INTRACELLULAR DEPOSITION OF SULFUR BY *SPHAEROTILUS NATANS*

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During the course of studies of the bacterial and algal flora of creek waters we were puzzled by the absence of any representatives of the genus *Thiothrix* from samples in which there was a regular and abundant growth of *Beggiatoa* species. Associated with Beggiatoa in many of these samples was the colorless sheathed organism *Sphaerotilus natans*. In view of the very gross similarity of Sphaerotilus morphologically to the recorded descriptions of Thiothrix, it was decided to test the ability of the organism to oxidize hydrogen sulfide in a manner similar to that recorded for the species of the genera *Beggiatoa*, *Thiothrix* and other filamentous sulfur bacteria.

MATERIALS AND METHODS

The strain of *Sphaerotilus natans* used was isolated from creek water on an agar medium containing 0.1 per cent peptone (Difco) in tap water.

In testing the ability of the organism to oxidize H_2S, it was grown in screw capped bottles containing one liter of 0.1 per cent peptone in tap water at pH 7.0, with an air space of *ca* 250 ml between the liquid surface and the cap. Cultures were incubated at 28 C for 3 to 6 days to permit a pellicle to develop. They were then exposed to H_2S by displacing the air in the bottles with an equal quantity of H_2S for a Kipps apparatus and then replacing the cap. Normally four cultures were exposed to H_2S and four held as controls. In addition, four flasks of uninoculated medium were also exposed to H_2S and subsequently treated in the same manner as the cultures.

The estimation of the material deposited intracellularly was made as follows. Cultures were examined microscopically until there appeared a large accumulation of material in the form of globules in the cell at which time there were no comparable globules visible in the medium external to the cells.

In the first experiment the contents of each set of flasks were filtered through weighed Whatman No. 541 filter papers and the latter subsequently washed free of H_2S with 500 ml of the sterile medium. The papers were dried at 80 C, weighed, and then extracted with redistilled CCl_4 in a Soxhlet apparatus. The solvent was cycled several times during a period of 2 to 3 hr. Preliminary tests had shown that CCl_4 readily removed the globules from the dried material and, provided sufficient material was used, sulfur could be crystallized from the extract.

Microscopical examination of the extracted cells showed that all globules had been removed.

The CCl_4 extracts were evaporated to dryness over a steam-bath and the residues oxidized with bromine in potassium bromide followed by concentrated HNO_3 according to the method of Allen and Bishof (Scott and Furman, 1939). Ten ml of concentrated HNO_3 were added to each flask and left at room temperature for 15 min. The flasks were then warmed over a steam-bath in a fume cupboard until all bromine was removed and then partially immersed in the steam-bath and evaporated to dryness. Ten ml of concentrated HCl were added and each flask evaporated to dryness again. The residue of each sample was dissolved in 1 ml of concentrated HCl and *ca* 25 ml of glass distilled water. Any insoluble material was filtered off. Five ml of 1 per cent $BaCl_2$ were added dropwise with shaking to each filtrate. The precipitated $BaSO_4$ was subsequently retained on Whatman No. 42 ashless filter papers, washed, dried, ashed at 800 C and weighed.

In a second experiment, in order to remove any possibility that results may be due to sulfides retained in the filaments, the pellicles were separated from the culture fluid by siphoning off the latter leaving the pellicles adhering to the glass. These were resuspended in 4 N HCl and boiled to remove any sulfide and then subsequently washed with distilled water. This treatment did not remove the globules as shown by microscopical examination. The treated cells were then dried and extracted with CCl_4. Further treatment followed procedures outlined above.

The medium + H_2S control in this experiment was treated as follows. The 4 L of medium exposed

to H_2S were collectively filtered through a 6 cm ashless filter paper. The suction dried paper was then boiled in 4 N HCl and the whole refiltered through a second filter paper, washed, dried and extracted.

Microscopical examination and photomicrographic technique. Microscopical examination was made mostly on material mounted under coverslips in the culture medium. For photomicrography, slides were first immersed in a 2 per cent clarified water agar and drained. Slides were stored in a humid chamber prior to use. In use, agar was removed from one side of the slide, and the specimen mounted in about the middle of the medium on the other side and covered with a coverslip. Excess fluid was expressed from beneath the coverslip by slight pressure with a piece of filter paper over the coverslip. Such preparations, sealed with a fingernail varnish, would keep for quite long periods. The thin film of agar acts as an adhesive and will draw excess water away giving a relatively flat specimen for photographic purposes. It also aided considerably in subsequent extraction procedures.

Both visual and photographic work was done almost exclusively with the Leitz Dialux microscope using the Leitz Fluorite 114/1.32 oil immersion objective for light-ground purpose and the Leitz Pv 10/0.25 with oil cap and Leitz Pv Apo Oil 90/1.15 coupled with the Heine condenser for phase contrast. Photomicrographs were made with a Leitz Periplan OK ×10 eyepiece on Perutz "Pergrano" and Ilford "MicroNeg Pan" using a green filter. Exposure times of 4 sec for light field and 20 sec for phase contrast (Zernike) and dark-field were used.

Comparative studies were made with crude cultures of Beggiatoa.

RESULTS

Microscopical and cultural observations on the organism. Morphological and cultural characteristics of our strain fit very closely those outlined by Stokes (1954). There was, however, no difficulty in distinguishing individual cell formation in young filaments on slides which had been partially immersed in the growth medium. In such cultures holdfasts became visible under phase contrast when filaments were a few cells long (figure 1). Although a sheath was difficult to resolve at this stage the spirally coiled flagellum shed by the organism was frequently visible projecting from the margin of the holdfast. It is interesting to note that in the feathery colonies grown on 0.1 per cent peptone agar the sheath could be clearly demonstrated in filaments which extended well out beyond the main growth but appeared to have dissolved in the more dense portions of the colony. Flagella were frequently visible within the sheath. The "flagellum" of a free cell, particularly after exposure to H_2S, resolved itself into its several components which were clearly visible under phase contrast. The active movement of flagella of completely immobilized cells has also been observed.

There was also a tendency for cells to arrange themselves in rosettes similar in some respects to those described by Harold and Stanier (1955) for Leucothrix (figure 2).

With the exception of granules occurring along the longitudinal axis and appearing black under phase contrast, intracellular inclusions were noticeably absent from young cells grown in 0.1 per cent peptone. Older cultures grown under normal "aerobic" conditions were frequently full of fat as recorded by Stokes (1954). These fat globules stained intensely with Sudan Black B. In unstained preparations examined by light field they appeared slightly yellowish. In phase contrast (Zernike) they appeared a dull white. They showed none of the characteristics attributed to sulfur globules.

The prominent granules occurring along the longitudinal axis and appearing black under phase contrast were most frequently four in number in mature cells. Their nature has not been determined but there was some evidence to indicate that they frequently become paired. The smaller cells appearing immediately after division frequently had only two (figure 2). They seemed to play a part in the development of the sulfur globules.

The synonomy of the genera *Sphaerotilus* and *Leptothrix*, studied by Pringsheim (1949), was quite easily demonstrated with the strain employed.

Development of sulfur globules. The first clear evidence of the accumulation of material in the cells exposed to H_2S was observed approximately 45 min after exposure. When the process was followed under phase contrast, the first change which became evident was a marked accentuation of a halo which appeared around the black granules located along the longitudinal axis of the cell. This was followed by the appearance of a bright bluish globule, usually to one side of and adjacent to a black granule. As the globules enlarged they ap-

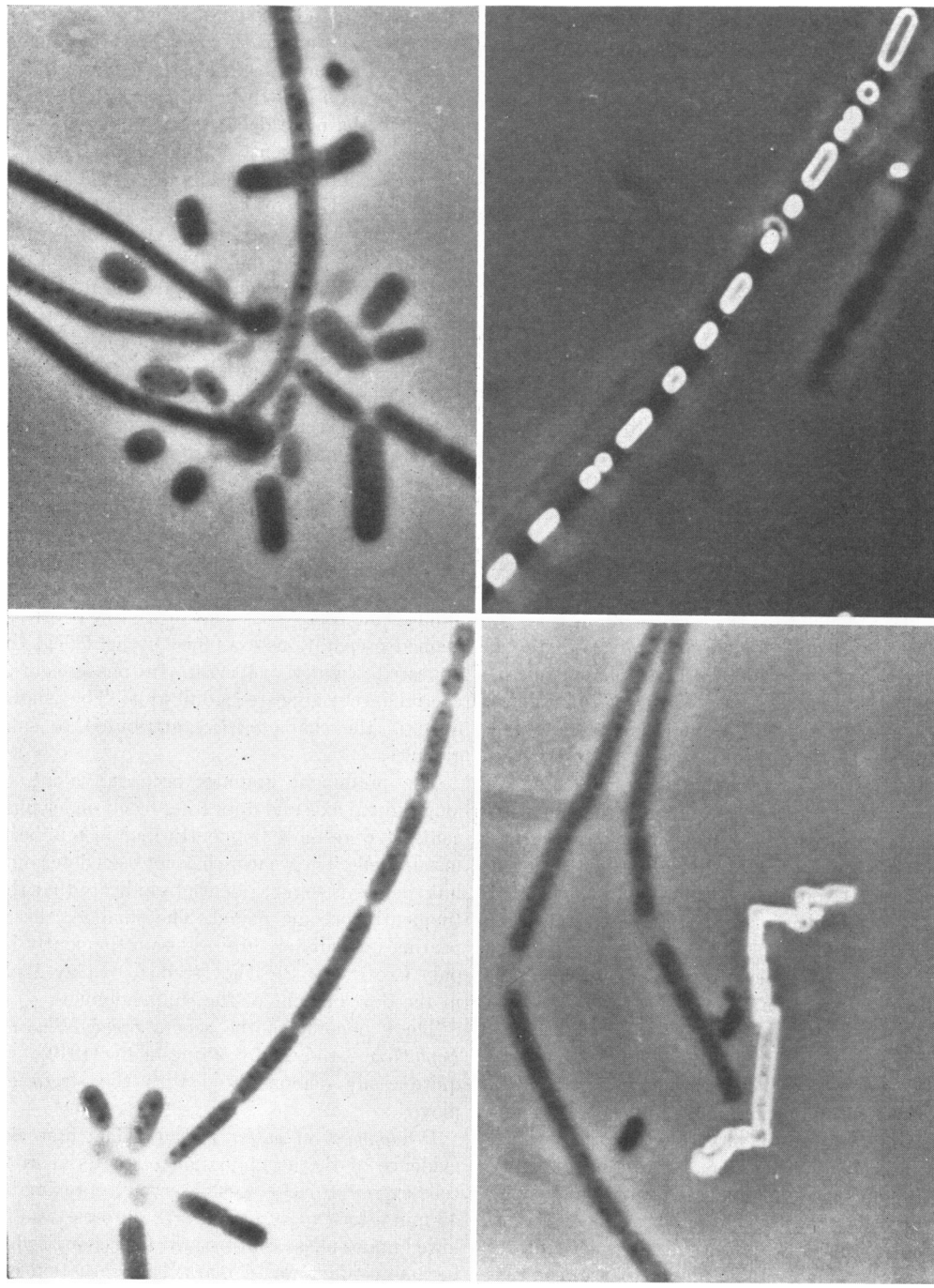
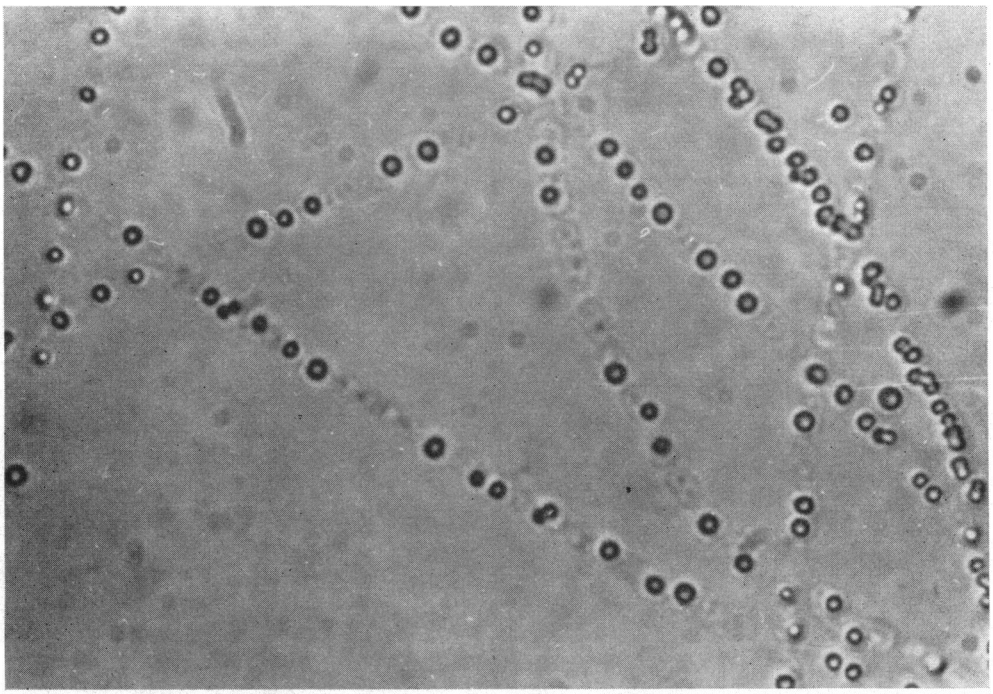


Figure 1 (upper left). Hold-fasts of *Sphaerotilus natans*. Phase contrast 900× (enlarged 4×).

Figure 2 (lower left). Microcolony of *Sphaerotilus natans* on a glass slide. Phase contrast 900× (enlarged 4×).

Figure 3 (upper right). Cells of *Sphaerotilus natans* heavily engorged with sulfur. Phase contrast 900× (enlarged 4×).

Figure 4 (lower right). Bizarre crystal mass formed in the pellicle of *Sphaerotilus natans*. After prolonged exposure to H_2S. Phase contrast 900× (enlarged 4×).



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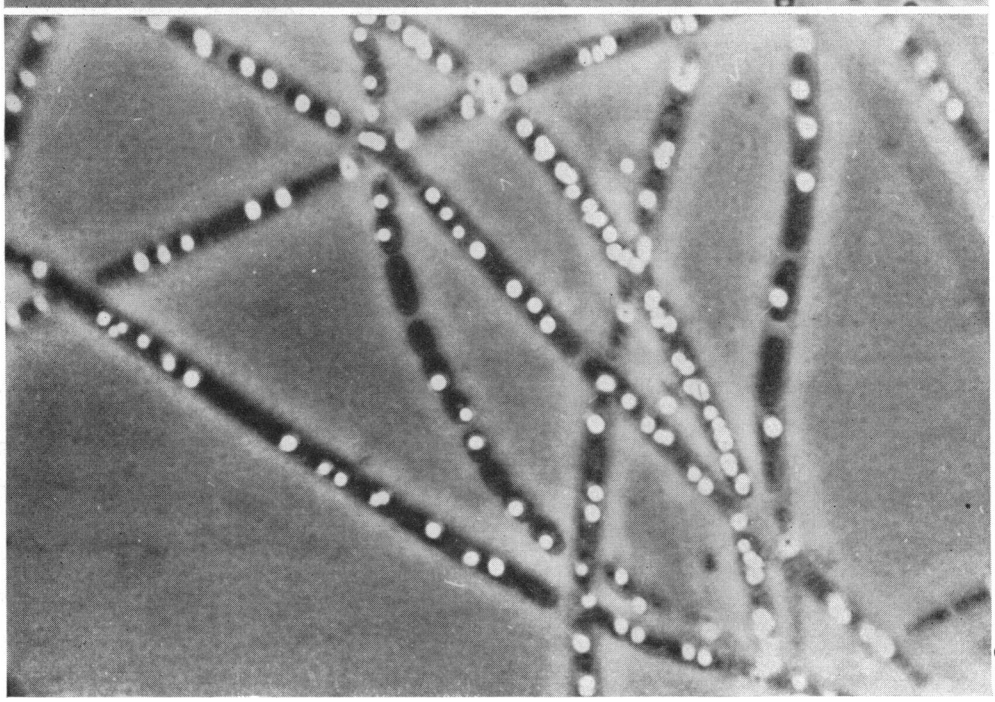

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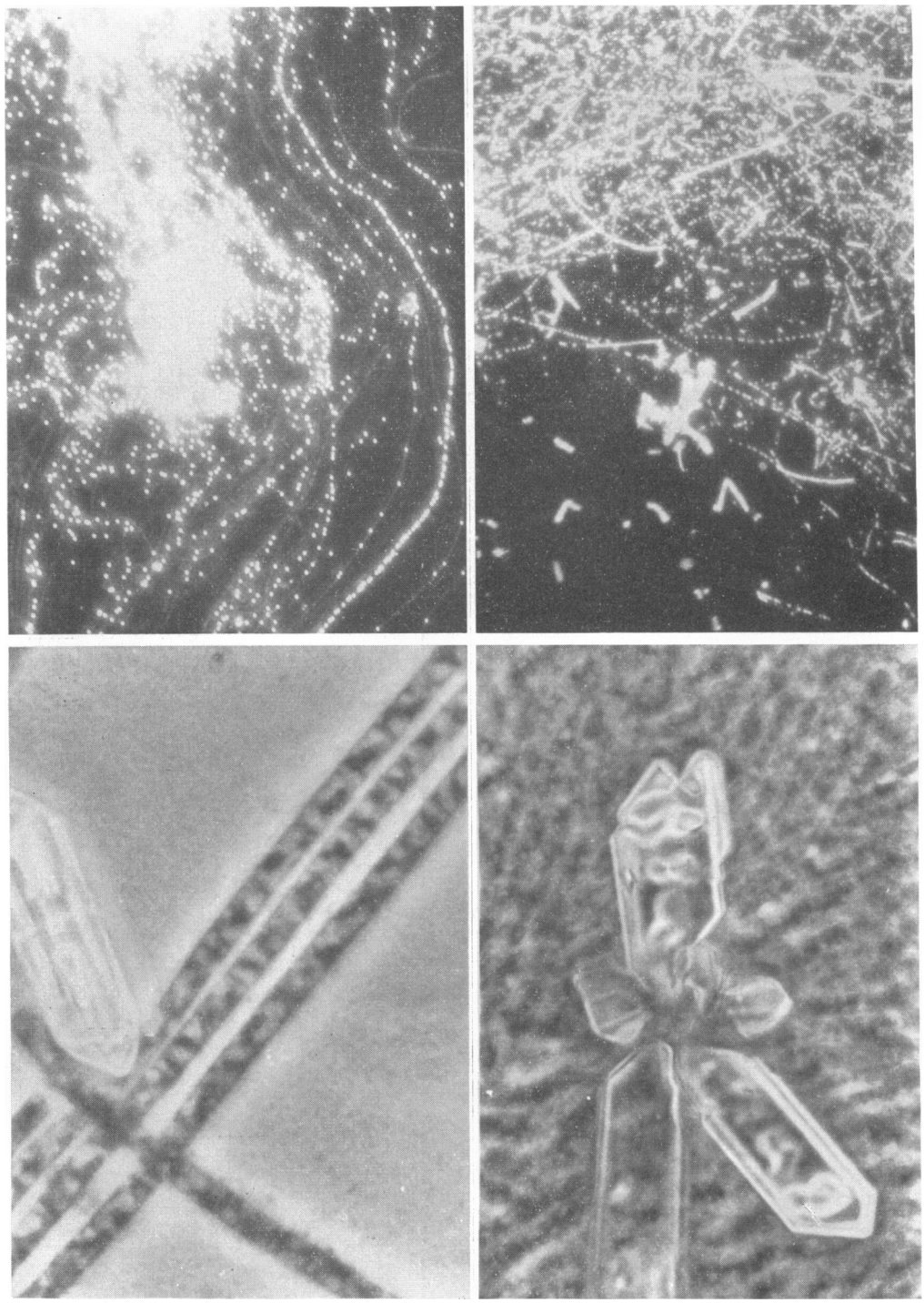
Figures 5–6. Light-field and phase contrast photomicrographs of the same field of *Sphaerotilus natans* 2 hr after exposure to H_2S. Light-field 1140×. Phase contrast 900× (enlarged 4×).



Figures 7–8 (upper left and right). Dark-field appearance of pellicle of *Sphaerotilus natans* 2 hr (7) and 3 days (8) after exposure to H_2S. 100×. (Enlarged 4×).

Figure 9 (lower left). Filaments of *Beggiatoa* species extracted with pyridine. Note vacuoles in extracted cells and sulfur crystal. Phase contrast 900× (enlarged 4×).

Figure 10 (lower right). Cluster of sulfur crystals formed during pyridine extraction of *Beggiatoa* species. Phase contrast 900× (enlarged 2×).

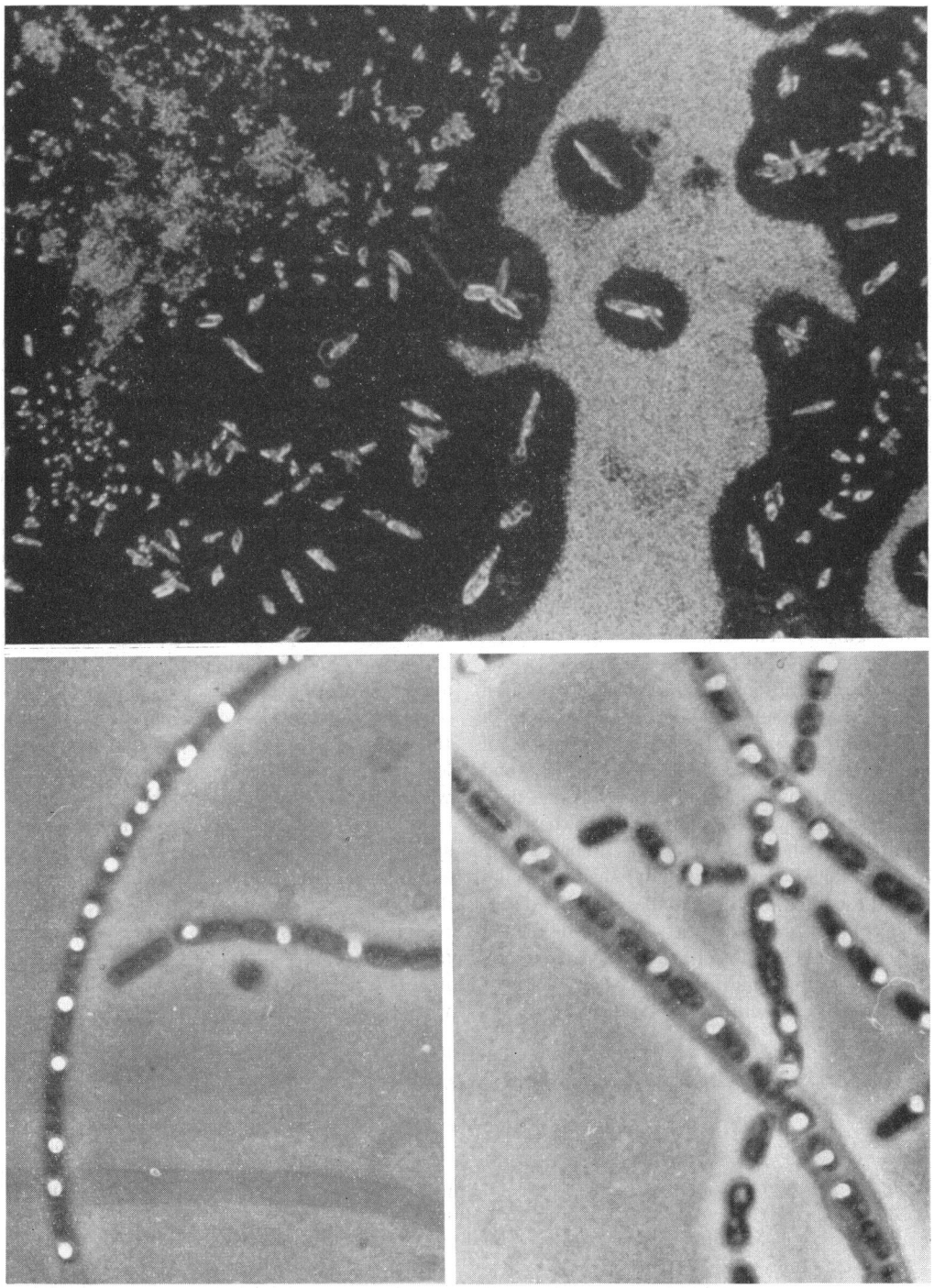


Figure 11 (upper). Crystal formation during extraction of a mass of *Beggiatoa* species with pyridine. The white band across the picture is a mass of unextracted cells. Darkfield 100× (enlarged 4×).

Figures 12–13 (lower left and right). Stages in the development of sulfur globules in *Sphaerotilus natans*. Phase contrast 900× (enlarged 4×).

peared a gleaming white and when fully developed exhibited a double ring effect similar to the large sulfur globules in Beggiatoa. The light emitted by the globules in dark phase contrast and dark field (figure 7) was brilliant (figures 3 and 6).

In the light field the globules showed the typical optical properties of sulfur globules of Beggiatoa (figure 5).

In the early stages of exposure to H_2S, frequently only one globule appeared in each cell and some cells did not produce any (figures 12 and 13). As the length of the time of exposure was increased some cells developed several globules (figure 5). In others a single globule appeared to have enlarged and completely obscured the cell (figure 3). The presence of cells under the latter condition could only be demonstrated by extraction methods which leave only the cells visible with large vacuoles and cytoplasm compressed in one or both ends.

Preparations examined at this stage under low power dark field exhibited dense masses of sulfur at isolated spots in the pellicle surrounded by long chains of bright spots representing the free filaments (figure 7). The dense masses are the focal points of development of floating colonies.

In older preparations the entire sheath appeared to be filled with sulfur, sometimes in a state of semicrystallization. At this stage aggregates of sulfur crystals appeared externally (figures 4 and 8). Further study is necessary to determine their origin. Preliminary observations suggest that they originate from the cells themselves. When young cells which had developed only small globules were exposed to air the globules rapidly disappeared. Where globules had developed to the optical exclusion of the cell, they appeared to remain on exposure to air or to slowly leave the cells on prolonged exposure and crystallize.

Conditions governing the development of globules of sulfur have not been critically studied. When slides were partially immersed in the culture fluid during incubation, growth appeared over the whole of the immersed portion of the slide but was concentrated at the liquid-air junction. Exposure of such cultures, undisturbed, to H_2S resulted in a rapid intracellular deposition of sulfur in the surface filaments. Appearance of globules in submerged filaments was markedly delayed. This may be due to slow penetration of H_2S or lack of sufficient oxygen.

Extraction of the globules. Attempts to extract the globules with CS_2 and CCl_4 in partially dried mounts proved unsuccessful due, no doubt, to the immiscibility of these solvents with water. Extraction with hot alcohol was slow but could be followed microscopically. The globules dissolved leaving a clear area in the cytoplasm. Owing to the presence of water, the extracted material appeared in globular form in the water as the alcohol evaporated at the edge of the coverslip.

The most successful procedure involved the use of pyridine. If specimens of Sphaerotilus or Beggiatoa were mounted in water under a coverslip and pyridine drawn under by placing a drop on one side and a strip of filter paper on the other, the globules of sulfur could be seen to disappear leaving a vacuole behind except when globules were initially very small (figure 9). If a film of clear water agar was first laid on the slide, the specimen placed on it and covered with a coverslip lightly pressed down, the transit of the pyridine was considerably delayed. Most of the residual water was taken up by the agar and crystallization of the sulfur occurred very soon after it was extracted from the cells. Cells were left vacuolated after extraction. The process is most

TABLE 1

Yields of $BaSO_4$ following oxidation of CCl_4 extracts of Sphaerotilus natans exposed to H_2S

Experiment	Treatment	Cells Washed with	Dry Wt of Cells	Yield of $BaSO_4$
			g	g
1	Cells alone	Medium	0.166	0.001
	Cells + H_2S	Medium	0.198	0.015
	Medium + H_2S	Medium	0.0001	0.001
2	Cells alone	4N HCl	Not recorded	0.0000
	Cells + H_2S	4N HCl	Not recorded	0.0025
	Cells + H_2S	4N HCl	Not recorded	0.0025
	Cells + H_2S	4N HCl	Not recorded	0.0025
	Medium + H_2S	4N HCl	Not recorded	0.0000

striking when viewed under low power dark field (figures 9–11). Illustrations are of Beggiatoa. Similar pictures were obtained with Sphaerotilus.

Extraction and precipitation as barium sulfate. The results of two experiments are shown in table 1. In the one in which the pellicles were washed with sterile medium, filtration through the slimy mass was difficult and not very thorough. Despite this, there were very significant differences between the exposed and unexposed cells and the exposed medium. No importance can be attached to the actual yields of $BaSO_4$ in relation to the dry weight of cells exposed. It was quite impossible to regulate the degree of exposure to obtain reproducible results.

In the experiment in which cells were exposed and subsequently boiled in 4 N HCl the differences between exposed cells and controls are quite clear.

DISCUSSION

We have been unable to locate any previous reference to intracellular deposition of sulfur by *Sphaerotilus natans*. Lack of mention of it in four recent publications, Pringsheim (1949), Stokes (1954), Harold and Stanier (1955) and Höhnl (1955) would suggest that it has not been reported. The present conclusion that the globules referred to in this paper are, in fact, sulfur is based on the following.

1. The globules are optically identical with those present in Beggiatoa.
2. They do not stain with Sudan Black B.
3. They are produced only when cells are exposed to H_2S.
4. They can be extracted under the right conditions with solvents which are known to dissolve sulfur.
5. They are insoluble in water and dilute mineral acids.
6. They can be extracted and recrystallized with ease using carbon tetrachloride on dry cells and pyridine on wet cells and subsequently yield crystals which resemble in all respects those obtained by dissolving and recrystallizing sulfur with these solvents.
7. On complete oxidation of the extracted globules, a precipitate is obtained on addition of $BaCl_2$ to a solution of the oxidized material in HCl.

No evidence has as yet been obtained concerning the possible function of this oxidation. Winogradsky (1888) noted that when filaments of Beggiatoa and Thiothrix were exposed to H_2S, the cells became completely filled in periods as short as ten min. Yet the generation time cited for Thiothrix is 24 hr. Either the energy liberated by the oxidation is rapidly converted to a form in which it can be stored or it is lost as heat. In the case of Sphaerotilus the accumulation occurs without any associated cell growth. It is extremely doubtful whether cells which have become engorged with sulfur are still viable. The possibility that the cells contain a mechanism for the rapid disposal of toxic H_2S by oxidizing it to sulfur must be seriously considered. No attempt has yet been made to determine whether any further oxidation to sulfate can take place. Owing to the ease with which Sphaerotilus can be cultured, it should be a useful tool for research into the oxidation process.

Evidence obtained on the actual conditions necessary for the oxidation to occur are as yet too fragmentary to warrant comment.

The possible relationship between the genera Sphaerotilus and Thiothrix is interesting. The authors have not encountered any identifiable specimens of *Thiothrix*. Winogradsky (1888) in his description of the genus states "*Thiothrix* is much more closely related to *Cladothrix* and the so-called *Leptothrix ochracea* than to Beggiatoa. It differs sharply from the *Leptothrix ochracea* because of its content of sulfur granules, the much weaker build of the sheath, the way the rods are built and divided, the peculiar movement of the rods as well as many other morphological characteristics in addition to *deep physiological* differences."

Fourment (1926) in his brief description of the cytology of Thiothrix states that "we have not found in the cells of *Thiothrix* any metachromatic granules nor nuclei nor central bodies resembling those of Cyanophyceae. The protoplasm of all elements presents big vacuoles which are occupied by very big spherules of sulfur which are placed in direct contact with siderophile granules. The very rudimentary organization of these organisms resembling more ordinary bacteria does not allow approach to the Cyanophyceae."

The authors are under the impression that Thiothrix forms a true trichome. If this is the case then the morphological resemblance of Thiothrix and Sphaerotilus is merely superficial. The evidence presented indicates that Winogradsky erred

with respect to the sulfur deposition. Many features of his description of Thiothrix, e. g., the isolation of rods, formation of the holdfast, the erect position of the rod when holdfasts are formed, are also features of Sphaerotilus. But here, apart from the similar deposition of sulfur, the resemblance would seem to end. Free cells of Sphaerotilus, when motile, are quite actively so, even after exposure to H_2S, and are clearly flagellated.

Fourment's (1926) descriptions and illustrations more nearly resemble those of *Sphaerotilus natans*. His mention of granules which appear to play a part in sulfur deposition is in close accord with the authors' limited observations. It is possible Fourment was observing the same organism.

It should be noted that the rosette-like colonies frequently formed by *Sphaerotilus natans* on glass slides, when exposed to H_2S, present a microscopic picture which is extraordinarily like that illustrated by Harold and Stanier (1955) for Thiothrix.

ACKNOWLEDGMENTS

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SUMMARY

Sphaerotilus natans, when exposed to H_2S, deposits intracellularly globules which have optical and chemical properties identifiable with sulfur.

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