# The Lime-Sulphur Bacteria of the Genus Hillhousia.

BY

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**With Plate X.**

IN 1909 the authors published a preliminary account[1] of a new organism which they named *Hillhousia mirabilis*, and which they regarded as a giant sulphur bacterium. Since that date further investigations have shown that the organism is without doubt a huge sulphur bacterium, but one that contains in addition a large amount of calcium carbonate.

Each individual organism is cylindrical with hemispherical extremities, and contains such a large amount of mineral matter that it has a specific gravity about equal to that of the small sand grains frequently associated with it.

It is a peritrichous bacterium with relatively short cilia, and it exhibits slow rolling movements of a rather irregular and often of a spasmodic character.

The cell-wall is very resistant to the passage of reagents, and has been shown to be lamellose.

The organisms are gregarious but not colonial. They have a tendency to adhere to the bottom of a glass vessel, probably by means of the small amount of mucus secreted by each individual.

They occur in the mud of freshwater pools, sometimes in very large numbers. Owing to their high specific gravity they can be obtained partially pure by gradually pipetting off the flocculent organic matter, when the organisms, mixed with a few small sand grains, remain. If the glass dish is carefully tilted the sand grains can be made to roll down, leaving the organisms as a greyish-white mass.

Although it is thus possible to obtain a pure 'collection' all attempts at pure cultures have failed. Such cultures would necessarily be of slow

[1] G. S. West and B. M. Griffiths: *Hillhousia mirabilis*, a Giant Sulphur Bacterium. Proc. Roy. Soc., B, vol. lxxxi, 1909.

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growth owing to the length of time (twenty-four to forty-eight hours) occupied by each cell-fission.

It has been found that a good fixative is 40 per cent. commercial formalin, which not only fixes the protoplast moderately well, but also removes the large globules of calcium carbonate, leaving only the protoplasmic reticulum with the numerous smaller grains of sulphur embedded in the threads. Formalin of this strength has, however, a tendency to affect adversely the protoplasmic reticulum, since it has to act for some hours in order to remove the larger globules. On the other hand, dilute acetic acid removes the larger globules at once with slight effervescence, leaving the sulphur grains unaffected, but again there is a slight injury to the protoplasmic reticulum.

The best fixative was one recommended by Professor J. B. Farmer, and consisted of a mixture of three parts of absolute alcohol and one part of glacial acetic acid. This not only removed the globules very rapidly, but also the reddish sulphur grains, and at the same time fixed the protoplasmic reticulum exceedingly well. By this method there was no appreciable shrinkage.

Since the original note, two distinct species of *Hillhousia* have been recognized. In addition to the large one—*H. mirabilis*—there is a smaller species not more than half the size. This small species, which we propose to call *H. palustris*, is distinguished not merely by its size, but also by the more rounded segments during the process of cell-division. Its protoplasmic reticulum is very little smaller than that of *H. mirabilis*, and therefore the cell contains comparatively few meshes.

Both species are widely distributed and appear to have rather different habitats. Doubtless they will be found to exist in many parts of the world.

THE PROTOPLAST. After the removal of the globules of calcium carbonate by means of formalin, the protoplast is seen to consist of a more or less uniform and rather coarse reticulum. There is no trace of a nucleus, and the reticulum is evenly distributed throughout the cell, as originally figured.[1] The whole reticulum is very finely granular and appears to contain rather larger granules in the corners of the meshes.

The sulphur grains are rather small (1–2 $\mu$ in diameter), of a reddish colour, and are situated within the threads of the protoplasmic reticulum between the much larger globules of calcium carbonate.

*Dry-staining* is carried out with difficulty owing to the highly resistant cell-wall. Specimens were fixed on cover-slips with 40 per cent. commercial formalin and allowed to dry in the air, the small amount of mucus on the outside of the organisms causing them to adhere. Fairly good stained preparations were obtained with safranin, iodine green, Ziehl's carbol-fuchsin,

[1] West and Griffiths, l. c., Fig. 19.

Giemsa's stain, and iron-haematoxylin (ferric alum and Heidenhain's haematoxylin).

In no case could any large deeply staining granules be detected, although very minute granules which stained rather more than the rest of the protoplasm were distributed throughout the reticulum. In dry-staining there was always an apparent concentration of the reticulum in the central part of the cell (Figs. 4 and 5), this being due to the flattening of the organism upon drying, and the consequent superposition of different parts of the reticulum.

*Wet-staining.* The water containing the living organisms was placed in small tubes and centrifuged, after which the organisms were fixed in three parts absolute alcohol and one part glacial acetic acid. This removed both the calcium carbonate and the sulphur. The collection was then washed thoroughly in alcohol to remove the acetic acid, and afterwards stained with the same stains as used in the dry-staining method. In no case could any true chromatin be detected, but, as before, minute granules staining rather deeper than the rest of the protoplasm were distributed throughout the reticulum. Those granules at the angles of the meshes were a little more conspicuous than the others.

No contraction of the reticulum was visible in any single instance (Fig. 6). We therefore regard the apparent concentration of stainable substance, which is observed after staining subsequent to fixation by drying, as an artifact.

The organism appears to be of a simple type in which as yet there is practically no differentiation of the protoplast.

The granules in the network consist, as previously stated,[1] of a nucleo-protein, and if they are of the nature of a chromatin substance, it is one which differs considerably from the chromatin of more highly organized cells. It has little, if any, affinity for the usual nuclear stains.

In the small species, *Hillhousia palustris*, the structure of the protoplast is precisely similar, but the meshes are fewer in number. (Compare Figs. 6 and 10.)

The Inclusions. The inclusions within the protoplast vary according to the amount of sulphuretted hydrogen and lime-salts present in the water. In a normal individual the cell is filled with refractive granules of variable size, which are of two distinct kinds.

1. *Globules of calcium carbonate.* These are large globules varying from 6 $\mu$ to 10 $\mu$ in diameter. They are of a steel-grey colour, highly refringent, and lie in the meshes of the protoplasmic reticulum, one only in each mesh (Fig. 1). They are plastic, being able to pass through the cell-wall without rupturing it. When the organism has been killed by reagents, such as a concentrated aqueous solution of sulphuretted hydrogen, iodine in

[1] West and Griffiths, l. c., p. 403.

potassium iodide, alcohol, or by drying and subsequently re-irrigating with water, the globules pass through the cell-wall and crystallize either on its exterior or in the water in the immediate vicinity. The exudation of the globules and their subsequent crystallization take about fifteen minutes. After prolonged drying this exudation does not occur. The crystals are of two forms, flat rhombohedra with angles 74° 55′ and 105° 5′, or rhombic prisms (*vide* Fig. 3).

The globules are readily soluble in sulphuric acid, hydrochloric acid, nitric acid, and dilute acetic acid. They are also dissolved by formalin in about an hour.

The crystals are readily soluble in all the above acids except sulphuric, when, after partial solution, dense tufts of small crystals (presumably of calcium sulphate) are formed and stop further action.

If the organisms are heated to redness on platinum foil, the globules remain apparently unaffected, and are soluble in the same reagents that dissolve the crystals.

In the Bunsen flame the organisms give the red calcium coloration.

If sulphuric acid is added to a collection of living organisms, gas is given off freely, and when passed into lime-water the gas causes a white precipitate. Therefore it consists of, or contains, carbon dioxide.

If to a collection of living organisms a solution of potassium permanganate is added, and then sulphuric acid, the permanganate is unaffected by the gas given off. There is no carbon monoxide given off, therefore, as in the case where calcium oxalate is similarly treated.

These tests indicate that the globules consist of calcium carbonate. This is confirmed by examination of the crystals with the polarizing microscope. It is found that their optical properties and crystalline form show them to be calcite. Such crystals may be produced chemically by adding a hot solution of ammonium carbonate to a hot solution of calcium chloride, when a mixture of flat rhombohedra and rhombic prisms is produced, identical with those formed from the crystallization of the plastic globules in the organism.

While within the organism the globules do not polarize.

The facts that sulphuric acid will completely remove the globules from the living organism, that gas is not given off very freely with dilute acetic acid, and that the globules will pass through the cell-wall without rupturing it, all appear to indicate that the calcium carbonate is possibly held in a colloidal form while within the organism. That there is a difference between the properties of the calcium salt in the living organism and in the dead organism is also shown by the very brisk effervescence when dilute acetic acid is added to specimens that have been subjected to prolonged drying, or to specimens incinerated on platinum foil.

2. *Sulphur grains.* Small granules varying in diameter from about 2 $\mu$

to mere specks are found lying in the threads of the protoplasmic reticulum. They are of a dull red colour and rather refractive in appearance. They remain in the protoplasm when the organisms are dried and re-irrigated with water, but if left for some days freely exposed to the air they slowly disappear.

They are soluble in hot potassium hydrate, in potassium cyanide, in carbon bisulphide, in chloroform, in strong nitric acid, and in glacial acetic acid. They are unaffected by the reagents that dissolve the globules of calcium carbonate.

If strong picric acid is added to organisms previously cleared of calcium carbonate, and subsequently washed thoroughly in water, the sulphur grains tend to run together and to become crystalline.

If the organisms are mounted on a slide in 40 per cent. formalin, the sulphur grains slowly crystallize in the course of a few weeks. If mounted in dilute acetic acid crystallization takes place in a few hours. The crystals are typical double pyramidal crystals of sulphur of a yellow colour (Fig. 7).

If individuals containing sulphur grains are treated with warm potassium cyanide and ferric chloride, a deep red coloration is produced. Organisms without sulphur grains do not produce this coloration.

When a quantity of organisms containing sulphur grains are burned on platinum foil, a very distinct odour of sulphur dioxide is noticed.

These experiments show that the reddish grains are of sulphur. The sulphur grains of *Beggiatoa* are precisely similar, in appearance and in their behaviour to reagents, to those of *Hillhousia*.

## Conditions necessary for Healthy Existence.

These lime-sulphur Bacteria are easily affected by slight changes in their environment. In order to remain in a healthy condition they require a sufficiency of lime-salts, sulphuretted hydrogen, and oxygen.

*Hillhousia* has been kept in a healthy condition for more than nine months in a glass dish six inches in diameter and two inches deep. The mud in which the organisms normally live was placed in tap-water in the dish, and water was added to replace that lost by evaporation. It was found necessary to stir up the mud frequently in order to get rid of the excess of sulphuretted hydrogen and to aerate the water.

If fresh water is allowed to run through a collection the organisms lose their sulphur within forty-eight hours. If the mud is then left undisturbed so that sulphuretted hydrogen can accumulate, the sulphur grains reappear. The addition of chemically prepared sulphuretted hydrogen to organisms removed from the mud and placed in fresh water also causes the sulphur grains to reappear, provided that the water has been

aerated. If there is not sufficient oxygen, the organisms exude their globules of calcium carbonate, leaving a long double line of crystals of calcite as they move slowly about; and subsequently death ensues.

### Comparison with Achromatium oxaliferum, Schewiakoff.

The large bacterium *Hillhousia* resembles in some respects the organism described by Schewiakoff[1] as *Achromatium oxaliferum*, but differs in many particulars, which may be summarized as follows:

| *Achromatium.* | *Hillhousia.* |
|---|---|
| Differentiation of protoplast into a peripheral zone with a small reticulum, and a larger central portion ('central body') with a much larger reticulum. | No differentiation in the protoplasmic reticulum. |
| Conspicuous reddish grains of chromatin (according to Schewiakoff) at the intersection of threads of central reticulum. | No definite chromatin recognizable by stains, but small granules, possibly of chromatin, in the threads of protoplasmic reticulum. |
| Globules of calcium oxalate in the meshes of the reticulum. | Globules of calcium carbonate in meshes of reticulum. |
| No sulphur grains. | Refringent reddish grains of sulphur in threads of reticulum. |
| Average size: 29 $\mu \times 15\mu$. | Average size:<br>*Hillhousia mirabilis*, 60 $\mu \times 26\mu$.<br>*Hillhousia palustris*, 25 $\mu \times 14\mu$. |

Eight years after the publication of Schewiakoff's paper, Massart[2] described and figured an organism as *Achromatium oxaliferum*. He asserted that 'en réalité la couche périphérique de petits alvéoles n'existe pas. . . . Je pense donc que même pour cette immense cellule il faut renoncer à l'espoir de trouver un corps central.' He also found that 'les petits grains très refringents qui sont engagés dans le réseau protoplasmique sont du soufre', and that '(la cellule) contient une substance qui, contrairement à ce que dit M. Schewiakoff, n'est certainement pas de l'acide oxalique ni un sel de calcium'. The size of the cell as figured is 30 $\mu \times 20\mu$.

There is little doubt that the organism described by Massart was not *Achromatium* but *Hillhousia palustris*. The remarks on the structure of the protoplast and the inclusions are in agreement with our observations on

[1] W. Schewiakoff: Ueber einen neuen bakterienähnlichen Organismus des Süsswassers, Heidelberg, 1893.

[2] Jean Massart: Recherches sur les organismes inférieurs. Sur le protoplasme des Schizophytes. Section C. Schizomycètes, *b*. Thiobactéries, pp. 259–60, Plate I, fig. 7. Recueil de l'Inst. Botanique, Univ de Bruxelles, tome v, 1901.

that organism, except that the large globules consist of a salt of calcium, viz. calcium carbonate.

An organism has also been described and figured by Virieux[1] under the name of *Achromatium oxaliferum*. He states that 'Schewiakoff y décrit un corps central à mailles plus larges que dans la couche périphérique : pas plus que West, je n'ai pu faire cette distinction'. He does not find that the large globules in the spaces of the reticulum are of calcium oxalate. He expresses doubt as to the composition of the refringent granules in the threads of the reticulum, but is inclined to think that 'ils sont très probablement constitués par du soufre'.

There is not the least doubt that the organism described by Virieux is *Hillhousia mirabilis*, and not *Achromatium oxaliferum*. His Fig. 1 shows the refringent granules of sulphur in the threads of the reticulum as seen after the removal of the large globules of calcium carbonate (consult Fig. 2 on Pl. IX). His Fig. 2 shows the minute granules in the threads of the reticulum after removal of both sulphur and calcium carbonate.

It appears, therefore, that neither Massart's nor Virieux's observations relate to *Achromatium*. That organism has several features in common with *Hillhousia*, the chief of which are the large inclusions of a salt of calcium and the smaller reddish grains in the threads of the reticulum. In *Achromatium*, however, the globules are asserted to be calcium oxalate and the reddish grains of the nature of chromatin. In *Hillhousia* the globules are certainly calcium carbonate (and not calcium oxalate), and the reddish grains consist of sulphur, similar to the sulphur grains in other sulphur Bacteria. The most profound difference is to be observed in the structure of the protoplast, which in *Hillhousia* consists of a uniform reticulum, whereas in *Achromatium* there is a differentiated peripheral zone with a much smaller reticulum.

A copy of one of Schewiakoff's figures of *Achromatium* is given (Fig. 15) for comparison with *Hillhousia mirabilis*.

## Summary.

The following is a summary of the genus and its two known species:

*Hillhousia*, West and Griffiths, 1909. A genus of Bacteria of relatively large size, with shortly cylindrical cells from two to three times as long as their diameter, extremities hemispherical. Protoplast consisting of a slender network with meshes of fairly regular size. Within each mesh is included a large amorphous globule of calcium carbonate, and numerous smaller grains of sulphur are located in the threads of the network in such a manner that they occupy the interstices between the globules of calcium carbonate. There is no differentiation of any nuclear body from the

[1] Virieux, J.: Sur l'*Achromatium oxaliferum*, Schewiakoff. Comptes Rendus, t. cliv, 1912.

remainder of the protoplasm. The protoplast contains phosphorus, and the nucleo-protein is diffused throughout the reticulum in small granules. These granules are probably a form of chromatin which has but small affinity for nuclear stains.

| | | *Known localities in British Isles.*[1] | *Habitat.* |
|---|---|---|---|
| *H. mirabilis*, West and Griffiths, 1909. | Length 42–86 μ.<br>Breadth 20–33 μ. | Stanklin Pool, Worcs.<br>Gt. Barr Park, Staffs.<br>Studley, Warwickshire.<br>King's Norton, Warwickshire.<br>Near Belfast, Ireland.<br>Near Edinburgh, Scotland. | In all cases in the mud of a freshwater pool. |
| *H. palustris*, sp. n. | From $\frac{1}{4}$ to $\frac{1}{2}$ the size of *H. mirabilis*.<br>Length 14–36 μ.<br>Breadth 11·5–18 μ.<br>During division the halves of the cell are much more rounded and the constriction more open. | Near Dewsbury, W. Yorks.<br>Near Bowness, Westmorland.<br>Cannock Chase, Staffs.<br>Fair Head, Antrim, Ireland.<br>Clare Island and near Westport, Mayo, Ireland. | In *Sphagnum* bogs. |

A point of considerable interest is the comparison of the results obtained by the wet-staining and the dry-staining methods. It is possible to obtain a correct idea of the cytological structure only by never allowing the organisms to become dry. Dry-stained specimens all show some kind of concentration of the protoplast, of very variable form, which is purely an artifact. Drawings of such specimens reduced by photography to a size approximately the same as those usually published of much smaller Bacteria show apparent nuclear masses very similar to those asserted to be found in the smaller species. In the case of *Hillhousia* these 'nuclear' masses are certainly artifacts due to the superposition of the protoplasmic threads on drying.

It is possible that similar effects may be produced when smaller Bacteria are prepared by the dry-staining method, and consequently some doubt must be thrown on any conclusions regarding the cytology of specimens prepared in that way.

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## DESCRIPTION OF PLATE IX.

Illustrating the paper by Professor West and Mr. Griffiths on the genus *Hillhousia*.

Fig. 1. Normal aspect of living specimen of *Hillhousia mirabilis*. × 500. The dark globules consist of calcium carbonate.

Fig. 2. Individual from which the calcium carbonate has been removed by dilute acetic acid. × 850. The protoplasmic reticulum is somewhat swollen and distorted, and the conspicuous granules are minute grains of sulphur.

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[1] We have also observed *H. palustris* in material collected by Professor H. H. W. Pearson from the sides of a spring at Henkriesfontein, in Little Namaqualand, S. Africa.

Fig. 3. Rhombic crystals of calcite obtained by allowing the organisms to dry and then re-irrigating with distilled water. × 850. The apparent triangular face on the side of the crystal away from the observer is an illusion due to refraction.

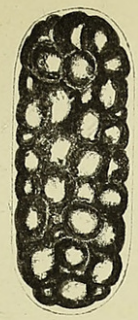
Figs. 4 and 5. Two specimens stained with safranin after fixation by drying (the usual dry-staining method for Bacteria). × 850. The central concentration of the protoplast is in each case an artifact.

Fig. 6. An individual stained with safranin by the wet-staining method after fixation in absolute alcohol and acetic acid. × 850.

Fig. 7. Two sulphur crystals obtained from solution in dilute acetic acid. × 1,200.

Figs. 8–15. *Hillhousia palustris.* × 850. 11–14, different examples showing stages of division; 15, cell immediately after division. The protoplasmic reticulum is only shown in Figs. 8–10 and 15, and both the calcium carbonate and sulphur have been removed.

Fig. 16. *Achromatium oxaliferum.* × 2,200. This figure is copied from one given by Schewiakoff (t. ii, f. 3 in his work) and shows the nature of the protoplasmic reticulum.



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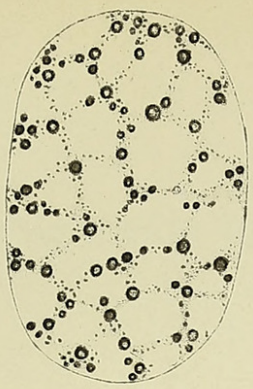

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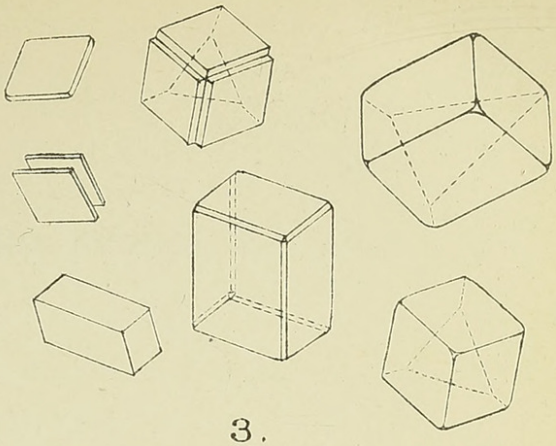

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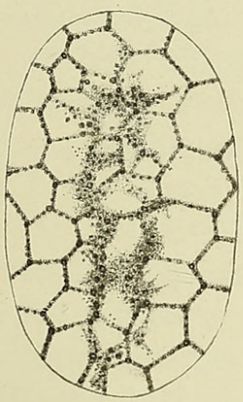

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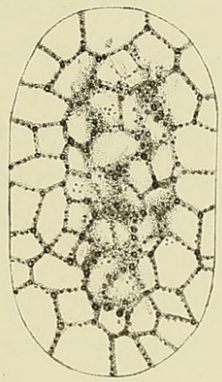

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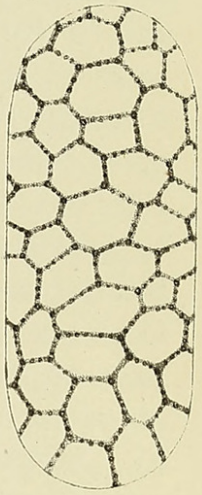

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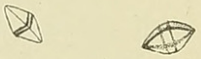

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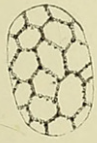

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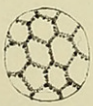

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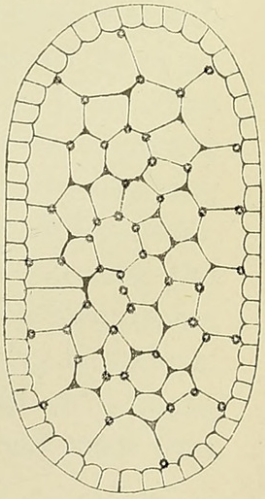

16.

G.S.West del.

Huth lith. et imp.

WEST & GRIFFITHS—HILLHOUSIA.