division is relatively slow, and in no observed instance was a single division completed in less than 24 hours, the time occupied in many cases being as much as 48 hours.

The Sulphur Globules.—The globules of sulphur are enormously larger and much more densely packed than in any of the other known sulphur bacteria. They are dissolved by glacial acetic acid,* by boiling in a solution of magnesium sulphate; and also by prolonged boiling in potassium chlorate. They turn almost black on boiling in ferrous sulphate; and a brownish-black precipitate is found in the cells after boiling in lead acetate. A $2\frac{1}{2}$-per-cent. solution of commercial formalin entirely removes the globules. Carbon bisulphide penetrates the cell-wall only with difficulty, but after penetration quickly dissolves the sulphur.

When the living organisms in a pure condition are placed in distilled water

* This was pointed out by Corsini in the case of *Beggiatoa*; *cf.* A. Corsini, "Ueber die sogenannter Schwefelkörnchen die man bei des Familie der 'Beggiatoacæ' antrifft," Centralbl. für Bakteriol.,' II, 1905.

the sulphur globules disappear in about 48 hours, the sulphur being oxidised in the process of respiration. The oxidation of the sulphur can best be demonstrated by allowing a culture to remain undisturbed for several days in a small quantity of water containing minute traces of lime. The organisms gradually lose all their sulphur, and a considerable deposit of granular crystals of calcium sulphate is formed.

By allowing the bacteria to dry on a slide, and then irrigating with water, the sulphur is obtained in small rhombic crystals (fig. 18). Crystals can also be obtained by allowing the formalin solution in which the bacteria have been fixed to slowly evaporate. These do not appear, however, to be crystals of pure sulphur.*

From the general behaviour of the sulphur globules, there are many reasons for supposing that the sulphur is not pure, but exists in some kind of loose combination, possibly with proteid material. The crystals obtained by drying the organisms and then irrigating with water are only formed *outside* the cells. After irrigation the colloidal sulphur, or sulphur-compound, passes through the wall of the cell, without causing the latter any injury, and crystallisation of the sulphur takes place in the surrounding medium.

Cultures.—Like other sulphur bacteria, *Hillhousia* will not grow on gelatine or agar.† We have obtained the best cultures in tap water containing minute traces of sulphuretted hydrogen.

Straining the mud through coarse muslin enables one to obtain almost all the bacteria, mixed only with various small living organisms, finely divided flocculent organic matter, and small sand-grains. The great weight of the bacteria can now be utilised to obtain a pure collection, as it is possible to remove the other organisms by means of a fine pipette, while the large sulphur bacteria and the small sand-grains remain as a sediment. If this sediment be now transferred to a watch-glass it is possible to separate the major portion of the sand-grains by a judicious tapping of the glass while held in a slightly inclined position. In this way the bacterium can be obtained mixed only with very minute sand-grains. We have not yet succeeded in obtaining a culture of the bacterium entirely free from these tiny fragments of silica, although some method of chemotaxis might possibly solve the

* After several hours' treatment with 40-per-cent. formalin, the individual bacteria are found to be encrusted with a thick deposit of radiating crystals. On the addition of more water to the solution the crystals are dissolved. If weaker formalin is used, the crystals are never formed, as the substance (some compound of sulphur) is dissolved as fast as it is produced.

† Winogradsky, in 'Ann. de l'Institut Pasteur,' 1889, pp. 49 and 50, has stated his inability to obtain cultures of *Beggiatoa*, *Thiothrix*, *Chromatium*, etc., on solid culture media.

difficulty. The culture should now be shaken up at intervals, and very small quantities of sulphuretted hydrogen water added every few days.

Such a culture will thrive for a time, although the multiplication of the organism is relatively very slow.

The organism thrives best when the flocculent organic matter, after straining, is allowed to remain in the water. Under these circumstances the bacterium remains healthy, and can be kept for a very long time without any addition of sulphuretted hydrogen water.

Experiments in obtaining pure cultures are still proceeding, and discussion of this part of the investigation is for the present deferred.

Keeping the organism in the original mud in which it is collected, without constant change of water, proves unhealthy and ultimately fatal. This is due to the accumulated excess of sulphuretted hydrogen in the water, which causes the bacteria to lose their sulphur. The addition of a strong solution of sulphuretted hydrogen to a culture also causes a solution of the sulphur and the death of the organisms.

Light is unnecessary for the perfect growth of this bacterium, cultures thriving as well in complete darkness as in diffuse light.

In mass and by reflected light a culture presents a greyish-white appearance.

Cytological Structure.—Formalin has been found the most useful fixing reagent on account of the fact that the sulphur globules are removed at the same time. A $2\frac{1}{2}$-per-cent. solution of commercial formalin will completely remove the sulphur in the course of a few hours.*

Individuals fixed in this way show *a network of protoplasm* which occupies the interstices between the sulphur globules, the position of the latter being indicated by the large clear spaces (figs. 5 and 19). *Embedded in the protoplasmic network are numerous minute granules* of very variable size. (These are well shown in fig. 19.)

The *cell-wall* is highly resistant to reagents, but becomes much more permeable after the organism has been dried. It contains no cellulose, and stains yellow on the addition of iodine. It dissolves only with difficulty in sulphuric acid, does not dissolve in an ammoniacal solution of cupric hydrate, and in many ways it is suggestive of fungus-cellulose. Its great resistance to reagents is probably due to the presence of a considerable proportion of chitin.

On the addition of 5-per-cent. carbolic acid to the living organisms, the cell-wall swells up and becomes lamellose, indicating that it is not of

* The removal of the sulphur is probably brought about by the small quantity of free formic acid present in commercial formalin.

homogeneous structure (figs. 13 and 14). There appear to be several firm layers, with intervening layers which become somewhat gelatinous on the addition of carbolic acid. The innermost layer is a firm one, but the outermost layer, which can only be demonstrated by special methods, is apparently gelatinous.*

Nothing of the nature of a definite nucleus is present in the cell, but as the protoplasmic network includes many conspicuous granules, very careful tests for nucleins have been made.

Staining has given very indefinite results. Erythrosin and methylene blue were of little use. Safranin and carbol-fuchsin were found the most useful stains, and double staining with safranin and gentian-violet gave good results. Carbol-fuchsin stains the protoplasmic network very well, but in no instances were the included granules distinctly brought out. Cover-glass preparations gave much better results than any other method. The granules of the network do not appear to have an affinity for any of the stains used, and they cannot be regarded as chromatin granules.

Microchemical tests for nucleo-proteids have been carefully repeated many times, using cultures of the organism fixed in $2\frac{1}{2}$-per-cent. commercial formalin. As stated before, by this means the sulphur globules are removed and the fixed protoplasmic network can be experimented upon. Owing to the impermeability of the cell-wall it was found necessary to allow the operations to extend over a considerable period.

Treatment with concentrated sodium carbonate removed fully nine-tenths of the granules, while the network remained clear and refractive (fig. 21).

A 10-per-cent. salt solution removed a large proportion of the granules, probably about five-sixths of them, and the network was again left clear except in the central part of the cell, where there appeared to be a concentration or shrinking together of the protoplasmic strands (figs. 10 and 11). For this reaction, and also the previous one, the cultures were exposed to the reagent for rather more than 14 days.

Treatment with dilute potash (5 per cent.) gave a variety of results due probably to the degree of penetration of the potash in different individuals. In most cases, after about 10 days, the granules were for the most part dissolved, and the network to a great extent disorganised (fig. 12).

A number of cultures were treated with acidulated pepsin-glycerin, and in these cases the network was for the most part digested, whereas the majority of the granules remained unchanged. Where the network had only

* There is evidence of this even in the active state of the organism, as the cells have a tendency to stick to the bottom of the vessel in which the culture is growing.

partly disappeared, the granules had the appearance of highly refractive beads strung on fine threads (fig. 20).

The above tests, taken collectively, furnish evidence which goes far to prove that a considerable proportion of the granules present in the general protoplasmic network consist of nucleo-proteids.

Lastly, the bacterium was tested for phosphorus. A considerable quantity of a culture (pure except for very minute grains of silica) was incinerated on platinum foil, and kept at a red heat for several minutes. The ash was then treated in small tubes with a reagent consisting of 10 c.c. of nitric acid to 1 gramme of ammonium molybdate, and kept at a temperature of 50° C. for a week, after which period numerous minute crystals of an intense yellow colour were present in all the slides prepared. These crystals belong to the cubic system, and there is every reason to regard them as crystals of ammonium phospho-molybdate. Slides of the reagent only, kept for the same period at 50° C., showed no trace of such yellow crystals.

From this test we conclude that phosphorus is present in the bacterium, and therefore that some nucleo-proteid is present.* The previous tests indicate that this nucleo-proteid is in the form of small granules in the protoplasmic network, and the staining proves the granules are not particles of chromatin.

Thus, Hillhousia *is a very primitive unicell in which chromatin has not been developed, and the particles of nucleo-proteid (possibly of the nature of linin) are scattered evenly through the whole protoplasmic network of the cell.*

Although the cytological structure of *Hillhousia* can be studied with comparative ease, it must not be assumed that other and less easily investigated bacterial cells have a similar structure. The sulphur bacteria may be of a low type, and it is quite probable that among the various known groups of the Schizomycetes there are bacteria in which the cytological structure is of a somewhat higher order.

The present investigation is only of a preliminary character, as much work yet remains to be done in obtaining pure cultures, and in further working out the cytology of the organism.

Summary.

Hillhousia mirabilis is a sulphur bacterium of giant proportions, and is much the largest solitary bacterium which has so far been discovered. Its average length is about 60 μ and breadth about 26 μ.

The organism is a peritrichous bacterium with a large number of short

* Galeotti (in 'Zeitschr. für physiol. Chemie,' vol. 25, 1898, p. 48) has definitely demonstrated the occurrence of a nucleo-proteid in certain bacteria.

cilia. It occurs among decaying organic matter in the mud of shallow fresh-water pools.

Each individual contains a protoplasmic network in the wide meshes of which large globules of sulphur (probably not pure, but in loose combination with proteid material), are located. The network includes numerous small granules, a considerable proportion of which consist of some nucleo-proteid. None of them are chromatin granules.

The cell-wall is firm and has great powers of resistance to reagents. It is not homogeneous, and 5-per-cent. carbolic acid demonstrates its lamellose character.

The multiplication of the organism is relatively slow, one division occupying upwards of 24 hours.

EXPLANATION OF PLATE.

FIGS. 1—16, each × 500 ; FIGS. 17—21, each × 1000.

FIGS. 1—3.—Drawings of living specimens of *Hillhousia mirabilis* to show the dark refractive sulphur globules practically filling the whole cell.

FIG. 4.—Specimen kept in tap water for one week. The sulphur globules have been almost entirely used up in respiration.

FIG. 5.—Individual after being in a 2½-per-cent. solution of commercial formalin for several days. The sulphur is completely removed and the protoplasmic network becomes very obvious. Note the numerous granules in the network.

FIG. 6.—Outline of a curved cell. These are rarely observed, the great majority of the cells being straight.

FIGS. 7—9.—Outline of three distinct specimens showing three of the principal stages in simple cell-fission. In fig. 9 the constriction is almost complete.

FIGS. 10, 11.—Two individuals after treatment for 14 days with 10-per-cent. NaCl. Many of the granules in the network have been removed, and there is a decided contraction or shrinking of the central parts of the network.

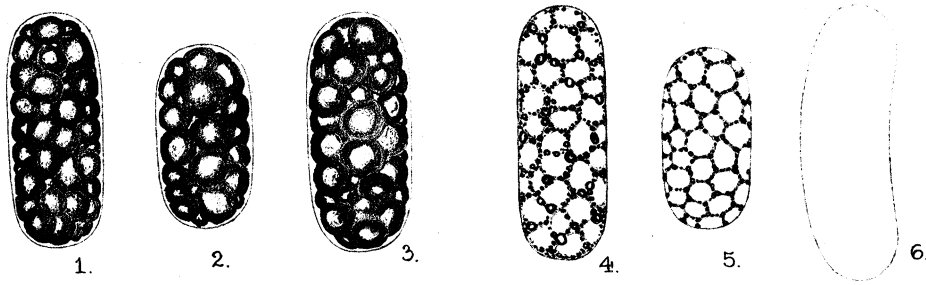
FIG. 12.—Individual after prolonged treatment with 2-per-cent. KOH. The protoplasmic network is largely disorganised, and a large number of the granules have been dissolved.

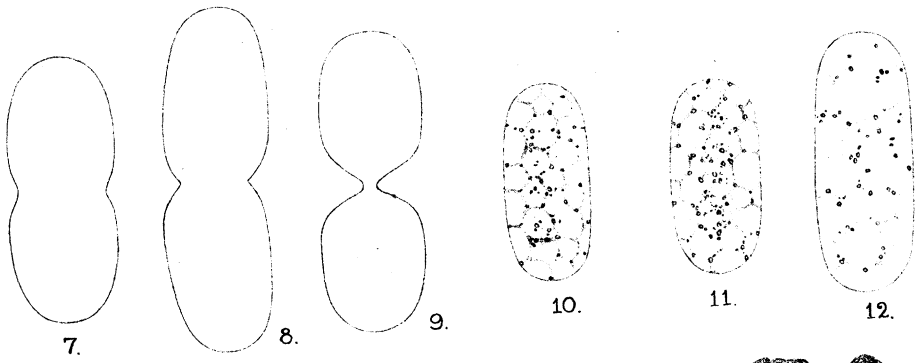
FIGS. 13, 14.—Two cells after treatment for about 15 minutes with 5-per-cent. carbolic acid. The cell-wall exhibits a lamellation, and the sulphur globules have coalesced into a central irregular mass.

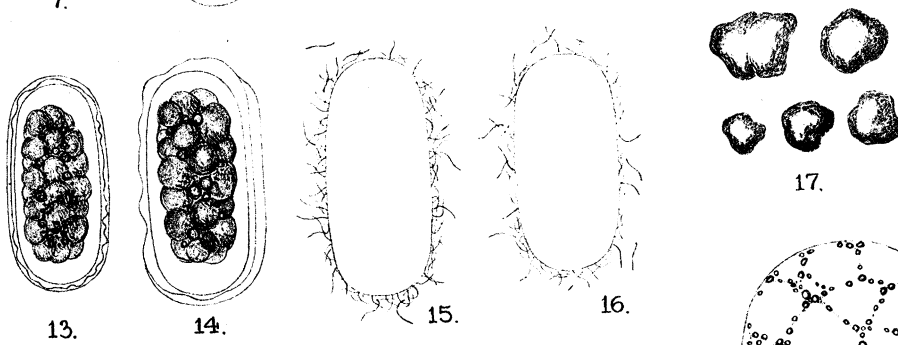
FIGS. 15, 16.—Two cells immediately on treatment with 40-per-cent. commercial formalin. The numerous short cilia are very readily observed for a brief period, and many of them can be seen to be thrown off into the surrounding liquid.

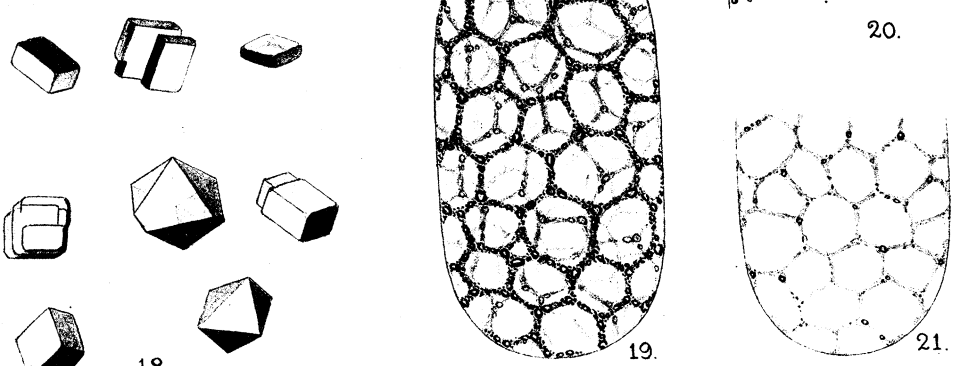
FIG. 17.—Isolated sulphur globules, showing their irregular form, obtained by crushing the living cell.

FIG. 18.—Small crystals (rhombic prisms and rhombohedra) of sulphur obtained by allowing the living organisms to completely dry up. These crystals are formed *outside* the organisms, the colloidal sulphur passing through the cell-wall to the outside after irrigating with water.









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HILLHOUSIA MIRABILIS, WEST & GRIFFITHS.

FIG. 19.—Single specimen after treatment with 2½-per-cent. commercial formalin, showing both the parietal and the more internal portions of the protoplasmic network. The granules are shown only in the upper parietal portion of the network.

FIG. 20.—One extremity of an individual after treatment for 14 days with acidulated pepsin-glycerin. Only the granules of the surface network are represented; these stand out very clearly, but the protoplasmic network itself has been for the most part digested.

FIG. 21.—One extremity of an individual after treatment for 14 days with a concentrated solution of Na_2CO_3. Only the surface network is represented. The protoplasmic network remains clear and distinct, but most of the granules have been dissolved.

The Development of Trypanosoma gambiense *in* Glossina palpalis.

By Colonel Sir DAVID BRUCE, C.B., F.R.S., Army Medical Service; Captain A. E. HAMERTON, D.S.O., and H. R. BATEMAN, Royal Army Medical Corps; and Captain F. P. MACKIE, Indian Medical Service. (Sleeping Sickness Commission of the Royal Society, 1908.)

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[PLATES 10 AND 11.]

The following experiment is so complete in itself that no apology is offered for publishing it by itself. In 1903 the Sleeping Sickness Commission of the Royal Society came to the conclusion that the carrying of infection from a sleeping sickness patient to a healthy person by the *Glossina palpalis* was a mechanical act, and required no previous development of the parasite within the fly. The Commission also held that the power of transferring the disease was lost to the fly 48 hours after it had fed on an infected person.

Koch and Stuhlmann, in German East Africa, described developing forms in *Glossina*, but did not succeed in infecting healthy animals by the injection of these forms.

Kleine, in German East Africa, at the end of 1908, succeeded first in showing that *Glossina palpalis* could convey *Trypanosoma brucei* some 50 days after the fly had fed on an infected animal.

It seems, at first, strange that this fact should have escaped notice for 15 years, and can only be accounted for by assuming that it is an event of the rarest for a fly to be found which fulfils the unknown conditions necessary for the development of the trypanosomes in its interior. If we assume that it is only one fly in a hundred or in a thousand in which this

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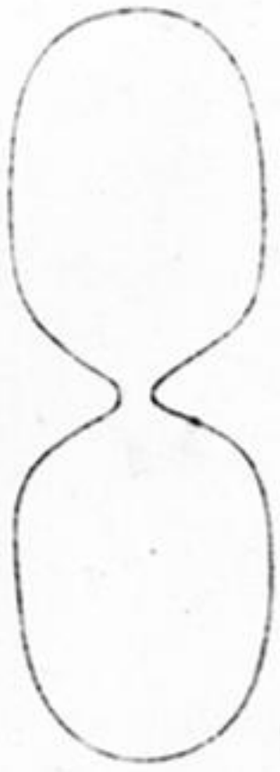

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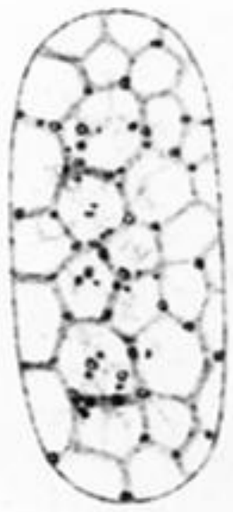

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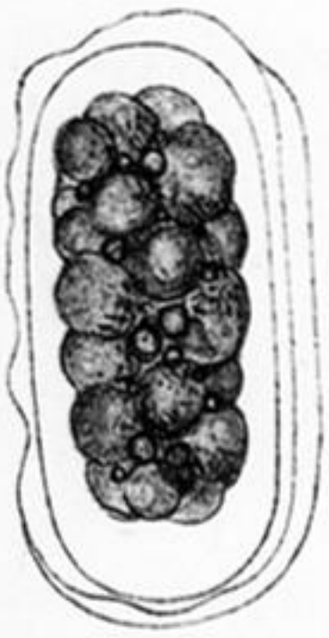

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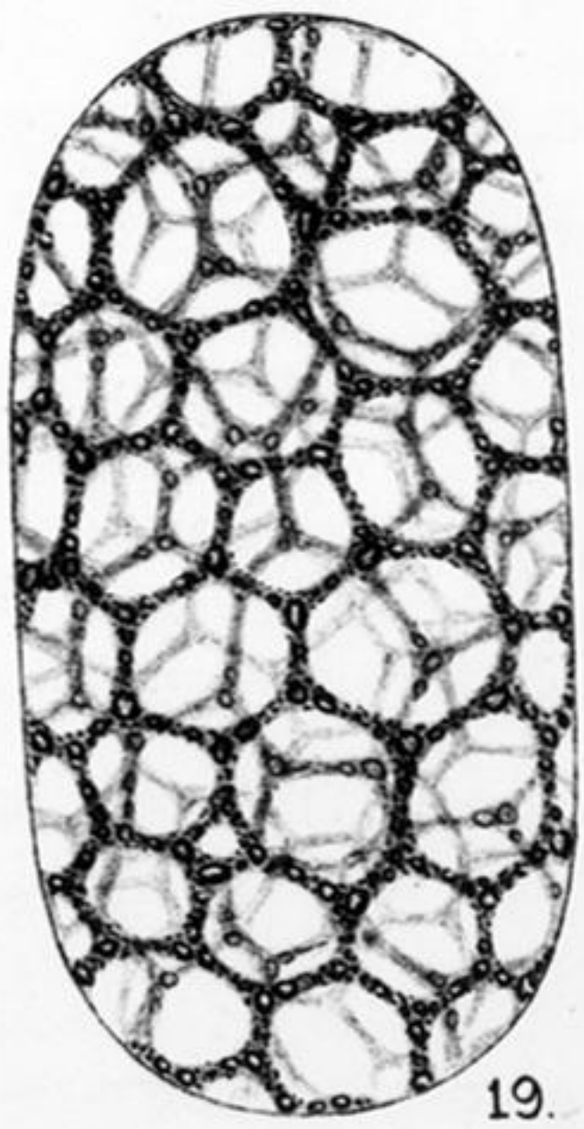

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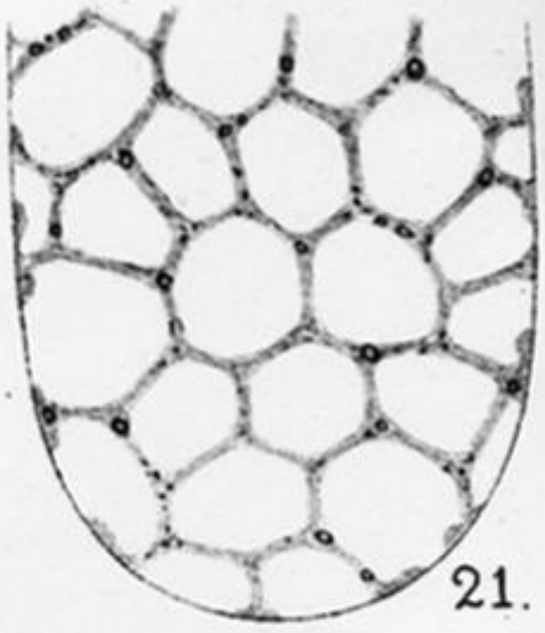

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