HYDROGEN-UTILIZING, SULFATE-REDUCING BACTERIA IN MARINE SEDIMENTS[1]

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Received for publication August 21, 1950

Molecular hydrogen is utilized under anaerobic conditions by several physiological types of bacteria (ZoBell, 1947), among which sulfate reducers appear to be the most important in marine sediments. Although the observations of Nikitinsky (1907), Kroulik (1913), and Niklewski (1914) suggested that certain sulfate-reducing bacteria oxidize hydrogen, it remained for Stephenson and Stickland (1931*a*,*b*) to establish the utilization of molecular hydrogen by such bacteria. The autotrophic activity of hydrogen-oxidizing sulfate reducers was reported by Wight and Starkey (1945) and by Butlin and Adams (1947).

Hydrogen-consuming sulfate reducers have been detected in water or soil by Stephenson and Stickland (1931*a*), Von Wolzogen Kühr and Van der Vlugt (1934), Pont (1939), Ménard and Berkalof (1940), Pomeroy (1945), Starkey and Wight (1943), Wight and Starkey (1945), Butlin *et al.* (1947, 1949), and Postgate (1949). Finding such bacteria in marine bottom deposits and in petroliferous sediments led ZoBell (1947) to believe that the activities of sulfate reducers might help to account for the general absence of free hydrogen in environments where the fermentation of organic matter and other chemical or physical processes tend to liberate hydrogen. Hydrogen-consuming sulfate reducers might also be important geochemical agents (ZoBell and Rittenberg, 1948). The present study is concerned primarily with a survey of the occurrence and abundance of hydrogen-utilizing, sulfate-reducing bacteria in marine sediments both recent and ancient. Pure cultures of such bacteria have proved to be species of *Desulfovibrio*.

MATERIALS AND METHODS

The bacteria were cultivated in sea water enriched with 0.5 per cent $MgSO_4 \cdot 7H_2O$ and 0.01 per cent $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. Following autoclave sterilization the reaction of the medium was adjusted to pH 7.5 and the redox potential to about E_h −100 mv by adding per liter 20 ml M/1 $NaHCO_3$, 5 ml M/1 KH_2PO_4, and 10 ml M/10 Na_2S. The sea water employed in the preparation of the mineral solution for demonstrating autotrophic sulfate reducers contained less than 1 mg per L of organic matter.

With aseptic technique, sterile 125-ml glass-stoppered bottles (figure 1) are filled three-fifths full of sterile mineral solution. Then, after the inoculum is introduced into culture bottle *A*, the bottles are connected with the glass siphon with all three stopcocks open. Air is displaced from bottle *A* by filling the latter

[1] Contribution from the Scripps Institution of Oceanography, New Series No. 490. This paper is a contribution from American Petroleum Institute Research Project 43A.

with solution from bottle B, after which hydrogen, passed through a train to remove oxygen and bacteria, is forced into bottle A until bottle B is filled to capacity with the mineral solution. The bottles are then incubated at 28 C with

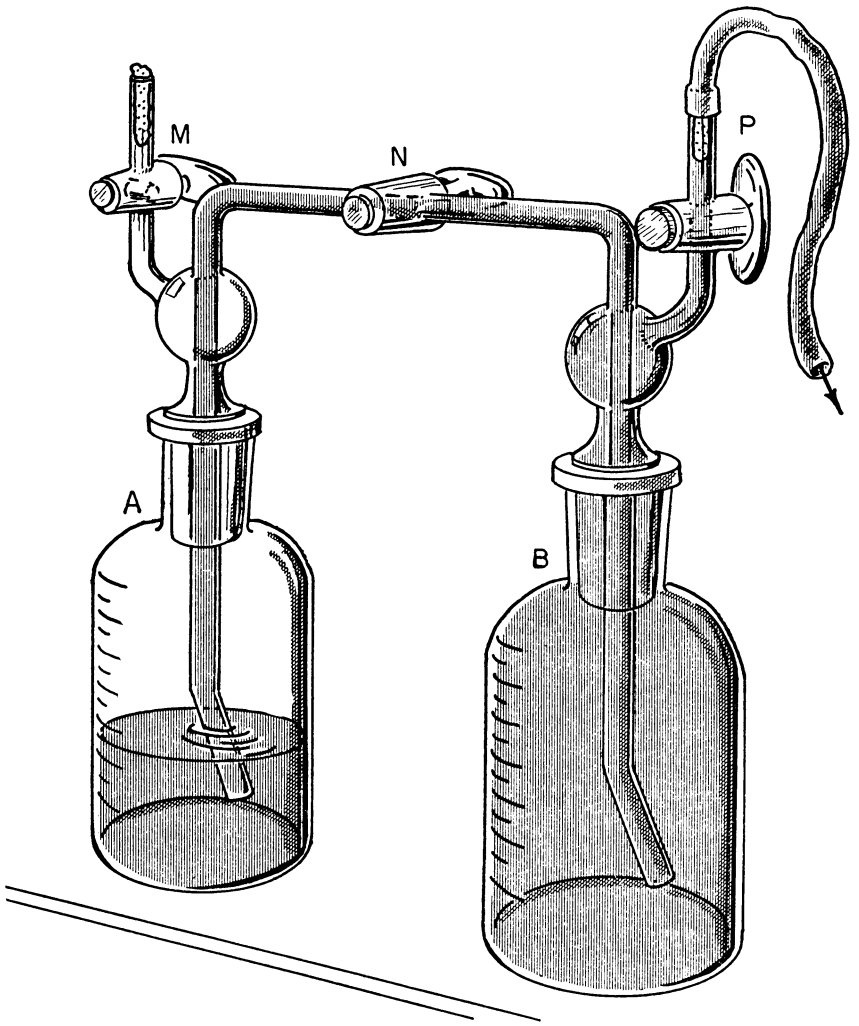

Figure 1. A pair of 125-ml glass-stoppered bottles with connecting siphon for following the utilization of molecular hydrogen by sulfate-reducing bacteria. The outlet tubes on stopcocks M and P are plugged with cotton in order to help prevent contamination. The outlet tube on stopcock P is connected with a train of wash bottles one of which contains alkaline pyrogallol to remove oxygen from air aspirated into reservoir bottle B as mineral solution is siphoned into culture bottle A when the culture consumes hydrogen.

stopcock M closed and stopcocks N and P open. As hydrogen is consumed, mineral solution is siphoned from bottle B into bottle A. Graduation marks on the bottles make it possible to read the volumes at constant temperature and corrected atmospheric pressure. Thus gas uptake can be followed without

interrupting the experiment. At the termination of our experiment the residual gas was analyzed by the Orsat apparatus or, in some cases, by mass spectrometer, the latter analyses being made by courtesy of the Richfield Oil Corporation laboratories at Wilmington, California.

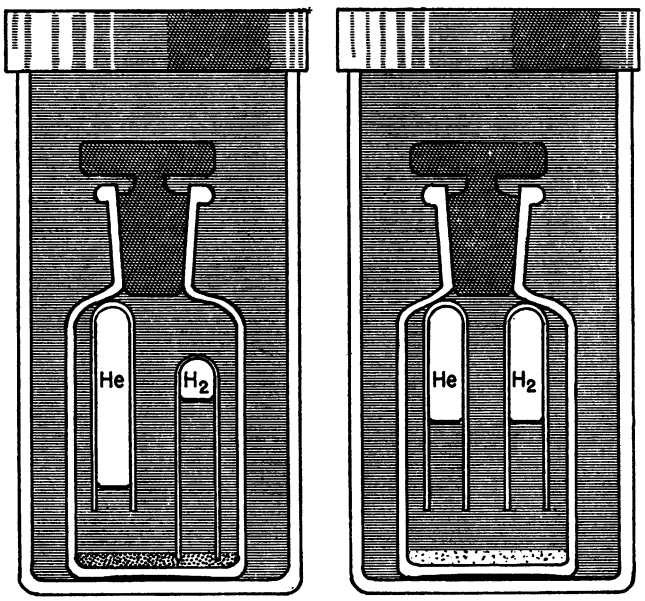

Figure 2. Glass-stoppered bottles containing vials of gas and mineral solution for the growth of autotrophic sulfate-reducing bacteria, incubated immersed in screw-cap jars containing the same solution. At the beginning of the experiment, as in uninoculated controls (*C*), one of the inverted vials is partly filled with helium and the other with hydrogen. When growth has occurred, most of the hydrogen is consumed, the helium is more rarefied, and a floc of ferrous sulfide intermixed with bacteria occurs in the bottom of bottle *A*.

Hydrogen sulfide resulting from the reduction of sulfate:

$$H_2SO_4 + 4H_2 \rightarrow H_2S + 4H_2O$$

reacts with ferrous iron in the mineral solution to render it progressively darker in color as black ferrous sulfide is formed:

$$H_2S + FeSO_4 \rightarrow FeS + H_2SO_4$$

The sulfide helps to maintain the anaerobic conditions so essential for the functioning of autotrophic species of *Desulfovibrio*.

Autotrophic hydrogen oxidation by sulfate reducers was also demonstrated in 60-ml glass-stoppered bottles, each containing two inverted 3-ml vials (figure 2), one containing hydrogen and the other helium. Since a negative pressure resulted from the consumption of hydrogen, these bottles were incubated immersed in wide-mouth, screw-cap jars filled with sterile mineral solution, thereby preventing the introduction of air into the system. Since the sulfate reducers are obligate

anaerobes, rigorous precautions must be exercised to maintain reducing conditions.

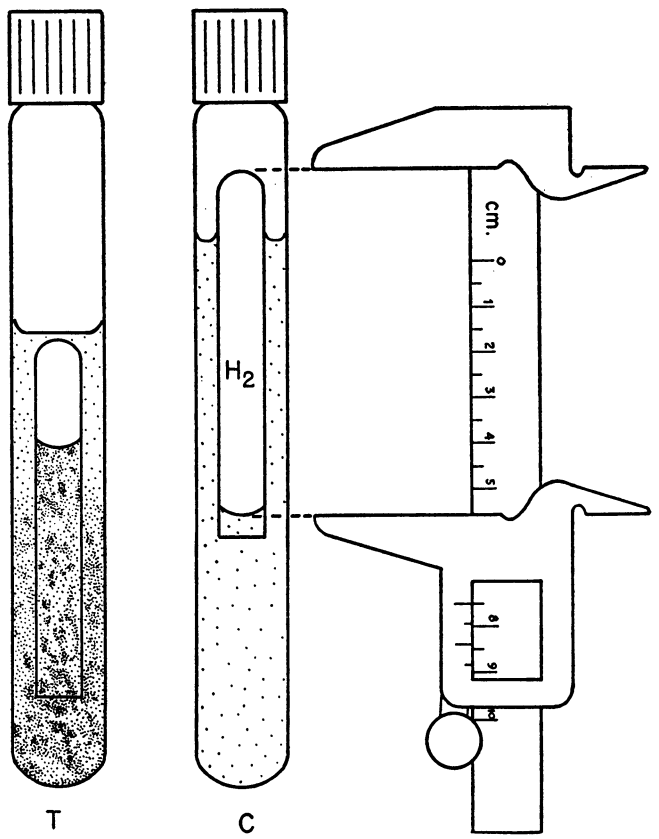

Figure 3. Screw-cap test tubes with inverted vials containing hydrogen alongside calipers for estimating the amount of hydrogen consumed by heterotrophic sulfate-reducing bacteria in tube *T* by comparing it with the uninoculated control *C*.

For the demonstration of hydrogen consumption by heterotrophic sulfate reducers a medium having the following composition was employed:

K_2HPO_4	0.2 g	Ascorbic acid	0.1 g
$MgSO_4 \cdot 7H_2O$	0.2 g	Calcium lactate	3.5 g
$(NH_4)_2SO_4$	1.0 g	Difco neopeptone	1.0 g
Na_2SO_3	0.1 g	Difco agar	3.0 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.1 g	Sea water	1,000.0 ml

The medium was dispensed in 30-ml, screw-cap test tubes containing inverted vials that were filled with hydrogen (figure 3). Hydrogen utilization was indicated by the disappearance of the gas from the inverted vials in an amount exceeding that in the uninoculated controls. Sulfate reduction was indicated by the blackening of the medium due to the formation of ferrous sulfide.

The two kinds of media were inoculated with approximately 1 gram of marine sedimentary material or dilutions (1:10, 1:100, etc.) thereof. A total of 245 carefully collected samples were examined in this manner. Recent sediments came from various parts of the Antarctic, Arctic, Atlantic, and Pacific oceans as well as from bays, estuaries, and marine marshes. Samples of ancient sediments were obtained from oil wells through the co-operation of oil companies and the American Petroleum Institute.

RESULTS

From 2 to 5 weeks' incubation at 28 C was required for the bacteria in the mud samples to consume detectable quantities of hydrogen (table 1). Occasionally selective media inoculated with mud exhibited evidence of hydrogen uptake only after 16 to 24 weeks' incubation, but the majority of the samples that ever became positive did so within a month. As soon as there was definite evidence of hydrogen uptake, a loopful of the culture was inoculated into another bottle of sterile inorganic medium in order to eliminate organic matter and to establish the autotrophic nature of the organisms, four or more such transfers being made seriatim. Such enrichment cultures generally consumed detectable quantities of hydrogen within a week.

The results of examining a total of 245 sediment samples in this way are as follows:

Hydrogen utilization in		*Sulfate reduction in*	
Inorganic medium	*Organic medium*	*Inorganic medium*	*Organic medium*
108 or 44%	150 or 61%	145 or 59%	187 or 76%

Finding sulfate reduction in a larger percentage of samples than hydrogen utilization in organic medium is a commentary on the fact that in these tests hydrogen sulfide formation was more readily detectable than hydrogen uptake. Since molecular hydrogen was the only available source of energy for bacteria in the inorganic medium, sulfate must have been reduced at the expense of such hydrogen. Actually subculturing the sulfate reducers from the inorganic medium established that they could utilize molecular hydrogen. As will be detailed in a subsequent paper, several cultures of sulfate reducers have been subcultured numerous times in inorganic medium enriched with molecular hydrogen as the only source of energy, and resting cells of some have been used to study the rate of hydrogen uptake in Warburg microrespirometers.

Finding a larger percentage of hydrogen utilization in organic medium than in inorganic medium has at least two explanations. First, the organic constituents might enhance the growth of certain hydrogen-utilizing sulfate reducers, and, second, bacteria are present in certain sediment samples that consume hydrogen by the reduction of organic compounds. Hoogerheide and Kocholaty (1938) have reported the relative rates at which various amino acids and related substances were hydrogenated by molecular hydrogen by suspensions of *Clostridium sporogenes*. The bacterial hydrogenation of fumarate and related organic compounds has been reported by Farkas and Fischer (1947) and Farkas and Schneidmesser (1947). Additional examples of the microbial reduction of organic

substances with molecular hydrogen are given by Wieland and Pistor (1938), Stephenson and Stickland (1933), and ZoBell (1947). Also, molecular hydrogen might be utilized in the reduction of carbon dioxide to methane, as discussed by Barker (1943) and Kluyver and Schnellen (1947). As a matter of fact, methane was quite commonly detected in our enrichment cultures that had grown in organic medium as well as in cultures developing in inorganic medium:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

Tests on enrichment cultures from marine sediments, however, showed that sulfate reduction accounts for most of the hydrogen consumption in both the organic and inorganic media used in these experiments.

Distribution of marine hydrogen utilizers. The widespread geographic distribution of hydrogen-utilizing sulfate reducers is illustrated by the data in table 2. The ancient marine sediment samples came from oil wells in Texas,

TABLE 1

H_2 uptake and H_2S production in different types of media inoculated with decreasing amounts of marine mud sample no. 43:11 after different periods of incubation at 28 C

AMOUNT OF MUD IN GRAMS	AUTOTROPHS IN MINERAL MEDIUM				HETEROTROPHS IN ORGANIC MEDIUM			
	Two weeks		Five weeks		Two weeks		Five weeks	
	H_2	H_2S	H_2	H_2S	H_2	H_2S	H_2	H_2S
1.0	+	+	+	+	+	+	+	+
0.1	+	+	+	+	+	+	+	+
0.01	+	+	+	+	+	+	+	+
0.001	−	−	+	+	−	+	+	+
0.0001	−	−	−	+	−	+	+	+
0.00001	−	−	−	−	−	−	+	+
None (control)	−	−	−	−	−	−	−	−

California, and Oklahoma, some from depths of several thousand feet. A good many of the samples of ocean bottom deposits came from depths of several hundred feet below the water surface and some from appreciable depths below the mud-water interface (table 3). Test borings for coastal pilings provided mud samples from depths as great as 181 feet, some of which contained hydrogen-consuming, sulfate-reducing bacteria. The marine marsh samples (tables 2 and 3) were collected along the coasts of California, Louisiana, and Cuba.

Some of the ancient marine sediment samples came from oil wells at depths as great as 9,000 feet. Sulfate-reducing bacteria found in such sediments, like some of those isolated from the deep-sea floor, have been found to be active at hydrostatic pressures isobaric with these depths, roughly 300 atmospheres at 9,000 feet. This observation tends to substantiate our belief that such baroduric (pressure-tolerant) bacteria are species indigenous to these great depths, since most bacteria isolated from near-surface material are inhibited by high pressure (ZoBell and Johnson, 1949). Certain sulfate reducers have proved to be obligate barophiles active only at pressures of 300 to 600 atmospheres.

TABLE 2

Number of samples of marine sediments from various geographic regions showing the presence of bacteria in 1-gram inocula that consumed molecular hydrogen or reduced sulfate, or both, in different kinds of media

SOURCE OF SEDIMENT SAMPLES	NUMBER OF SAMPLES EXAMINED	H_2 UTILIZATION IN		SO_4 REDUCTION IN	
		Organic medium	Inorganic medium	Organic medium	Inorganic medium
Arctic Ocean	40	24	11	31	15
Antarctic Ocean	5	2	0	1	0
Pacific Ocean	53	32	23	43	34
Caribbean Sea	2	2	1	2	1
Gulf of Mexico	24	24	16	24	18
Marine marshes	63	48	41	63	54
Ancient marine	58	18	16	23	23

TABLE 3

Vertical distribution of hydrogen-consuming and sulfate-reducing bacteria detected in 1-gram samples of marine sediments from various sources

DEPTH IN FEET	NUMBER OF SAMPLES EXAMINED	H_2 UTILIZATION IN		SO_4 REDUCTION IN	
		Organic medium	Inorganic medium	Organic medium	Inorganic medium
Water depth					
0–50	59	47	43	49	49
50–500	43	27	17	24	21
500–10,000	13	8	5	6	6
Core depth					
0–1	90	72	64	83	71
1–3	20	10	8	7	7
3–10	16	4	2	2	2
10–181	22	9	9	11	11

TABLE 4

Occurrence of hydrogen-utilizing and sulfate-reducing bacteria in 1-gram samples of a core of marine sediments (29:186) representing different core depths

CORE DEPTH IN FEET	DESCRIPTION OF SEDIMENT	H_2 UTILIZATION IN		SO_4 REDUCTION IN	
		Organic media	Inorganic media	Organic media	Inorganic media
1	Sandy clay	+	+	+	+
17	Sandy clay	+	+	+	+
37	Sandy silt	+	+	+	+
77	Blue clay & sand	−	+	+	+
97	Blue clay	−	+	+	+
117	Sand	−	−	+	−
137	Clay & sand	−	−	−	−
157	Clay & sand	+	−	+	−
181	Clay & sand	−	+	+	+

A larger percentage of positive results was obtained with the topmost portions of marine mud samples than in those from greater depths. Hydrogen-utilizing sulfate reducers appear to occur quite commonly in the upper layers of recent marine sediments, but at greater depths such bacteria occur only sporadically and in far smaller abundance. The minimum dilution method indicated the presence of from 0 to 100 viable hydrogen utilizers per gram of mud taken from depths below 1 foot as compared with counts ranging up to 10,000,000 per gram in the topmost foot of mud.

The sporadic occurrence of hydrogen-utilizing and sulfate-reducing bacteria in a long core of recent marine sediments is illustrated by the data in table 4. This core material was collected by the Humble Oil and Refining Company at Tiger Pass in the tidewater area of Louisiana. Aseptic precautions were exercised in handling the material, and only radially central subsamples of the core untouched by anything except laboratory-sterilized instruments were used to inoculate enrichment media.

DISCUSSION

Evidence for the general occurrence of sulfate reducers in marine sediments has been summarized by ZoBell and Rittenberg (1948). Many of these bacteria have been shown to be able to utilize molecular hydrogen. That sulfate-reducing bacteria are physiologically active *in situ* is indicated by a decreasing concentration of sulfate and an increasing concentration of sulfide, with depth or geological age of marine sediments and brines associated with petroleum. Though there may be other mechanisms to account for the reduction of sulfate, the presence of living bacteria offers the most plausible explanation, particularly since the nutrients and environmental conditions in the sediments have been shown to be conducive to the activity of sulfate reducers. The latter may also help to account for the observed decrease with depth or modification of the organic content of sediments. Besides being able to utilize several kinds of organic compounds (Baars, 1930), which may occur in marine sediments, certain sulfate-reducing bacteria attack petroleum hydrocarbons (Tausson and Aleshina, 1932; Tausson and Vesselov, 1934; ZoBell, 1950).

Bastin and his co-worker (1926, 1930) found sulfate-reducing bacteria in 28 oil field waters out of 30 sampled in Illinois and in 15 out of 37 sampled in California at depths ranging from 400 to 1,866 feet. Similar observations were made by Gahl and Anderson (1928) and by Ginter (1930, 1934). Upon finding such bacteria in 14 out of 15 samples of oil sands from the Grozny marine formation, Ginsburg-Karagitscheva (1933) attributed the abundance of hydrogen sulfide and the low concentration or lack of sulfate in water from oil wells to the activity of sulfate reducers. Such bacteria may utilize sulfate as a hydrogen acceptor while oxidizing organic matter or petroleum hydrocarbons as an energy source. The present studies show that molecular hydrogen may also serve as an energy source.

Hydrogen may be formed in marine sediments from the fermentation of the organic remains of plants and animals, or it may result from a variety of chemical

or physicochemical reactions (ZoBell, 1947). Its general, though not complete, absence from natural gases in marine sediments where one might expect to find free hydrogen may be due in part to the activities of sulfate-reducing bacteria that consume hydrogen.

ACKNOWLEDGMENTS

The authors wish to thank Helen H. Whelply, Margaret Knight, Nance Fountain, and Rose Marie Cassady for technical assistance. The suggestions and criticisms of Dr. D. L. Fox and Dr. D. M. Updegraff are also acknowledged. The investigations were aided by grants from the American Petroleum Institute and especially by co-operating oil companies that have provided samples and analytical services.

SUMMARY

Methods are described for demonstrating the utilization of molecular hydrogen by sulfate-reducing bacteria under strictly anaerobic conditions.

Hydrogen-utilizing sulfate reducers were found in about half of the 245 one-gram samples of marine sediments examined from different parts of the world. Such bacteria were more abundant in sediments near the mud-water interface than at greater depths, although some were found a few thousand feet below the surface.

Autotrophic sulfate reducers that utilize hydrogen as a source of energy were demonstrated in several enrichment cultures. Pure cultures have proved to be species of *Desulfovibrio*.

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