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Physiological and Morphological Observations on *Thiovulum* sp.†

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Cell suspensions of *Thiovulum* sp., collected from enrichment cultures, were grown, maintained, and harvested for periods up to 7 months. In open-flow cultures run with aerated seawater, a continuous supply of hydrogen sulfide was provided by diffusion through a semipermeable membrane from either a live culture of *Desulfovibrio estuarii*, neutralized sodium sulfide, or a N_2-H_2S gas mixture. Attempts to grow *Thiovulum* in pure culture failed despite variation in concentrations of dissolved oxygen and hydrogen sulfide in stratified as well as in completely mixed systems. Uptake of $^{14}CO_2$ and some organic compounds by purified cell suspensions was measured, and values were corrected for the activity of heterotrophic as well as autotrophic contaminants as determined in control experiments. Cell populations exhibited maximum uptake activities during formation of the characteristic veils. Substantial uptake of CO_2 in air-saturated seawater was coincident with an optimal concentration of hydrogen sulfide of about 1 mM. Glutamate and a selection of vitamins (B_{12}, biotin, and thiamine) did not significantly affect the uptake of CO_2. No substantial uptake of carbon from acetate, glutamate, mannitol, and Casamino Acids was found. Within the range of error indicated, the data are consistent with acceptance of a chemolithotrophic nature for *Thiovulum*.

Of all chemolithotrophic organisms, the aerobic sulfur-oxidizing bacteria represent the most diverse group. Apart from the well-studied thiobacilli, there are a number of conspicuous genera of colorless sulfur bacteria that have never been cultivated, although they have been known for a long time for their relatively large size, striking morphological features, and common occurrence in sulfur springs, salt marshes, etc. Their specific requirements—different from those of thiobacilli—for sulfide as well as free oxygen imply growth in a chemically unstable environment and, in principle, account for the difficulties of mass cultivation. They seem to enrich best in organic-rich media, and, with the exception of the thermophilic *Sulfolobus* (2), the truly chemolithotrophic nature of these organisms has never been proven.

Apart from general microbiological interest, the motivation for our renewed attempt at studying growth and basic physiological properties of *Thiovulum* was the need for information on the particular biochemical processes facilitating the successful competition for hydrogen sulfide as an electron donor in the presence of chemical autooxidation. Similar studies with other genera of this group of organisms, such as *Thiospira*, *Thiophysa*, and *Achromatium*, are even more difficult at this time because no suitable enrichment procedures are known.

In 1786, Müller (14) described *Volvox punctum* as a colorless, egg-shaped organism 10 to 25 μm in diameter. He mistook the sulfur inclusions for small daughter cell assemblages characteristic of *Volvox*. Warming (22) recognized the nature of this organism as a sulfur bacterium and named it *Monas mülleri* and later, according to Lauterborn (11), *Thiovulum mülleri*. In 1913, Hinze (7) did a thorough study of this genus and described two more species, *T. majus* and *T. minus*, which were included in Bavendam's (1) monograph on the colorless and purple sulfur bacteria. Whereas Hinze (7) observed a peritrichous flagellation in silver-stained preparations, Metzner (13) deduced from the peculiar motility of *T. mülleri* the existence of an equatorial zone of cilia.

More recent work was initiated when van Niel (21) drew attention to these organisms, emphasizing their chemotactic and microaerophilic characteristics. His suggestions were followed up by LaRivière (9, 10), who devised a technique for obtaining purified cell suspensions from enrichments and transferring into sterile media. In

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1965, Starr and Skerman (20) reviewed recent literature, which is limited to a number of morphological observations. Faûré-Fremiet and Rouiller (5) and de Boer et al. (4) describe a thin (less than 50 nm) cell wall separated from a double-layered cytoplasmic membrane. The former authors discovered a polar body composed of fibrils about 100 nm wide and 3,000 nm long, arranged perpendicularly to the external wall. Hinze's (7) original observations of peritrichous flagellation were confirmed electron microscopically, but Metzner's (13) cilia were not found.

On rare occasions the characteristic *Thiovulum* veils visible to the naked eye can be observed in nature, more commonly in neglected aquaria, where they are draped over the surface of decaying organic matter. Enrichment cultures which provide basically the same conditions are easily obtained within days, dependent on the state of decay, hydrogen sulfide production, and optimal concentration of oxygen manifested by a slow flow of the overlaying seawater. An addition of sulfide-containing mud provides a rapid start of sulfate reduction and probably an inoculum of *Thiovulum*. Vigorously growing cells are arranged in visible veils of slimy material. They usually contain sulfur globules which disappear after the water becomes devoid of sulfide. Only Lauterborn (11) observed freshwater forms. This may simply reflect the more regular and widespread occurrence of sulfate reduction in marine than in freshwater environments.

That almost 200 years after its first observation and 100 years after its recognition as a sulfur bacterium the true physiological nature of *Thiovulum* is still uncertain must be explained by the absence of a pure culture technique. LaRivière (9) continued earlier unpublished work by van Niel, Wijler, and Lascelles (16) and described a simple enrichment technique using *Ulva* thalli as the source of organic matter. He was then able to purify cell suspensions obtained from enrichments and to transfer them into sterile media providing a sulfide-oxygen gradient. As LaRivière (9) states: "The success of the described method depends on the degree of contamination of the enrichment material and the capacity of the cells to withstand a sufficient number of puification steps."

In addition to obtaining pure cultures, cell growth has to be achieved to maintain the cultures for continued transfers. We have not been successful in obtaining and maintaining pure cultures of *Thiovulum*, and the reasons are most likely those stated in the passage quoted above. We have been able, however, to maintain enriched populations for prolonged periods of time. The present paper reports on maintenance techniques and some physiological studies with partially purified cell suspensions of *Thiovulum* sp.

MATERIALS AND METHODS

Enrichment. Wijler's enrichment technique, described by LaRivière (9) was used to obtain fresh cell suspensions of *Thiovulum* whenever needed. Open beakers or jars 1 to 5 liters in volume were half filled with *Ulva*, inoculated with black mud from a local marsh, and left in the dark. When decay of the packed-down plant material became apparent after a few days, a slow flow of fresh seawater was admitted into the vessel and permitted to overflow. *Thiovulum* veils developed in the area between the bottom layer and the rim of the enrichment jar and replenished quickly when harvested repeatedly for a period of a week to 10 days. An initial addition of neutralized Na_2S accelerated the appearance of *Thiovulum* veils and produced larger enrichment populations.

Purification of cell suspensions. The procedures of separating *Thiovulum* populations from protozoa and from bacterial and fungal contaminants were based either on chemotactic responses or on filtration methods. LaRivière (9) used a combination of both, by passing the collected cell suspension through a filter of cheesecloth and permitting the veil to re-establish in the filtrate. This could be repeated three to four times before the cells died; this coarse filtration removes particles larger than *Thiovulum* cells (protozoa, nematodes, slime aggregates) and the chemotactic response of the cells during veil formation is hoped to leave small contaminants behind. However, our success with this technique was limited, and we resorted to using only freshly formed veils free of large contaminants. This made the cheesecloth filtration unnecessary. For collecting, the visibility of fine *Thiovulum* veils was enhanced using a flashlight in a darkened room. The veils were harvested with a pipette connected by silicone tubing to a 100- to 250-ml suction flask. The sample of 20 to 50 ml was then poured into a large test tube, thereby adding more oxygen to the water and causing the cells to settle to the bottom. Decanted or removed by a pipette, the cells survived two or three more repetitions of resuspending and settling in sulfide-free and sterile seawater. When cell suspensions were transferred from enrichments into water deoxygenated by bubbling with nitrogen gas, they migrated near the surface and could be collected and resuspended in fresh sterile medium.

Membrane filters (Millipore) with a porosity of 8 μm were used for purifying cell suspensions by filtration. Samples taken from the enrichments were concentrated over the filter (2.5-cm diameter) to about 2 ml, washed with sterile seawater, and filtered again. This procedure was repeated three to five times.

By either of these three procedures, cell suspensions were freed of gross contamination, especially from bacteria attached to or associated with the slimy material which provides the matrix for the typical *Thiovulum* veils. The cell suspensions were then checked for motility and transferred either into prepared sulfide-oxygen gradient systems for population maintenance studies or used directly for the experiments

measuring the uptake of radiolabeled materials.

The degree of purity of *Thiovulum* cell suspensions was estimated by (i) plating aliquots on nutrient- (Difco 2216) and thiosulfate-seawater agar and (ii) measuring the uptake of radiolabeled substrates in controls filtered through membranes of 8-μm porosity.

LaRivière (9) attempted to grow purified suspensions of *Thiovulum* cells in a sulfide-oxygen gradient in a seawater-filled Erlenmeyer flask with a layer of sulfide-containing agar on the bottom and a constant stream of air bubbles near the surface. He was able to show that inoculated *Thiovulum* cells formed a veil in the midsection of the culture vessel. Initially, with LaRivière's participation, we continued these studies with the aim of achieving actual growth in such a system. Our results were not satisfactory, and we attempted a number of other approaches.

Long-term maintenance of purified cell suspensions was obtained in a dual Bellco Spinner vessel(s) separated by a nylon membrane of 0.45-μm porosity (see Fig. 1). Initially one of the jars was filled with decaying *Ulva* and inoculated with fresh sulfide-containing mud. The other jar was filled with membrane-filtered seawater. When sulfide started to penetrate the membrane and to appear in the seawater jar, *Thiovulum* cell suspensions were introduced. After the formation of a veil, a slow flow of sterile-filtered seawater was introduced in the center of the vessel providing a continuous supply of oxygen. The rigid structure of the veil prevented the cells from being washed out of the overflow. We later replaced the sulfide-producing, decaying algal matter by a pure culture of *Desulfovibrio aestuarii* periodically supplied with sterile sodium sulfate-lactate medium.

In another system, we avoided the addition of unknown organic solutes to the culture by supplying neutralized sodium sulfide from an immersed glass tube closed off by a dialysis membrane (see Fig. 2). The flow-through medium was membrane-filtered (0.45 μm) seawater. A suction flask provided a convenient overflow arrangement. After inoculation, veils formed readily. It turned out to be advantageous to keep the level of the neutralized sodium sulfide solution slightly above that of the seawater. This culture system was also used in an attempt to grow an inoculum of *Thiovulum* on sodium thiosulfate instead of sodium sulfide as a source of reduced sulfur.

To avoid the washout of cells in certain experiments, the medium was recirculated by metering pumps (see Fig. 3). With respect to the supply of oxygen (dropping vessel open to cotton-filtered air) and sulfide, the system is not closed. It was used for studying growth of *Thiovulum* in the absence of soluble organic materials, or for testing the utilization of labeled organic and inorganic substrates and the role of vitamins or chelating agents.

Another procedure to supply dissolved sulfide in low concentrations used submerged silicone capillaries filled with hydrogen sulfide gas. This approach was developed by K. Eimhjellen (Department of Biochemistry, Technical University of Norway, Trondheim) when working in our laboratory. A loop of capillary silicone tubing attached to glass tubing was coiled on the bottom of a jacketed culture vessel and connected to a slow flow of nitrogen gas containing 1% hydrogen sulfide (see Fig. 4). Oxygen was introduced by a flow of fresh seawater as above. This system was used with or without continuous overflow. It was applied either for maintenance of a *Thiovulum* veil or, providing a stirring bar in the center of a loop of silicone rubber tubing, in the attempt to grow *Thiovulum* in homogeneously mixed cell suspension.

Analytical procedures. For the measurements of CO_2 uptake, stock solutions of sodium carbonate (55.7 mCi of ^{14}C per mmol) were added to oxygenated artificial seawater which contained 200 μg of sodium bicarbonate per ml. The medium for experiments measuring the uptake of radiolabeled substrates was artificial seawater (Seven Seas Mix) supplemented with 100 mg of NH_4Cl and 10 mg of KH_2PO_4 per liter. The labeled compounds (L-[*U*-^{14}C]glutamic acid, 200 mCi/mmol; D-[*U*-^{14}C]mannitol, 22 mCi/mmol; [2-^{14}C]acetic acid, 52 mCi/mmol; uniformly ^{14}C-labeled amino acid mixture, 1.0 mCi/mg) were added to unlabeled carrier stock solutions to give a final concentration of 10 μg/ml in the test cultures.

The uptake experiments were conducted with 1-ml sample volumes, containing 3×10^5 to 5×10^6 cells for 2 to 18 h at 21 ± 1°C. Cells were counted microscopically in an aliquot of the sample in a Petroff-Hauser chamber at a magnification of ×120. The amount of radioactivity incorporated into cell material was determined after filtration through 0.45-μm membrane (Millipore) filters, triple washing with artificial seawater, and counting the filters in 10 ml of Bray solution in a scintillation spectrometer (Intertechnique SL-20). Quenching was corrected for by the channels ratio method. Uptake measurements were corrected for activity due to residual contamination by subtracting values obtained from a parallel sample incubated after filtration through a 8-μm membrane filter. Also, to account for radiolabeled substrate adsorbed to cells and not removed by washing, Formalin controls of both the cell suspension and the 8-μm filtrate were conducted. The actual uptake of purified cell suspensions was calculated according to: gross uptake minus (i) adsorption by Formalin-treated cells and (ii) uptake by the 8-μm filtrate from which a Formalin-treated 8-μm filtrate was subtracted.

In some experiments vitamins (biotin, B_{12}, and thiamine) were added in quantities of 20 μg/liter each. When the pH was measured in the vicinity of the veil, either combination electrodes were introduced directly or small samples were withdrawn. The latter was done for the quantitative determination of sulfide concentrations using F. Pachmayr's procedure (Ph.D. thesis, University of Munich, 1960).

Electron microscopy. For transmission electron microscopy, a clump of cells from a freshly collected veil suspended in 70 ml of seawater was prefixed by the addition of 5% glutaraldehyde in 1/7 M Veronal-acetate buffer at pH 7.0 for 45 min. It was then washed in buffer and fixed in 1% osmium tetroxide overnight at 4°C. The cells were then stained with 0.5% uranyl acetate for 30 min at room temperature, washed with distilled water, dehydrated through an ethanol series and embedded in Epon. Thin sections were poststained with both uranyl acetate and lead citrate and examined by a Philips 300 electron microscope. Scanning electron microscopy was done with air- and crit-

ical point-dried and palladium-coated specimens in a Jeolco model U-3 microscope.

RESULTS

Enrichment and maintenance. Freshly developed veils of *Thiovulum* sp. were more or less rigid and contained the highest concentration of motile cells consisting of up to 20% of dividing stages. Two ranges of cell size were observed, 9 to 10 μm and 15 to 18 μm (mean diameter), which might relate to Hinze's (7) description of the two species *T. majus* and *T. minus*. The two types occurred in veil populations either as a mixture or in separate enrichment cultures. Veils lasted usually between 4 and 8 days. If harvested frequently, the production of cells increased considerably. If an established veil changed its location due to a change in the flow of water, the rigid slime matrix stayed behind and shrank within a short time to a thin thread made visible by adhering sulfur particles.

Prior to the decay of a *Thiovulum* veil in an enrichment or maintenance culture, the brief occurrence of "swarming" cells can usually be observed. Although hardly distinguishable from cells collected from veils, they are somewhat smaller, contain less or no sulfur globules, show very few or no division stages, and occur in the typical "bioconvection" pattern described by Platt (17) in *Tetrahymena* and by Pfennig (16) in *Chromatium okenii*. This typical movement in vertical directions can hardly be related to oxygen-sulfide interfaces, and we assumed originally a succession of two physiologically different species. However, extended observations of a similar behavior in maintenance cultures and the frequent changes between veil-forming and swarming cells at certain intervals suggested two stages of the same organism. Depending on the change of H_2S production and the amount of organic matter released, the enrichment cultures were ultimately overgrown by types of thiobacilli and often by gelatinous masses reminiscent of the genus *Thiobacterium* (3).

Of all the attempts to maintain a freshly collected cell suspension over an indefinite period of time, the dual vessel arrangement (Fig. 1) brought the best results. With a growing culture of *D. estuarii* in one of the jars as a sulfide-generating system, veils could be maintained for as long as 220 days without reinoculation and at the same time frequently harvested. At irregular intervals of 2 to 4 weeks, the veils disintegrated and swarming cells occurred. To prevent washout of these nonattached cells, it turned out to be practical to disrupt the remaining veil structure mechanically while the flow of seawater was momentarily stopped. As a result, the veils

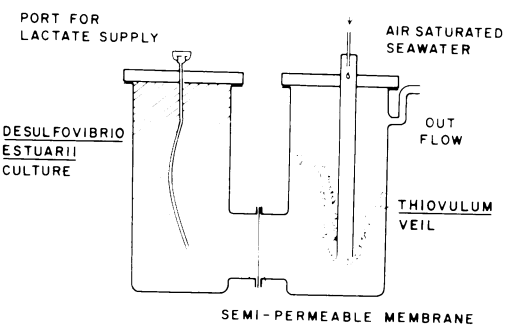

FIG. 1. *Double-culture vessel for maintenance of purified cell suspensions of Thiovulum sp. (modified from reference 8).*

re-established within periods of 5 to 10 min before the water flow could be continued. Harvesting of cells had a similar rejuvenating effect.

Within 4 to 5 weeks, a brownish layer of colloidal sulfur occurred on the bottom of the vessel beneath the *Thiovulum* veils. It was siphoned off periodically. Lactate medium was supplied to the *Desulfovibrio* cultures in intervals of 2 to 4 weeks. The supply of sulfate by diffusion through the membrane appeared sufficient.

Sulfide concentrations around the well-developed veils ranged from 0.03 to 1.40 mM and the pH ranged from 6.9 to 7.4. Neither the rate of H_2S supply nor the flow of oxygenated seawater was constant at any one location. In fact, the formation of the more or less rigid slime structure of the veils prevents this constancy, and this might explain why the periodic disruption of the structure appears to be a necessity for the prolonged maintenance of the *Thiovulum* population.

The maintenance culture technique described in Fig. 2 kept populations growing for about 3 to 4 months. The range of sulfide concentrations in the vicinity of the veils was 0.12 to 1.12 mM. The neutralized sodium sulfide solution was replaced every 2 to 3 weeks. The accumulation of colloidal sulfur was also apparent here. It is unclear at this stage whether or not the lack of an organic component is responsible for the shorter maintenance period as compared to the system which supplied sulfide by means of a living culture of sulfate-reducing bacteria or a decaying *Ulva*-mud mixture.

In the attempt to use thiosulfate as a source of reduced sulfur, it was added to the inflowing seawater at a concentration of 5 mM. Before inoculation with *Thiovulum* cells and start of the seawater flow, sodium thioglycolate was added to the culture vessel at a final concentration of 0.012% to lower the initial redox potential to the approximate value of the sodium sulfide

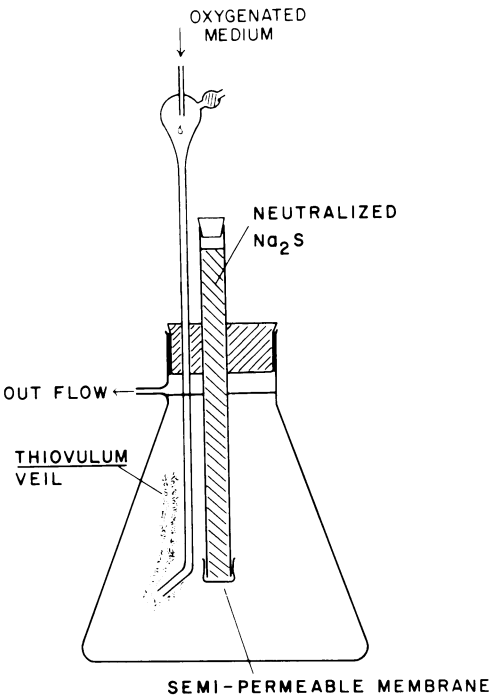

FIG. 2. *Flow-through culture vessel providing a slow supply of sodium sulfide for the maintenance of purified cell suspensions of Thiovulum sp.*

culture. After inoculation, veils established briefly before the cells settled to the bottom; these cells were no longer motile and were not revived in a sodium sulfide-providing system.

Equipped with a circulating medium flow (Fig. 3), this system has also been used successfully in maintaining freshly harvested *Thiovulum* populations. It is suitable for the study of swarming cells but has not yet been used extensively for this purpose.

The continuous culture set-up described in Fig. 4 permitted maintenance of a veil for a brief period only. Swarming cells were washed out quickly. Studies using various organic nutrients or growth factors such as *Ulva* extract, mud extract, acetate, glucose, and vitamins have not resulted in sustained or increased growth rates. The same experiments were done with complete mixing of the culture. Again cells washed out with the rate of dilution, i.e., no growth could be demonstrated.

Attempts have also been made to culture *Thiovulum* on solid and semisolid agar media supplemented with a variety of substrates and reduced sulfur compounds under aerobic to strictly anaerobic conditions. In soft agar (0.3%), the freshly inoculated cells formed a band near the surface (reduced oxygen concentration) but did not remain viable for longer than 24 h.

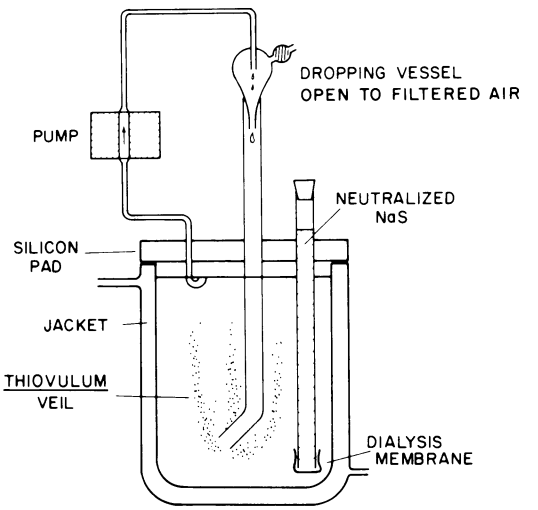

FIG. 3. *Flow-through, closed system culture vessel for maintenance of purified cell suspensions of Thiovulum sp.*

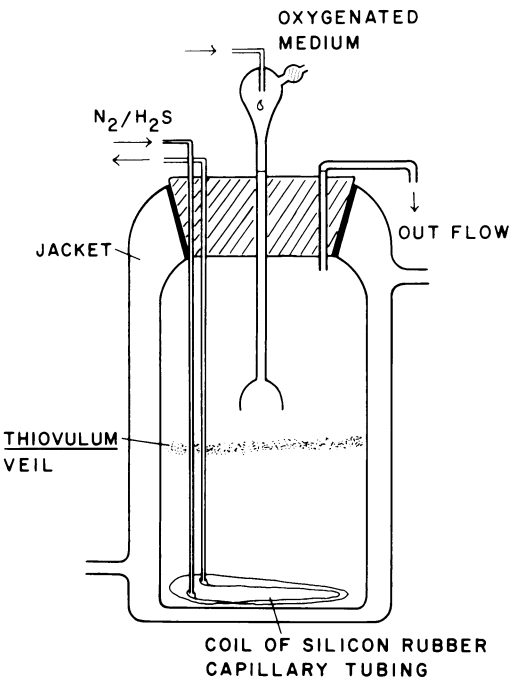

FIG. 4. *Flow-through culture vessel, chemostat type, providing dissolved sulfide from a N_2-H_2S gas mixture through a thin-wall silicone tubing for growth of Thiovulum sp. in mixed cell suspensions.*

Uptake experiments. Table 1 represents data from a series of orienting CO_2 uptake experiments conducted with purified populations of *Thiovulum* in the closed and recirculating culture system described in Fig. 3. When the

TABLE 1. *Uptake of CO_2 by whole maintenance populations of Thiovulum sp.*

Vol filtered (ml)	Incubation period (h)	CO_2 uptake (μg/ml)	Initial pH	Final pH
350 (total)	18	4.58	7.2	6.1
12 (veils only)	5	38.90	7.4	6.3
338 (remaining)	5	0.71	7.4	7.15
350 (total) (control)	5	0.011	7.4	7.35

entire culture, after establishment of the veils, was incubated for 18 h in the presence of ^{14}C-labeled bicarbonate, considerable uptake was measured, accompanied by a drop of pH. If, after a 5-h incubation experiment, the CO_2 uptake per milliliter of the veil-associated part of the population, selectively collected by a siphon, was compared to the rest of the culture, a 54-fold difference was recorded. As a control, the 8-μm filtrate showed practically no CO_2 uptake, thus demonstrating the absence of a major population of small chemolithotrophic bacteria, such as thiobacilli. The pH changes, attributable to the formation of sulfate, correspond to the CO_2 uptake activities.

The following experiments were conducted with purified cell suspensions in 1-ml subsamples collected from maintenance cultures and incubated at 20°C. The data in Tables 2 through 4 and in Fig. 5 are corrected for activity due to the remaining contamination as described above. The contamination, expressed in percent plate count on Difco 2216 or thiosulfate agar, respectively, of the microscopic count of *Thiovulum* cells, ranged from 0.06 to 4.8% for heterotrophic bacteria and from 0 to 0.014% for thiobacilli. The corrected data were calculated uniformly for concentrations of 10^6 *Thiovulum* cells/ml. Removal of *Thiovulum* cell suspensions from maintenance cultures and transfer into small tubes for uptake experiments in the absence of veil formation resulted in a cell die-off after 4 to 6 h. The data shown in Fig. 5 suggested that a 2-h incubation period was most suitable for further experimentation. Changes of the initial cell counts during this period of incubation were negligible.

Data on the effect of different sulfide concentrations on the CO_2 uptake are given in Table 2. Uptake in the absence of sulfide can be attributed to the oxidation of intracellular sulfur to sulfate. Although the disappearance of intracellular sulfur from cells in sulfide-free enrichment or maintenance media can be observed, it is impossible at this state of our cultivation capabilities to control either the sulfur content of *Thiovulum* cells or the growth phase or physiological state of a population. It is reasonable, therefore, to compare primarily those data which have been obtained from identical batches of cell suspensions, (i.e., within the sections A to C in Table 2). Variations between these batches are considerable. In general, however, an increase of CO_2 uptake with increasing sulfide concentrations is observable with a wide-range optimum around 1 mM. There is a noticeable decrease of uptake activity at about 3 mM and a definite inhibition of 8.5 mM sulfide. A slight stimulation of CO_2 uptake by Casamino Acids and the vitamin mixture appears, at least as far as the latter is concerned, to depend on the presence of sulfide.

TABLE 2. *Uptake of CO_2 as effected by different concentrations of sulfide in Thiovulum sp.*[a]

	Sulfide (mM)	CO_2 uptake (μg/10^6 cells per h)
A	0	0.26
	0.72	1.29
B	0	0.139
	0.085	0.615
	0.85	1.22
	8.55	0.046
	0 + vitamins	0.124
C	0	0.137
	2.85	0.293
	2.85 + vitamins	0.495
	2.85 + Casamino Acids	0.556

[a] The cell count ranged from 7.5×10^5 to 5.4×10^6/ml. The initial concentration of sodium bicarbonate in the air-saturated artificial seawater was 200 μg/ml. The 2-h incubations were conducted with 1-ml sample volumes in the absence of a gas phase. A to C indicate three different batches of freshly collected cells. Vitamins were 20 μg each of biotin, B_{12}, and thiamine per liter. Casamino Acids were added in a quantity of 25 μg/ml.

TABLE 3. *Uptake of CO_2 by Thiovulum sp. as effected by different concentrations of sodium glutamate in the absence and presence of sulfide*[a]

Sodium glutamate (μg/ml)	Sulfide (mM)	CO_2 uptake (μg/10^6 cells per h)
0	0	0.116
10	0	0.247
100	0	0.164
1,000	0	0.108
0	0.32	0.892
10	0.32	0.686
100	0.32	0.903
1,000	0.32	0.839

[a] The initial cell count was 1.88×10^6/ml. The initial concentration of sodium bicarbonate in the air-saturated artificial seawater was 200 μg/ml. The 2-h incubations were conducted with 1-ml sample volumes in the absence of a gas phase.

TABLE 4. *Uptake of four organic substrates at different concentrations of sulfide by Thiovulum sp.*[a]

Substrate	Sulfide (mM)	Net uptake ($\mu g/10^6$ *Thiovulum* cells per h)	% of gross uptake by 8-μm filtrate
Casamino acids	0	0.005	65
Casamino acids	0.40	0.025	55
Casamino acids	2.85	0	
Sodium glutamate	0	0	
Sodium glutamate	0.40	0.023	41
Sodium glutamate	2.85	0	
Mannitol	0	0	
Mannitol	1.2	0	
Acetate	0	0	
Acetate	0.21	0	
Acetate	0.39	0	

[a] The initial cell counts ranged from 2.8×10^5 to 5.4×10^6/ml. The 2-h incubations were conducted with 1-ml sample volumes in air-saturated artificial seawater and the absence of a gas phase.

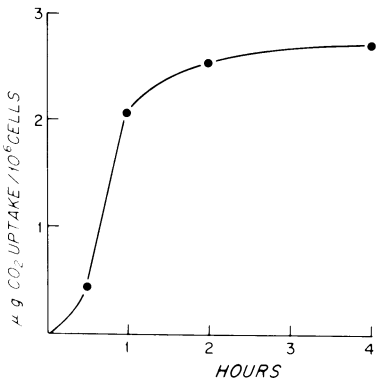

FIG. 5. *Uptake of CO_2 by Thiovulum sp. plotted versus time. The initial cell count was 2.9×10^6/ml; the initial concentration of sulfide was 0.9 mM.*

To study this point further, the effect of sodium glutamate on the CO_2 uptake of *Thiovulum* was determined in the absence and presence of sulfide. No stimulation could be recorded in either case (Table 3). The high variability of the uptake values within each of the two sets of data makes it probable that the apparent stimulation by Casamino Acids and the vitamin mixture is not real. This is corroborated by a similar set of data showing that yeast extract at concentrations of up to 40 μg/ml did not affect the CO_2 uptake.

In the attempt to substantiate these data, the uptake of ^{14}C-labeled organic carbon, using four different substrates, was measured at various sulfide concentrations (Table 4). Whereas the residual contamination of *Thiovulum* cell suspensions was always less than 1% of the total CO_2 uptake values, it was substantially higher when the uptake of labeled organic carbon was determined. Considering the low relative degree of contamination in terms of biomass, this 41 to 65 percent contribution to the total activity measured indicates the low significance of the data for organic carbon uptake attributable to *Thiovulum*. There was no uptake of organic carbon by *Thiovulum* in any of the other cases.

In view of our general observation that maximum activity of *Thiovulum* populations was usually found in well-established veils, two experiments on the uptake of labeled acetate during the reaggregation of veils were conducted: (i) in a 10-ml tube and (ii) in the closed, recirculating system described in Fig. 3. Indeed, the only significant uptake of organic carbon was detected in these experiments, namely, 0.074 and 0.015 $\mu g/10^6$ cells per h for (i) and (ii), respectively.

Morphological observations. The Faûré-Fremiet and Rouiller (5) organelle (Fig. 6a and b) was observed most frequently in cells collected from freshly established veils. The typically gram-negative cell wall appears to surround the organelle. The material regularly appearing on the outside of the fibrilla, which are perpendicularly oriented toward the external wall, suggests the formation of a "tail," the slime thread observed by de Boer et al. (4). In view of the peritrichous flagellation demonstrated by the same authors, the tumbling but polarly oriented motion of cells freshly collected from veils might simply be caused by the drag of the slime thread. It was this motion which prompted Metzner (13) to speculate on the existence of an equatorial zone of cilia. Remsen and Watson (19) observed the 1-μm-diameter base of a tufted flagellum with a helical substructure in a freeze-etch preparation made from a compacted, nonpurified *Thiovulum* veil. Such an organelle would present the most fitting explanation for the type of cell motion observed. However, in spite of its size, it had not been seen in the earlier stained preparations by Hinze (7) and others, nor can it be found by phase microscopy, which usually suffices for observing flagella of similar size, for instance, in *Chromatium*.

It is probable that the excreted slime threads form the matrix of the veils and that swarming cells do not excrete slime. Too few observations on sectioned preparations exist for a definite statement on the presence or absence of the organelle in swarming cells.

The large transparent areas within the cell (Fig. 5a) represent sulfur inclusions dissolved during the dehydration process. They appear to be encased by a nonunit membrane unlike the

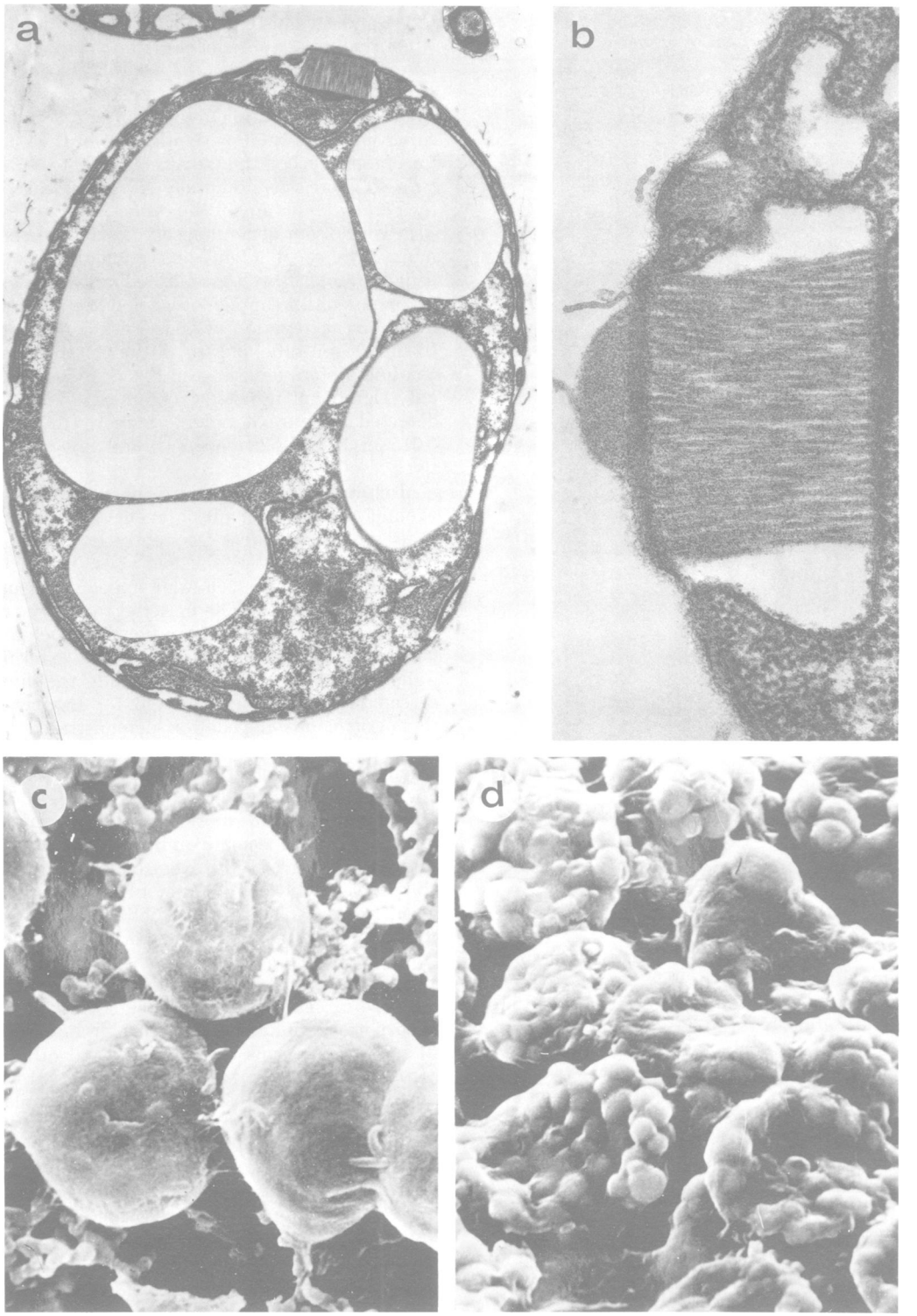

FIG. 6. *Electron micrographs of Thiovulum sp. (a and b) Sections of cells freshly collected from active veils, magnification × 14,200 (a) and × 64,300 (b); (c and d) critical point- and air-dried preparations, magnification × 2,600, the former showing a number of rods and spirilla attached to Thiovulum cells.*

abundant other membrane-bound invaginations.

The appearance of sulfur globules in collapsed cells as demonstrated in the scanning electron micrograph (Fig. 6d) was caused by air drying of glutaraldehyde-fixed preparations. Critical point drying led to a clear surface image of the cells (Fig. 6c), but it failed to show peritrichous flagella. This procedure also revealed some rod-shaped organisms which may or may not have been attached to the surface of the living *Thiovulum* cells. Pieces of peritrichous flagella, which are best shown in negative stain preparations (4), can be seen on Fig. 6a and b near the cell wall.

DISCUSSION

A number of conclusions can be drawn from several observations of *Thiovulum* populations in enrichment or maintenance culture. Veil-forming populations disperse within minutes throughout the culture vessel when the flow of seawater is stopped. The veils re-establish upon resumption of the flow. This chemotactic response can be interpreted to indicate a sensitivity toward a critical concentration of oxygen as well as hydrogen sulfide. Most active growth was observed within intact veils, i.e., in O_2-H_2S interfaces. This is in accordance with a generalization made by Pringsheim (18), who, for the purpose of isolating microorganisms in pure culture, describes the usefulness of chemo-, photo-, and geotactic movements and concludes: "They always indicate healthy cells and should be attended to with deliberation." We failed to maintain *Thiovulum* populations under anaerobic conditions in the presence of various reduced sulfur compounds. Nitrate was not used as an alternate electron acceptor. Values of dissolved oxygen obtained with electrodes near and within active veil populations scattered widely, probably reflecting a gradient in the immediate vicinity of metabolizing cells. A considerable pH tolerance was found, as can be expected in sulfate-producing organisms. The pH measured near active veil populations was as low as 6.1. Growth on thiosulfate or on solid or semisolid media was not obtained, which would greatly simplify these and future studies.

Successful enrichments have been obtained from various inocula such as anaerobic sulfide-containing muds, aerobic sediments, or just *Ulva* thalli as collected from estuarine waters. This fact indicated the existence and wide distribution of viable cells of *Thiovulum* which must be less sensitive to variations of the O_2-H_2S regime than those cells which are collected from active veils. Commonly the swarming cells occur when the veils disappear at the end of an enrichment culture. Morphologically they do not appear to be different from veil-forming cells except for the virtual absence of dividing stages and sulfur inclusions. Their swarming behavior could be explained by a cessation of slime excretion and chemotactic responses toward H_2S and/or O_2. The typical "biconvection" in populations of a number of motile microscopic organisms has been more recently described mathematically by Levandowsky et al. (12) and Hartman and High (6), who assume interactions between geotropism and chemotaxis. If the swarming stage of *Thiovulum* is indeed transitory to a stage of nongrowing and more resistant cells, the veil-forming cells appear to represent the short-living reproductive stage. This hypothesis would offer an explanation for the fact that *Thiovulum* appears to persist well under unfavorable growth conditions as indicated by the ease with which enrichments can be obtained from aerobic as well as anaerobic samples of mud or plant material.

Since the most successful media for the enrichment of *Thiovulum* contain high concentrations of organic substrates, doubt concerning this organisms' truly chemolithotrophic nature appeared justified. To clarify this point, pure cultures seemed to be necessary. The reasons for our failure to separate *Thiovulum* cells from bacterial contaminants, although we used a large variety of approaches including LaRivière's (9) technique, are twofold: growing cells are associated with a matrix of slime threads in an enrichment medium which also supports abundant growth of other chemolitho- as well as chemoorganotrophic bacteria, and second, any prolonged mechanical purification procedure disrupts the specific O_2-H_2S gradient and appears to impair growth of the *Thiovulum* cells irreversibly.

In view of the large biomass of *Thiovulum* populations, however, as compared to that of contaminants still remaining after two to three washings, it appeared feasible to conduct some basic physiological experiments with purified cell suspensions. The corrected CO_2 uptake data indicate that the number of thiobacilli-type contaminants in fresh veils is low. The values of organic carbon uptake are less significant due to larger numbers and the quick response of heterotrophic bacteria. Based on these controls, the data suggest that growth of *Thiovulum* sp. does not significantly depend on any of a number of organic substrates tested. This does not preclude the possibility that a gradation of types from strictly autotrophic to just incidental users of sulfur compounds, as known from the thiobacilli, does also occur among the large colorless sulfide-oxidizing bacteria.

A number of separate observations on motility and chemotactic behavior of *Thiovulum* are not reported here because of the lack of quantification. Further and more detailed studies require the well-controlled pure culture of cells in sufficient quantity.

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