HYDROGEN UTILIZATION BY SOME MARINE SULFATE-REDUCING BACTERIA[1]

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Sulfate-reducing bacteria which oxidize molecular hydrogen have been detected in water or soil by various workers (Starkey and Wight, 1945, ZoBell, 1947). Sisler and ZoBell (1950) found such bacteria to be widely distributed in marine sediments, where they are believed to be important geochemical agents. Pure cultures proved to be species of *Desulfovibrio*, some of which are capable of an autotrophic existence in mineral solutions with molecular hydrogen serving as the sole source of energy.

The autotrophic activity of H_2-oxidizing sulfate reducers was first demonstrated by Wight and Starkey (1945) and confirmed by Butlin and Adams (1947). The enzyme which activates H_2 was designated hydrogenase by Stephenson and Stickland (1931*a*,*b*). The part played by H_2-consuming *Desulfovibrio* species in the anaerobic corrosion of iron has been discussed by Starkey and Wight (1943, 1945).

This paper is concerned with a study of the physiology of several cultures of *Desulfovibrio* isolated from marine sediments, with particular reference to their hydrogenase activity under various conditions.

MATERIALS AND METHODS

Heterotrophic sulfate reducers were tested for their ability to utilize H_2 in screw-capped test tubes of semisolid lactate agar containing inverted vials filled with H_2 (figure 3, Sisler and ZoBell, 1950). Inoculated tubes were incubated at 28 C along with sterile tubes of similar medium containing vials of H_2, which served as controls. Lactate medium having the following composition was used for this purpose:

Calcium lactate	3.5 g	Dibasic potassium phosphate	0.2 g
Neopeptone	1.0 g	Ferrous ammonium sulfate	0.1 g
Yeast extract	1.0 g	Agar	3.0 g
Ascorbic acid	0.1 g	Sea water	750.0 ml
Magnesium sulfate	0.2 g	Distilled water	250.0 ml

The ascorbic acid helped to maintain reducing conditions (Kligler and Guggenheim, 1938) essential for the initiation of growth of sulfate reducers. In inorganic media employed for the growth of autotrophs, sodium sulfide was substituted for the ascorbic acid to create reducing conditions. Once the sulfate reducers start to grow, they produce enough H_2S to provide a reducing environ-

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ment. The reaction of the sulfide with ferrous iron blackens the medium to a degree that is roughly proportional to the amount of sulfate reduced. The pH of the medium was 7.5 following autoclave sterilization.

For studying autotrophic sulfate reducers a similar mineral medium, free of organic matter, was dispensed in pairs of glass-stoppered bottles connected with a siphon in such a way that the utilization of H_2 in the inoculated bottle resulted in a like volume of medium being siphoned over from the reservoir bottle (figure 1, Sisler and ZoBell, 1950). At the termination of the experiment the residual gas in the inoculated bottle was analyzed by a Fisher unitized precision model Orsat apparatus (Matuszak, 1934) or by mass spectrometer, the latter determinations being made by the Richfield Oil Corporation, Wilmington, California.

Cell suspensions for chemical analysis and for use in Warburg respirometer experiments were grown in 20-liter lots of mineral salts solution over-layered with H_2 purified by passage through alkaline pyrogallol solution and sterile cotton. The cells were harvested with a Sharples supercentrifuge.

Enrichment cultures of sulfate reducers were purified by preparing serial dilutions for inoculating nutrient agar deeps. From the highest dilution showing growth of sulfate reducers, further serial dilutions (1:10, 1:100, etc.) were used to inoculate tubes of nutrient agar which, in the molten state, was drawn into sterile tubes of 2 mm inside diameter and 60 cm long. After the agar had solidified, the ends of the tubes were coated with sterile paraffin, and the tubes were incubated at 28 C. Tubes showing typical black discrete colonies were selected for the isolation of pure cultures. By breaking the tube near such colonies, they could be transferred aseptically with an inoculating needle to appropriate media or to dilution water. Microscopic examination and cultural tests established the purity of the isolated cultures.

EXPERIMENTAL RESULTS

Hydrogenase activity of sulfate reducers. Out of a total of 39 cultures of sulfate reducers isolated from marine sediments, 33 exhibited hydrogenase activity after 9 weeks' incubation at 28 C as indicated by their ability to utilize H_2 in lactate medium. Although there was evidence of growth and H_2S formation by all cultures after 1 week's incubation, only a few consumed detectable quantities of H_2 during this period. The rate at which heterotrophic sulfate-reducing bacteria consumed H_2 is illustrated by data summarized in table 1.

Some cultures exhibited hydrogenase activity only after 20 to 24 weeks' incubation at 28 C in nutrient medium enriched with H_2, which suggests that hydrogenase may be an adaptive enzyme. Our results indicate that not all strains of sulfate-reducing bacteria utilize H_2, some cultures having been maintained for several months in the presence of H_2 without exhibiting any hydrogenase activity.

A few cultures have been observed that utilize H_2 only in the absence of lactate or other organic hydrogen donors, but such obligate autotrophs are in the minority. Eleven of the 39 cultures tested utilized H_2 in purely inorganic medium; of these only 3 failed to utilize H_2 in lactate medium.

Many anaerobes found in marine sediments besides sulfate reducers consumed H_2 in nutrient medium containing lactate, peptone, and other organic compounds or their decomposition products, presumably with the reduction of carbon compounds (ZoBell, 1947). Among the possible hydrogen acceptors of such cultures are CO_2 and other one-carbon-atom compounds known to be reduced to methane (Barker, 1943; Kluyver and Schnellen, 1947; Stephenson and Stickland, 1933) and various organic or amino acids (Farkas and Fischer, 1947; Farkas and Schneidmesser, 1947; Hoogerheide and Kocholaty, 1938; Lascelles and Still, 1946). To date, however, the only substances found to serve as hydrogen acceptors for pure cultures of sulfate-reducing bacteria are sulfate,

TABLE 1

Average amount of H_2 consumed after different periods of incubation at 28 C by duplicate cultures of sulfate-reducing bacteria in tubes containing 20 ml of semisolid lactate agar and 2.5 ml of H_2

CULTURE NUMBER	H_2 CONSUMED AFTER			
	1 week	4 weeks	7 weeks	9 weeks
	ml	*ml*	*ml*	*ml*
29:57-1	0.4	2.1	2.5	2.5
29:65-128	0.0	0.0	0.0	0.0
29:66-26A	0.0	0.4	0.6	1.4
29:66-27	0.4	2.5	2.5	2.5
29:129-5	0.0	0.2	1.7	2.1
29:130-2	0.0	0.0	0.2	0.9
29:130-4	0.2	2.3	2.5	2.5
29:136-1	0.2	1.9	2.3	2.5
29:136-4	0.0	0.0	0.4	1.6
29:136-9	0.0	0.2	0.9	1.4
29:193-7	0.3	1.7	2.0	2.5
None (control)	0.0	0.0	0.0	0.0

sulfite, thiosulfate, sulfur, CO_2 or carbonate, certain unsaturated organic acids, and nitrogen.

CO_2 reduction by autotrophic sulfate reducers. Evidence has been obtained for the reduction of CO_2 by sulfate-reducing bacteria by cultivating pure cultures for several serial transfers in mineral salts solution. The growth or multiplication of the bacteria resulted in an accumulation of organic metabolic products in amounts that were proportional to the decrease in the total CO_2 content of the medium. Moreover, the quantity of H_2 consumed was more than the amount required to account for the amount of H_2S formed from the reduction of sulfate, the latter requiring 4 volumes of H_2 for each volume of H_2S produced:

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

Whereas the H_2/H_2S ratio approximated 4 for "resting" cells in sulfate medium containing no CO_2, the H_2/H_2S ratio ranged roughly from 5 to 10 for most actively growing cultures of sulfate-reducing bacteria. If sulfate is reduced only to free sulfur, as has been claimed for certain cultures by Datta (1946), or

if it is reduced to some product intermediate between sulfate and sulfur, as postulated by Baars (1930), the H_2/H_2S ratio would be less than 4. The representative results summarized in table 2 were obtained by incubating cultures in 100 ml of mineral salts solution overlayered with 100 ml of H_2 in glass-stoppered bottles. The volumes of residual gases, including total CO_2, calculated at standard conditions, were determined at the termination of the experiment.

The $(H_2\text{-}H_2^s)/CO_2$ ratios given as the last column in table 2 are especially significant. These values represent the amount of H_2 available for the reduction of CO_2 after subtracting the amount of H_2 required (indicated by H_2^s) to account for the H_2S produced. Assuming that H_2 is utilized for the reduction of either sulfate or CO_2, the only substances in the medium capable of being reduced, and assuming that the CO_2 is reduced only to a primary building block of bac-

TABLE 2

H_2 consumed, H_2S produced, and CO_2 reduced in 100 ml of purely mineral medium by cultures of autotrophic sulfate-reducing bacteria in 3 weeks at 28 C

BOTTLE NUMBER	CULTURE NUMBER	H_2 CONSUMED	H_2S PRODUCED	CO_2 REDUCED	H_2/H_2S RATIO	$(H_2\text{-}H_2^s)/CO_2$ RATIO
		ml	*ml*	*ml*		
1	43:70-12	23.8	2.2	4.6	10.8	3.3
2	43:70-12	18.5	1.7	3.3	10.9	3.5
3	43:74-54	9.1	1.7	0.8	5.3	2.9
4	43:74-54	9.3	1.7	1.0	5.5	2.5
5	43:74-58	33.1	4.5	6.6	7.4	2.3
6	43:74-58	29.9	3.9	6.4	7.7	2.2
7	43:74-71	34.7	4.8	5.0	7.2	3.1
8	43:74-71	34.5	5.0	4.6	6.9	3.2
9	None (control)	0	0	0		
10	None (control)	0	0	0		

H_2^s = H_2 required to account for H_2S formed.

terial cell substance represented by (CH_2O), the theoretical $(H_2\text{-}H_2^s)/CO_2$ ratio would be 2:

$$CO_2 + 2H_2 \rightarrow (CH_2O) + H_2O$$

However, all cultures gave $(H_2\text{-}H_2^s)/CO_2$ ratios exceeding 2, which suggests that substances more reduced than (CH_2O) were formed. Such substances have proved to consist at least in part of lipids and hydrocarbons. There is considerable variation in the competition for H_2 by sulfate and CO_2 among different cultures as indicated by the ratios in the last two columns in table 2, and also considerable variation was found in the amounts and kinds of reduced carbon compounds produced by H_2-consuming sulfate reducers.

Products of autotrophic metabolism. The formation of methane by a small percentage of our cultures of H_2-consuming sulfate reducers, which appeared by the usual criteria to be pure, was indicated by Orsat analyses and confirmed by mass spectrometer examination of residual gases.

Mass spectrometer analyses also helped to establish that hydrogenase-producing sulfate reducers may, after prolonged incubation, bring about the quantitative removal of H_2 from media containing an excess of sulfate or carbonate. Culture 48:43, for example, removed the last detectable trace of H_2 from 100 ml of mineral medium to which 100 ml of H_2 had been added, during five weeks' incubation at 28 C while producing, among other reduction products, 0.7 ml of methane.

The small amount of methane produced by autotrophic sulfate reducers (table 3) shows that methane formation is only an incidental and not the principal part of their metabolism. In fact, the cultures recorded in table 3 are the only ones among 39 studied to date which have been found to produce any methane.

During 50 days' incubation at 32 C in 15 liters of mineral medium, culture no. 45:10-12 consumed 13,416 ml of H_2, the volume being corrected for standard conditions. The good agreement between the amount of H_2S produced, namely

TABLE 3

Methane detected by mass spectrometer in cultures of sulfate-reducing bacteria after 3 weeks' incubation at 28 C in 100 ml of mineral medium enriched with H_2

CULTURE NUMBER	H_2 CONSUMED	H_2S PRODUCED	CH_4 PRODUCED
	ml	*ml*	*ml*
43:0-13	28.4	3.1	0.3
43:0-99	42.7	5.8	0.6
43:100-109	36.5	4.0	0.3
43:101-105	19.8	1.8	0.2
43:103-102	43.1	6.4	1.4
48:43	24.0	2.4	0.2
None (control)	0.0	0.0	0.0

1,032 ml, and the amount of sulfate reduced, 4.92 g, indicates that this culture reduced sulfate almost completely to H_2S, the theoretical amount of H_2S that could be produced from the complete reduction of 4.92 g of sulfate being 1,123 ml. An H_2/H_2S ratio of 13 (13,416/1,032) shows that something besides sulfate was reduced, and indeed it was found that a total of 3,910 ml of CO_2 (carbonate, bicarbonate, and CO_2 all calculated as CO_2) had disappeared from the system.

Although no gaseous hydrocarbons were produced by culture no. 43:10-12, in the experiment described in the preceding paragraph, 0.72 g of CCl_4-soluble material was extracted from the bacterial cell substance that developed in 16 liters of medium. Of this, 0.21 g proved to be an oily unsaponifiable material consisting largely of combined hydrogen and carbon. This could account for the $(H_2\text{-}H_2^*)/CO_2$ ratio of 2.37.

The remarkably high lipid yield is of special interest. The heat of combustion of 0.72 g of bacterial lipids is 6,830 cal, assuming that it is comparable to animal and vegetable oils having an average heat of combustion of 9,500 cal per g (Hodgman, 1947). Combustion of 13,416 ml of H_2 to water yields 40,700

cal. Thus 17 per cent of the energy of the H_2 oxidized by the autotrophic sulfate reducer could be accounted for as energy stored as lipids.

Spectroscopists, working under the supervision of Dr. L. M. Henderson of The Pure Oil Company, Crystal Lake, Illinois, who examined an aliquot of the unsaponifiable material produced by the autotrophic sulfate reducer reported that, "The infrared spectrums of the sample furnish partial, but not complete, evidence that the compounds present in the sample are composed for the most part of saturated —CH_2— groups with possibly some C—CH_3 groups. No other groups were evidenced. This would indicate that the compounds present are paraffinic or naphthenic hydrocarbons." The infrared spectrums showed no evidence for the presence of C—O, C=O, or O—H linkages, but the sample was not large enough to establish that these linkages were not present in very small percentage amounts.

TABLE 4

Results of growing sulfate reducer no. 45:117 for 45 days at 32 C in 16 liters of mineral salts solution overlayered with H_2

Total amount of H_2 consumed (standard conditions)	19,500 ml
Total amount of CO_2 consumed (including carbonates)	4,110 ml
Total amount of H_2S produced (standard conditions)	2,155 ml
Ratio of H_2/H_2S (19,500/2,155)	9.1
Ratio of $(H_2—H_2^*)/CO_2$ (19,500-8,620/4,110)	2.6
Terminal pH of medium (from initial pH 7.7)	8.4
Terminal E_h of medium (from initial E_h near 0)	−310 mv
Weight of cell substance recovered (dry basis)	3.67 g
Weight of CCl_4-soluble fraction	0.75 g
Weight of unsaponifiable material	0.148 g

It is not to be concluded from these and similar observations that autotrophic sulfate reducers produce crude oil, although traces of oily, CCl_4-soluble, unsaponifiable compounds, definitely more reduced than (CH_2O), are produced as part of the bacterial cell substance. The production of paraffinic hydrocarbons by heterotrophic cultures of sulfate-reducing bacteria was reported by Jankowski and ZoBell (1944), and the observations of Oakwood (1944) suggest the rather common synthesis of hydrocarbons by micro-organisms as part of their cell substance.

Table 4 summarizes the results of an experiment similar to that previously described. The CCl_4-soluble unsaponifiable material produced by H_2-consuming sulfate reducers growing in inorganic medium had a consistency and appearance of amber colored grease. Spectroscopic examinations indicated that at least part of it consisted of paraffinic or naphthenic hydrocarbons.

The ability of autotrophic sulfate reducers to utilize CO_2 as the sole hydrogen acceptor was demonstrated by growing cultures for several generations (seriatim transfers) in mineral salts solution containing less sulfate than one part per million (calculated from impurities in reagents used to prepare medium). Neither growth nor H_2 uptake, however, was nearly as rapid in sulfate-free media as in

similar media enriched with sulfate (table 5). In order to minimize the amount of sulfate carried into the medium in the first transfer, washed cells were used, which may account for activity slower than that in the second and subsequent transfers. There was no evidence for the cultures becoming acclimatized to sulfate-free medium nor did they lose their ability to reduce sulfate. When transferred back into sulfate medium, the cultures produced H_2S vigorously.

Hydrogenase activity by "resting" cells. Washed cells suspended in mineral solution in Warburg microrespirometer flasks were found to utilize sulfate almost exclusive of CO_2 or carbonate as hydrogen acceptor. Cells of H_2-consuming sulfate reducers were harvested by centrifugation and then washed repeatedly in phosphate-saline buffer solution containing a trace of sodium sulfide to maintain reducing conditions essential for the stability of hydrogenase. The washed cells were placed in the respirometer flasks with magnesium sulfate or sodium bicarbonate solution to serve as hydrogen acceptor in one of the side arms of the flask. Filter papers soaked with KOH were placed in alternate

TABLE 5

H_2 consumed and H_2S produced by culture 43:11 in 2 weeks at 28 C per liter of mineral media containing sulfate or carbonate

HISTORY OF CULTURE	CO_2 OR CARBONATE ONLY		CARBONATE PLUS SULFATE	
	H_2 consumed	H_2S produced	H_2 consumed	H_2S produced
	ml	*ml*	*ml*	*ml*
First transfer	1	0.0	46	4.1
Second transfer	12	0.0	106	11.3
Third transfer	14	0.0	87	12.0
(Sterile control)	0	0.0	0	0.0

flasks for H_2S absorption. The flasks were agitated for 15 minutes while flushing pure H_2 through the system. The manometers were closed while filled with H_2 and then equilibrated. After further agitation, the sulfate or bicarbonate solution was tipped into the flasks containing the washed cells, and the flasks were again agitated. Periodic readings were taken to determine change in volumes from which the amount of H_2 consumed and H_2S produced was calculated.

In systems to which no carbonate had been added, it was found that the endogenous metabolism of the washed cells resulted in the liberation of a little CO_2. This observation, coupled with finding no H_2 uptake in the absence of sulfate or other hydrogen acceptor, indicated that CO_2 is not readily reduced by "resting" cells of sulfate reducers. The H_2/H_2S ratios of most cultures of autotrophic sulfate reducers approximated the theoretical value of 4 for sulfate, 3 for sulfite, and 2.5 for thiosulfate (table 6).

Warburg microrespirometer experiments confirmed tests with actively growing cultures that H_2-consuming sulfate reducers utilize sulfite and thiosulfate more readily than sulfate. The sodium salts of these substances were used in the experiments summarized by the data in table 6. Elementary sulfur failed

to serve as an hydrogen acceptor in the Warburg experiments, although we have confirmed the observations of Baars (1930) that certain actively growing cultures of heterotrophic sulfate reducers reduce free sulfur.

Washed cells of sulfate reducers with H_2 in Warburg flasks were found to reduce fumarate considerably more rapidly than sulfate. Fumarate is believed to be reduced to acetic acid:

$$HC—COOH{=}CH—COOH + 2H_2 \rightarrow 2CH_3COOH$$

H_2 consumption by cultures of hydrogenase-producing sulfate reducers, growing

TABLE 6

H_2 consumed and H_2S produced at 30 C per hour per mg (dry basis) of washed cells of sulfate reducers in microrespirometers containing different hydrogen acceptors

LOT	CULTURE NUMBER	HYDROGEN ACCEPTOR	H_2 CONSUMED	H_2S PRODUCED	H_2/H_2S
			mm³	*mm³*	
A	41:38	Sulfate	3.4	0.9	3.8
A	41:38	Carbonate	0.0	0.0	—
A	41:38	Sulfate + Carbonate	4.1	0.9	4.5
B	43:76	Sulfate	8.5	2.4	3.5
B	43:76	Carbonate	0.0	0.0	—
B	43:76	Sulfate + Carbonate	7.9	2.0	3.9
C	43:38	Sulfate	10.6	2.5	4.2
C	43:38	Sulfite	22.8	7.4	3.1
C	43:38	Thiosulfate	20.7	7.8	2.6
D	41:76	Sulfate	6.5	1.5	4.3
D	41:76	Sulfite	17.8	5.4	3.3
D	41:76	Thiosulfate	14.2	5.2	2.7
E	43:6	Sulfate	13.0	3.1	4.2
E	43:6	Fumarate	29.4	0.0	—
F	43:63	Sulfate	8.7	2.1	4.1
F	43:63	Fumarate	12.3	0.0	—

in media enriched with either linolenic or linoleic acid as the only hydrogen acceptor, indicated that both of these unsaturated fatty acids were reduced, but we have not yet confirmed the reduction of these substances with "resting" cell technique.

Nitrogen fixation by sulfate reducers. The reduction or fixation of nitrogen by hydrogenase-producing sulfate reducers has been reported by Sisler and ZoBell (1951) in which inverted vials were filled with N_2 to serve as controls alongside vials filled with H_2; a diminution in the volume of N_2 was observed in inoculated media. For this reason helium was adopted for routine use as the inert gas in controls because its volume remained constant except in so far as its solubility

was influenced by temperature or pressure changes. Mass spectrometer analyses established that N_2 actually disappeared along with the consumption of H_2 by sulfate-reducing bacteria.

Mass spectrometer data also showed that the ratio of N_2 to argon in gas mixtures decreased under the influence of H_2-consuming sulfate reducers. Since the N_2/A ratio for the atmosphere is fairly constant, any decrease in the ratio in a biological system is suggestive of nitrogen fixation.

Although calling for confirmation by conventional Kjeldahl procedures, these observations on the disappearance of N_2 in cultures of hydrogenase-producing sulfate reducers assume some significance in the light of the recently expressed belief of Lindstrom *et al.* (1949, 1950) that all hydrogenase-producing bacteria can fix nitrogen.

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SUMMARY AND CONCLUSIONS

Most of the pure cultures of sulfate-reducing bacteria isolated from marine sediments produce hydrogenase as indicated by their ability to utilize H_2, 33 out of 39 cultures tested showing this ability in lactate medium. Eleven of them multiplied and consumed H_2 as the sole source of energy in purely inorganic medium. A few cultures developed the ability to utilize H_2 after being cultivated in its presence for several generations, which suggests that hydrogenase may be an adaptive enzyme for *Desulfovibrio* species.

The ratio of H_2 consumed to H_2S produced approximated the theoretical value of 4 for the reduction of sulfate by "resting" cells, but greatly exceeded 4 in cultures growing in inorganic medium, thereby proving the reduction of something besides sulfate. The reduction of CO_2 was established both by a decrease in the CO_2 content of inorganic medium and also by the synthesis of bacterial cell substances. An appreciable part of the latter was more reduced than (CH_2O) as indicated by $(H_2\text{-}H_2^s)/CO_2$ ratios exceeding 2, H_2^s being the quantity of H_2 required to account for the H_2S formed.

Up to 30 per cent of the bacterial cell substance was CCl_4-soluble, of which fraction 10 to 20 per cent, or up to 3 to 6 per cent of the cell substance, was an oily unsaponifiable material, probably consisting at least in part of paraffinic or naphthenic hydrocarbons. Traces of methane were produced by 6 pure cultures of autotrophic sulfate reducers.

Warburg microrespirometer experiments with "resting" cells confirmed cultural tests that *Desulfovibrio* species consume H_2 more readily in the presence of sulfite or thiosulfate than sulfate. Fumarate was likewise more readily reduced than sulfate.

The loss of N_2 from cultures, as well as observed decreases in the nitrogen/argon ratio demonstrated by mass spectrometer analyses, indicates that certain hydrogenase-producing bacteria fix nitrogen.

The ability of certain sulfate-reducing bacteria found in marine sediments to consume H_2 may help to account for the general absence of H_2 in natural gases.

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