ON THE CULTURE AND GENERAL PHYSIOLOGY OF THE GREEN SULFUR BACTERIA

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Green sulfur bacteria were first obtained in pure culture and studied under controlled conditions by van Niel (1930, 1931). He isolated several strains which, on the basis of their morphology, were identified as *Chlorobium limicola* Nadson (1912). Physiologically the species was characterized as strictly anaerobic, obligatorily photosynthetic, requiring H_2S for growth. The sulfide was quantitatively oxidized to sulfur with concomitant reduction of CO_2. The assimilatory process could be approximately expressed by the equation:

$$CO_2 + 2H_2S \xrightarrow{\text{light}} (CH_2O) + H_2O + 2S \qquad (1)$$

in which (CH_2O) denotes the average reduction level of the assimilation products.

Subsequently van Niel (1935, 1949*a*, 1949*b*) has developed a more detailed concept of the photosynthetic mechanism, suggesting that H_2S is used as electron donor for the continued reduction of an enzyme which is oxidized during the photoactivation of H_2O, while a simultaneously generated reduced enzyme serves for the reduction of CO_2.

The purple sulfur bacteria carry out a similar type of photosynthesis (van Niel, 1930, 1931). However, they oxidize H_2S, as well as some other reduced sulfur compounds, completely to H_2SO_4, and also can use other electron donors such as H_2 and various simple organic substances. These differences between the purple sulfur bacteria and *C. limicola* need not apply to the green sulfur bacteria in general. It has been pointed out that "it is entirely possible that organisms with characteristics intermediate between these groups can be found" (van Niel, 1941, p. 281).

This paper presents evidence for the existence of green sulfur bacteria with such intermediate characteristics.

CULTURES

Enrichment cultures for green sulfur bacteria were prepared by inoculating a sterile medium of appropriate composition in completely filled glass stoppered bottles with samples of marine and fresh water mud, and incubating the bottles at 28 to 30 C with continuous illumination from ordinary 25 to 50 watt incandescent lamps. The medium consists of tap water with 0.1 per cent each of NH_4Cl, KH_2PO_4, and $Na_2S \cdot 9H_2O$; 0.05 per cent $MgCl_2$, 0.2 per cent $NaHCO_3$,

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and NaCl as required; the initial pH is adjusted to 7.3 (van Niel, 1931). This medium is rather selective for green sulfur bacteria; at a higher pH or lower sulfide concentration a preferential development of purple sulfur bacteria is likely to occur.

Five out of nine mud samples so treated yielded cultures of green sulfur bacteria. Two samples produced cultures of purple sulfur bacteria; in the bottles with the remaining two samples no photosynthetic bacteria developed.

This result suggests that the last two samples contained very few or no green and purple sulfur bacteria, and that in the other two samples the purple bacteria so far outnumbered the green bacteria that, even though the medium used favors development of the latter, the purple bacteria can exhaust the sulfide supply rapidly enough to prevent visible growth of the green bacteria.

From such mud samples one may, however, obtain cultures of green bacteria by using large amounts as inoculum and ensuring a continuous supply of H_2S. For this purpose the Winogradsky technique (1887) is admirably suited. The mud is mixed with $CaSO_4$ and some insoluble organic matter, e.g., cellulose, poured into a tall glass cylinder, whereupon the cylinder is filled completely with water containing 0.1 per cent NH_4Cl and 0.1 per cent phosphate buffer at pH 7.3, and incubated with continuous illumination. The enriched mud serves as a continuous H_2S generator (bacterial sulfate reduction), and the photosynthetic sulfur bacteria soon appear as colored spots at the mud-glass interphase. The two mud samples which, in bottle cultures, had yielded only purple sulfur bacteria caused by this method the appearance of a few green among innumerable purple spots. Inoculation of bottle cultures with material from a green patch invariably resulted in satisfactory growth of green sulfur bacteria. The superiority of the Winogradsky method in such cases is not only due to the fact that it permits the use of large amounts of mud as inoculum; additional advantages are the continued formation of H_2S and the localization in space of the photosynthetic bacteria, thus facilitating their detection in the form of "colonies".

Enrichment cultures in bottles with media containing potential electron donors other than H_2S (sulfur, thiosulfate, thioglycolate, and cysteine supplied in molar concentrations corresponding to that of the sulfide in the original medium) provided evidence for the existence of green sulfur bacteria which can use thiosulfate in place of sulfide. Similar observations had been made by Dr. D. L. Ray at the University of Washington (personal communication). With one single exception, the organic sulfur compounds failed to give rise to green bacterial cultures. The exception pertains to a single case in which green bacteria developed in a cysteine medium. However, this was not transferable; it seems most probable that in the first culture the green bacteria had appeared as a secondary flora, feeding on H_2S produced from cysteine by other microbes.

Pure cultures were obtained by preparing shake cultures in the earlier mentioned sulfide medium which was solidified with 2 per cent agar (van Niel, 1931). Three successive transfers through shake cultures, each time from a single, well-isolated, and microscopically homogeneous colony, were made before an isolate was considered pure. In this manner pure cultures were secured,

and two strains have been studied in detail. The characteristics of one of these strains suggest its identity with *C. limicola*, although it should be emphasized that some differences have been noted. But because the present investigation has led to distinct improvements in the methods for culturing green sulfur bacteria, it seems probable that the early descriptions of *C. limicola* are based on observations with "unhealthy" cultures. Hence, it is considered premature to use these discrepancies as a justification for a "specific" differentiation between *C. limicola* and the new isolate. The latter, therefore, will be referred to as *C. limicola*.

The other strain presents one strikingly aberrant feature and is described here as a new species under the name *Chlorobium thiosulfatophilum*.

Synthetic medium. The first enrichment cultures usually produced intensely green, evenly suspended, heavy growth of the green sulfur bacteria. In transfers these organisms developed far more poorly, the bacteria forming a yellowish green, slimy precipitate. Thus, it appeared that the mud contributed some factor favorable for growth of the green bacteria lacking in the synthetic medium. Attempts to improve the latter by addition of trace elements revealed that $FeCl_3$ exerted a striking influence; in media with added iron salts growth was faster as well as much more "normal"; it compared favorably with that in the mud-containing cultures. Analyses showed that the amount of green pigment formed per unit growth increased with increasing iron concentration up to a maximum at 20 μg per cent Fe. This indicates that iron plays a role in the biosynthesis of the particular chlorophyllous pigment of the green sulfur bacteria, just as it is known to do in the chlorophyll synthesis of green plants.

In media prepared with glass distilled instead of tap water, growth was poor even in the presence of iron. Such media, however, were rendered fully satisfactory by the addition of $CaCl_2$ (0.01 per cent), and trace metals as follows: $FeCl_3$, 160 μg per cent; B, 10 μg per cent; Zn, 10 μg per cent; Co, 5 μg per cent; Cu, 0.5 μg per cent; and Mn, 0.5 μg per cent. Special studies on the effect of omitting one or more trace elements other than Fe and Ca have not been carried out; it is possible that some, at least, of the ingredients could be left out without affecting the results. For culturing marine strains NaCl must be added; 1 per cent generally suffices. If media with electron donors other than H_2S are to be used, it is advisable to add 0.01 per cent $Na_2S \cdot 9H_2O$ to ensure anaerobic conditions from the start; this small amount is not sufficient to cause appreciable growth of green bacteria in the absence of a satisfactory electron donor. The bicarbonate, sulfide, and ferric chloride are separately sterilized, as are also the solutions of other electron donors. The pH of the final medium is adjusted to 7.3.

Under proper conditions it was possible to obtain yields up to 2.5 g of bacteria (wet weight) per liter of culture in 36 to 48 hours.

MORPHOLOGICAL DESCRIPTION

In adequate media both strains develop as nonmotile, ovoid cells and short rods, 0.9 to 1.5 by 0.7 μ. Under unfavorable conditions, e.g., at low pH or excessive H_2S concentrations, involution forms appear in the shape of greatly

elongated, irregularly curved cells. Club-shaped and spiral structures such as described for *C. limicola* (Nadson, 1912; van Niel, 1931) have never been observed. Whether these growth forms, heretofore considered characteristic of *C. limicola*, might appear under special conditions remains to be determined.

Both strains also secrete elementary sulfur in sulfide media. According to van Niel's observations the excreted sulfur is not further oxidized by *C. limicola*. In the present investigation a gradual disappearance of initially precipitated sulfur has often been noted. This is true especially in cultures of *C. thiosulfatophilum;* in old cultures of this species the typical sulfur droplets or crystals can no longer be detected. But it appears that *C. limicola*, too, can carry out the further oxidation of sulfur under favorable conditions. More will be said about this phenomenon hereafter.

A visual criterion by which the two new strains can be differentiated pertains to the appearance of their colonies in sulfide agar. *C. limicola* grows rapidly, producing colonies of about 1 mm diameter in 5 to 6 days. They are disc-shaped, yellowish green, with a halo of sulfur globules (or later, crystals). *C. thiosulfatophilum* forms colonies of this size only after incubation for several weeks. They rarely develop a halo of sulfur and present a deep green to bright yellow color. Single colonies may be partly green and partly yellow, and it is probable that the sulfur excretion in localized areas is responsible for this variegated appearance.

The most striking difference between the two strains is physiological, as the next section will show.

PHYSIOLOGICAL EXPERIMENTS

Qualitative. Pure cultures of the two strains in completely filled bottles with sulfide media developed with excretion of sulfur, the latter disappearing with time. When growth had ceased, the cultures gave strongly positive tests for sulfate. This shows that elementary sulfur can be used by both strains as electron donor for photosynthesis.

In thiosulfate media *C. limicola* does not grow, and thiosulfate remains unaltered, whereas *C. thiosulfatophilum* produces heavy growth accompanied by the disappearance of the thiosulfate and the formation of copious amounts of sulfate. Thus, thiosulfate can be used as electron donor by the last named organism.

Cultures in mineral media without reduced sulfur compounds but continuously provided with a current of hydrogen gas containing 2 per cent CO_2 revealed that *C. thiosulfatophilum* can grow readily under these conditions. Evidently molecular hydrogen, too, can be used as electron donor by this organism. Similar experiments with *C. limicola* have not been carried out yet. From the fact that the latter species does oxidize hydrogen with the simultaneous reduction of CO_2 in the light, as shown in manometric experiments, it seems clear, however, that growth could be expected in such cultures.

Attempts to grow the green sulfur bacteria in darkness also have failed. The earlier mentioned designation of green sulfur bacteria as obligatorily photosynthetic organisms, therefore, still holds good.

Quantitative. Analyses of cultures of the two green sulfur bacteria and of simultaneously prepared but uninoculated media have been conducted to determine the quantitative relations between CO_2 assimilation and oxidation of sulfide. CO_2 was estimated by van Slyke's manometric method; when the sample to be analyzed contained sulfide, a solution of HCl saturated with $HgCl_2$ was used to liberate gaseous CO_2. Sulfide, thiosulfate, and sulfite were each determined by three subsequent iodine titrations according to the methods of Kurtenacker and Goldbach (1927); sulfate gravimetrically as $BaSO_4$. Qualitative tests for polythionates were conducted by Starkey's methods (1934).

The results showed that *C. thiosulfatophilum* oxidizes sulfide completely to sulfate, with a simultaneous assimilation of CO_2 in amounts that can be approximately expressed by the following equation:

$$2CO_2 + H_2S + 2H_2O \xrightarrow{\text{light}} 2(CH_2O) + H_2SO_4 \qquad (2)$$

Table 1 supplies the experimental data. The values for CO_2 assimilation in column 7 have been calculated from equation (2) on the basis of H_2S actually

TABLE 1

Chemical transformations in cultures of Chlorobium thiosulfatophilum using sulfide as electron donor

EXPERIMENT NO.	M MOL H_2S OXIDIZED	M MOL H_2SO_4 FORMED	M MOL H_2SO_4 CALCULATED	FOUND IN % OF CALCULATED	M MOL CO_2 ASSIMILATED		FOUND IN % OF CALCULATED
					Found	Calculated	
1	3.29	3.29	3.29	100.0	5.93	6.58	90.1
2	3.42	3.51	3.42	102.7	6.22	6.84	91.0
3	3.37	3.52	3.37	104.4	6.05	6.74	89.8

All figures are calculated "per 1,000 ml culture".

oxidized. That the experimental values are somewhat smaller than the "theoretical" ones indicates that the cell material formed during the assimilation process is slightly more reduced than (CH_2O), a result obtained also in the case of photosynthesis by purple sulfur (Muller, 1933) and purple nonsulfur bacteria (van Niel, 1941).

In cultures of *C. limicola* the amount of sulfide-sulfur which disappeared has never been quantitatively recovered as sulfate. Occasionally, the analysis of such cultures has yielded results that agree with van Niel's early observations, demonstrating a quantitative conversion of sulfide only to free sulfur, accompanied by the assimilation of somewhat less than 0.5 mol of CO_2 per mol of H_2S oxidized. Far more frequently, however, sulfate is encountered as an oxidation product, though generally not more than 70 per cent of the sulfur can thus be accounted for. Special tests for soluble S-compounds other than sulfate in the culture media have indicated the absence of sulfide, thiosulfate, sulfite, and polythionates. The most reasonable interpretation of the observed discrepancy between sulfide disappeared and sulfate recovered appears to be that the primarily excreted sulfur is only partially oxidized to sulfate, a fraction of it escaping further oxidation, possibly because conditions unfavorable for

this process develop in cultures of *C. limicola* more readily than in those of *C. thiosulfatophilum*. This hypothesis is considerably strengthened by the fact that careful microscopic examination of *C. limicola* cultures has never failed to reveal the presence of elementary sulfur. It also accounts for the results of van Niel with green sulfur bacteria cultures; they would simply imply that unfavorable conditions existed in his cultures from the start, not an improbable situation in view of the inadequate composition of the media used.

The results of chemical analyses of some representative cultures of *C. limicola* are summarized in table 2. The figures in column 4 (m mols of S left) have been computed from the difference between the amount of H_2S disappeared

TABLE 2

Chemical transformations in cultures of Chlorobium limicola using sulfide as electron donor

EXPERIMENT NO.	M MOL H_2S OXIDIZED	M MOL H_2SO_4 FOUND	M MOL S LEFT, CALCULATED	M MOLS CO_2 ASSIMILATED		FOUND IN % OF CALCULATED
				Found	Calculated	
1	3.25	2.22	1.03	4.62	4.96	93.0
2	3.59	2.36	1.23	5.28	5.34	98.8
3	3.31	2.38	0.93	5.43	5.23	103.8

All figures are calculated "per 1,000 ml culture".

TABLE 3

Chemical transformations by resting cells of Chlorobium thiosulfatophilum using thiosulfate as electron donor

EXPERIMENT NO.	μMOL $Na_2S_2O_3$ OXIDIZED	μMOL CO_2 ASSIMILATED		FOUND IN % OF CALCULATED
		Found	Calculated	
1	4.00	7.36	8.00	92.1
2	3.46	7.10	6.92	102.6
3	3.46	6.68	6.92	96.6
4	3.46	6.94	6.92	100.2
5	3.46	6.80	6.92	98.3
6	3.46	6.65	6.92	96.1

and sulfate formed. Similarly the numbers in column 6 (m mols CO_2 assimilated) have been calculated from the data on sulfur oxidation on the basis of equations 1 and 2. The agreement of the calculated and found values supports the interpretation here proposed.

Manometric experiments. Suspensions of green sulfur bacteria from actively growing cultures of the organisms have been used in attempts to determine the relation between substrate oxidation and CO_2 assimilation in experiments of short duration by means of manometric techniques. Cell suspensions were prepared in M/40 phosphate buffer (pH 7.0) with appropriate amounts of NaCl added; when thiosulfate or tetrathionate were used as electron donors, known amounts of substrate were placed in one side arm, the other containing 10 per cent citric acid. The gas phase consisted of O_2-free N_2 with 5 per cent CO_2. After equilibration, initial bound CO_2 was determined in a control vessel; final

bound CO_2 was calculated from manometer excursions after addition of acid to the main compartment at a time when no further pressure changes occurred in the vessels to which substrate had been added. The vessels were illuminated from below. The light intensity was such as to ensure saturation of the photosynthetic mechanism.

TABLE 4

Chemical transformations by resting cells of Chlorobium thiosulfatophilum using tetrathionate as electron donor

EXPERIMENT NO.	μMOL $Na_2S_4O_6$ OXIDIZED	μMOL CO_2 ASSIMILATED		FOUND IN % OF CALCULATED
		Found	Calculated	
1	1.96	6.80	6.86	99.0
2	2.00	6.45	7.00	92.2
3	2.00	6.23	7.00	89.0

TABLE 5

Chemical transformations by resting cells of Chlorobium thiosulfatophilum using molecular hydrogen as electron donor

EXPERIMENT NO.	μMOL H_2 OXIDIZED	μMOL CO_2 ASSIMILATED		FOUND IN % OF CALCULATED
		Found	Calculated	
1	14.03	5.96	7.02	84.9
2	14.69	6.65	7.35	90.5
3	14.05	6.25	7.03	88.9
4	15.03	6.56	7.52	87.3
5	11.10	5.40	5.55	97.3
6	10.69	4.98	5.35	93.1
7	10.85	4.71	5.43	86.7

Tables 3 and 4 present the results obtained with thiosulfate and tetrathionate as substrates. It is clear that the assimilatory process can be approximated by the following equations:

$$2CO_2 + Na_2S_2O_3 + 3H_2O \xrightarrow{\text{light}} 2(CH_2O) + Na_2SO_4 + H_2SO_4 \quad (3)$$

and

$$7CO_2 + 2Na_2S_4O_6 + 13H_2O \xrightarrow{\text{light}} 7(CH_2O) + 2Na_2SO_4 + 6H_2SO_4 \quad (4)$$

Similar experiments showed that *C. thiosulfatophilum* cannot use trithionate, dithionate, or sulfite as electron donor. *C. limicola* shows the same deficiency with respect to trithionate and dithionate. Sulfite was not tested. In addition it is unable to use thiosulfate and tetrathionate.

However, both strains can use molecular hydrogen. Manometrically this was tested with cell suspensions in the presence of an atmosphere of H_2 with 2 per cent CO_2. Total CO_2 assimilated was determined by gas analysis. Taking into account the pressure changes observed during the experiment the amount of H_2 disappeared could be computed. Table 5 presents the results of several

experiments with suspensions of *C. thiosulfatophilum*. *C. limicola* behaves similarly. The data show that photosynthesis with hydrogen can be expressed by equation (5):

$$2CO_2 + 2H_2 \xrightarrow{\text{light}} (CH_2O) + H_2O \qquad (5)$$

CHARACTERIZATION OF CHLOROBIUM SPECIES

The results of the physiological experiments described herein are summarized in table 6. They provide convincing evidence for the existence of at least two different types of green sulfur bacteria, distinguishable by their behavior towards thiosulfate and tetrathionate.

Previously published descriptions of green sulfur bacteria leave little doubt that the organisms used in the present investigation must be considered as members of the genus *Chlorobium* Nadson. However, there is considerable

TABLE 6

Utilization of inorganic electron donors by the two species of the genus Chlorobium

SPECIES	ELECTRON DONOR			
	Used	Oxidized to	Not used	Not tested
Chlorobium limicola	H_2S S H_2	SO_4^{--} and S SO_4^{--} H_2O	$S_2O_3^{--}$ $S_4O_6^{--}$ $S_3O_6^{--}$ $S_2O_6^{--}$	SO_3^{--}
Chlorobium thiosulfatophilum, n. sp.	H_2S $S_2O_3^{--}$ $S_4O_6^{--}$ S H_2	SO_4^{--} SO_4^{--} SO_4^{--} SO_4^{--} H_2O	$S_3O_6^{--}$ $S_2O_6^{--}$ SO_3^{--}	

room for doubt as to whether either or both types can be identified with the one species of the genus so far recognized. The diagnosis of *C. limicola* as given by van Niel in 1931, and again in the 6th edition of *Bergey's Manual of Determinative Bacteriology* (1948) does not correspond fully with the behavior of either strain. A judicious evaluation of the situation has led the author to the conclusion that it is advisable to propose only one new species at the present time. This is based on the following considerations.

In the first place, previous ecological studies have tended to show that *C. limicola* is the one representative of the green sulfur bacteria that can be obtained readily by enrichment cultures in sulfide media (van Niel, 1931). Nevertheless, in spite of the fact that several pure cultures were isolated in the course of the present study, not one of them has exhibited characteristics in complete conformity with those of the previously named species. It is true that only one strain was studied in detail, but there are good reasons for asserting that the other pure cultures of nonthiosulfate utilizing green bacteria are essentially

similar. Hence, one might be led to conclude that *C. limicola* was never encountered in my own work.

While this is, of course, not impossible, a second line of thought makes it unnecessary to adhere to so extreme a position. It follows from the fact that the published descriptions of *C. limicola* have been based largely on observations of cultures in media that are now recognized as "deficient". Naturally, such cultures may present features not shown by cultures grown under optimum conditions.

The differences referred to are morphological as well as physiological. The former pertain to the shape of "involution forms". Now, in spite of the insistence of Nadson and van Niel that spirally-wound structures are characteristic for *C. limicola*, one may justifiably question the significance of such morphological abnormalities for diagnostic purposes. Obviously, if such abnormalities are not encountered under "favorable" conditions, this hardly constitutes a valid reason for insisting on a specific differentiation.

Physiologically, the nonthiosulfate utilizing, or "sulfide," strain differs from *C. limicola* in that the former does, whereas the latter presumably cannot, oxidize elementary sulfur. As has been mentioned, however, an oxidation of sulfide only as far as sulfur has been observed occasionally in this investigation, and it is quite possible that van Niel never observed sulfate formation by his cultures simply because his media were deficient.

Only on the basis of a careful comparison with "authentic" cultures of *C. limicola* can the problem of identity of the sulfide strain be definitively settled. This is not possible now, since van Niel's original cultures are no longer available. If, at some future date, "typical" cultures of *C. limicola* are isolated, and sufficiently distinctive differences with the strain under discussion established, the creation of a new species for the latter might become desirable. Until such time it has, however, appeared advisable to regard this strain as a culture of *C. limicola*, and to emphasize the differences with previously described strains.

Description of *Chlorobium limicola:*

Cells: Small ovoids to short rods, 0.9–1.5 by 0.7 μ. Involution forms greatly elongated and irregularly curved rods. Club-shaped and spirally-wound involution forms have been described, but not encountered in the present study. Nonmotile, not producing endospores, gram negative.

Color: Intensely green in healthy cultures. Poor pigmentation in media deficient in iron.

Growth: In healthy liquid cultures evenly dispersed, not clumped or stringy, though generally somewhat mucoid. Cells do not settle rapidly.

Physiology: Strictly anaerobic, obligatorily photosynthetic. Can use H_2S, elementary sulfur, and molecular hydrogen as electron donors for CO_2 assimilation. Oxidizes sulfide and sulfur to sulfate, though under certain conditions sulfur may appear as the end product. Unable to use thiosulfate, tetrathionate, or organic substances for growth.

Habitat: Mud and stagnant water containing H_2S.

Description of *Chlorobium thiosulfatophilum*, n. sp.:
Cells: Indistinguishable from *C. limicola*.
Color: As above.
Physiology: Distinguishable from *C. limicola* by its ability to oxidize thiosulfate and tetrathionate in a photochemical CO_2 assimilation.
Habitat: As above.

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SUMMARY

The isolation from natural inocula of green sulfur bacteria, including one new species, *Chlorobium thiosulfatophilum*, is described. A synthetic medium has been developed which permits rapid growth and yields dense, healthy cultures. The new isolates are distinguished from the classical species, *Chlorobium limicola*, by their ability to oxidize elementary sulfur. *C. thiosulfatophilum* is physiologically characterized by its ability to utilize thiosulfate and tetrathionate as electron donors in photosynthesis. Evidence has been presented to show that both strains can use molecular hydrogen.

It is pointed out that the existing descriptions of *C. limicola* may be inadequate because they have been based on observations in "deficient" media.

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