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Recent Advances in the Study of the Sulfate-Reducing Bacteria

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INTRODUCTION

The sulfate-reducing bacteria form a specialized group of microbes that use sulfate as terminal electron acceptor for their respiration. Though many microbes generate H_2S metabolically, sulfate often being the primary source of that H_2S, the process is normally a small-scale one involving the incorporation of sulfur into cell protein and its subsequent degradation by catabolic and autolytic processes. Dissimilatory (or respiratory) sulfate reduction is a direct process that involves 10- to 100-fold greater turnovers of sulfur. It is of great physiological interest because of the analogies sulfate respiration provides with oxygen and nitrate respiration; for example, the metabolism of these bacteria is essentially oxidative, involving a cytochrome system and respiratory chain phosphorylation, yet to the bacteriologist they are among the most awkward and exacting of anaerobes. Their carbon metabolism is generally incomplete, yielding fatty acids as well as CO_2, yet with certain substrates (pyruvate, choline), some strains can conduct "sulfate-free" growth analogous to the facultatively anaerobic growth of an aerobe. Recently a most exciting vista of anaerobic assimilatory processes, such as that performed with *iso*-butanol, has been uncovered.

Despite their anaerobic habit, representatives of the group tolerate some of the most extreme terrestial conditions of heat, cold, salinity, and pressure, so that, in an era when science may be poised on the discovery of a true exobiology, knowledge of bacteria so firmly independent of the common terrestial environment assumes renewed interest. In more mundane terms, the ubiquity of these bacteria and their proneness to generate large quantities of H_2S lead to a variety of impressive industrial, economic, and ecological effects, and for this reason papers relevant to them tend to be scattered over journals ranging from purely academic to those devoted to detailed technology. During the past 20 years, considerable advances have been made in our understanding of these bacteria; yet, no doubt because of its scattered character, information about these bacteria is not always readily accessible, and, as a glance at any text book of microbiology will show, this information has not penetrated far beyond the circle of those directly concerned with sulfate-reducing bacteria.

In two previous reviews (137, 139) I covered several aspects of academic knowledge of sulfate-reducing bacteria and attempted to deal exhaustively with their economic activities. Because of space limitations, certain aspects of the

academic side were not covered, notably their nutrition, hydrogen metabolism, chemical composition, and inhibition. The present review, then, will attempt to cover these topics exhaustively but will otherwise bring together mainly such information as has come to my attention between the dates of publication of my previous reviews and the end of 1964. A general survey of these bacteria was given by Starkey (184); reviews of more limited aspects of their behavior will be cited under the appropriate heading below.

CLASSIFICATION

Coleman (49, 50) described a new mesophilic, sporeforming organism isolated from the rumen of a hay-fed sheep. It had superficial resemblances to *Desulfovibrio orientis* but was not curved; it has since proved, by serological and deoxyribonucleic acid (DNA)-composition tests, to be a mesophilic relative to the sporogenous thermophile *Clostridium nigrificans* (147), the base composition of which is remote from that of six other clostridia (160). A slight serological relationship between *D. orientis* and the other two sporogenous types was observed; it seems likely that *D. orientis* was incorrectly assigned to the genus *Desulfovibrio* by Adams and Postgate (55). A case has been made for classifying all three sporogenous types in a separate genus for which the name *Desulfotomaculum* has been proposed by Campbell and Postgate (46). This name will be adopted for the rest of the present review; the three known species of this genus with their new names follow: *Desulfotomaculum nigrificans*, earlier *Clostridium nigrificans*; *Desulfotomaculum orientis*, earlier *Desulfovibrio orientis*; *Desulfotomaculum ruminis*, earlier "Coleman's organism."

The name *Desulfovibrio* will be retained for the nonsporulating types. A new species of nonsporulating vibrio was described and named *Desulfovibrio gigas* by LeGall (109). It is large, granular in appearance, and shows lophotrichous flagellation. A survey of the buoyant density of DNA from many strains of *Desulfovibrio* by Saunders et al. (159) showed three categories: group 1 [61% guanine + cytosine (G + C)], group 2 (52% G + C), and group 3 (45% G + C); by one of the remarkable coincidences that sometimes advance science, Saleh (157) was surveying the resistance to inhibitors of an almost identical group of strains and observed that they fell into three categories according to their tolerance of a proprietary bactericide Hibitane (chlorhexidine; bis-*p*-chlorophenyldiguanidohexane diacetate). With one exception the chlorhexidine resistance categories overlapped those of DNA composition, suggesting that these properties will be of value for compiling a rational classification of *Desulfovibrio* species. Attempts to reisolate *Desulfovibrio rubentschikii*, all unsuccessful, were published (161), and renewed doubts were cast on the real existence of this species. A choline-fermenting anaerobe ("*Vibrio cholinicus*," 74) was identified by growth, serology, and DNA-composition tests as an ordinary *Desulfovibrio* (13, 166, 171).

GROWTH AND NUTRITION

Growth Curves and Yields

Desulfovibrio cultures normally show linear growth and, indeed, provide excellent examples of such growth; typical growth curves are available in an early publication by Senez (162), and figures that may be found in the literature purporting to be exponential are often seen to be linear or indeterminate on careful inspection of the experimental points. Linear growth may arise for three reasons. (i) Sulfide accumulates in the cultures during growth and acts as a growth inhibitor. (ii) Sulfide precipitates iron, which is required as a micronutrient, and thus decreases its availability. (iii) Sulfide evaporates during growth, thus lowering the *p*H value of the culture. This *p*H change alters both growth rate and yield and, in extreme cases, can cause alkaline lysis of the population.

A fourth, practical, problem in determining growth rates and yields arises because media containing sufficient iron salts for optimal growth darken as the culture grows, thus making photoelectric turbidimetry difficult.

Unpublished experiments by L. L. Campbell and myself indicate that sulfide inhibition takes place at least partly by mechanism (ii), because nonlinear growth curves can be obtained if citrate or ethylenediaminetetraacetate (EDTA) is added to the medium. Use of a weak-base sulfate, together with a continuously renewed atmosphere containing CO_2 to buffer the medium and remove sulfide, permits exponential growth in a defined medium at a minimal doubling time of about 3 hr to produce cell yields in the region of 0.75 mg (dry weight) of organism per ml. LeGall and Senez (110) and Senez (164) quoted exponential-growth rates and final cell yields, but they have so far published insufficient experimental data for one to judge whether these parameters were optimal in their experiments. Quantitative data based on growth rates and yields of sulfate-reducing bacteria should be interpreted with reserve unless the above considerations have been explicitly allowed for.

Inorganic Nutrition

Butlin, Adams, and Thomas (43) showed that *Desulfovibrio* has an absolute requirement for inorganic iron. Postgate (136) determined the optimal concentration at 10 to 15 μg-atoms of Fe per liter; his strain was capable of growth without sulfate in pyruvate media and had no absolute iron requirement in those circumstances. Hata (69) confirmed a requirement for iron. Hata (70) showed that marine *Desulfovibrio* required Na^+, K^+, Mg^{2+}, SO_4^{2-}, Cl^-, CO_3^{2-}, and $H_2PO_4^-$ for growth but not Ca^{2+} or Br^-. Senez and Pascal (166) stated that *Desulfovibrio* requires HCO_3' for growth with choline; a requirement for HCO_3' in complex media was also recorded (134). The sporeforming sulfate-reducing bacteria contain cytochromes, and, thus, an iron requirement is to be expected; it has been reported in the case of *Desulfotomaculum ruminis* (50).

Organic Nutrition

Stimulation of growth or sulfide yield, or both, by materials such as yeast extract or peptone has been reported often (19, 38, 43, 119, 134). Postgate (134) analyzed the effects of peptone and yeast extract by replacement studies and attributed them to their content of serine, ornithine, isoleucine, and cysteine; his early experiments were confused by use of an impure culture, but the finding was still valid for purified strains of *Desulfovibrio* (135). Grossman and Postgate (66) then observed that the effect of cysteine had nothing to do with nutrition: it poised the E_h value of the medium at a level favorable to growth and could be, for example, replaced by Na_2S. Unpublished work by the writer indicates that ornithine, serine, and isoleucine can be replaced by the chelating agent EDTA; consequently these amino acids may simply render inorganic iron available in the presence of sulfide. That amino acids and peptones increase the solubility of FeS has been known for some years (122). Thus, the situation returns to the position held in 1949: inorganic iron partly accounts for the growth-stimulating effect of yeast extract; the other stimulants are unidentified and may not, in fact, be real in a nutritional sense. Similar criticisms must be applied to some recent work in this field. Kadota and Miyoshi (93, 94) found that 18 amino acids plus adenosine triphosphate (ATP) would replace ammonium for a salt-water strain of *Desulfovibrio* (strain "*maizuru*"). In view of the involvement of ATP in sulfate activation (below), unqualified evidence that ATP has growth factor activity would be of great interest; unfortunately Kadota and Miyoshi's reports must be criticized on four grounds: (i) there is no evidence that chelating effects on traces of iron were considered; (ii) the buffering power of their medium was so slight as to render yield experiments dubious; (iii) despite the report to the contrary of Kimata, Kadota, and Hata (100), their strain grows well with ammonium as sole nitrogen source in other laboratories; and (iv) preparation of their basal medium involved filtration after autoclaving (93), a procedure which is known to remove iron by a coprecipitation mechanism. Although one must doubt the evidence so far presented by Kadota and Miyoshi, it is noteworthy that Macpherson and Miller (116) claimed stimulation of growth of a fresh-water strain by ATP and amino acids. The question requires re-examination, and at present the status of ATP as a micronutrient growth stimulant is doubtful.

Several published studies have used sulfide rather than cell yield as an index of growth. Stimulation of sulfide yield by excess ferrous salts (38), and ascorbate or cysteine (148) has been reported. These effects can reasonably be attributed to the reducing action of the additives on the media. Iya and Sreenivasaya (87) noted that urea, peptone, or casein hydrolysate accelerated sulfide formation by a salt-requiring strain [Postgate (134) found no effect of urea on growth yield]. Bunker (38) and Miller (119) showed that a wide variety of organic materials accelerated sulfide formation; Wiken and Ghose (196) found a similar effect with biotin at 25 μμM [biotin at this concentration did not affect the cell yield of the Hildenborough strain (Postgate, *unpublished data*)]. Sulfide yields depend stoichiometrically on the carbon source and are influenced both by chelating agents and by the E_h of the medium; the significance of studies on sulfide yields to understanding the nutrition of these bacteria is therefore marginal.

Coleman (50) showed that *D. ruminis* required iron, *p*-aminobenzoic acid, and biotin for growth; otherwise, the nutrition of *Desulfotomaculum* species has not been studied systematically.

Autotrophy

Though sulfate-reducing bacteria are widely believed to be facultative autotrophs, the reports of Mechalas and Rittenberg (117) and Postgate (140) cast doubt on their autotrophic status. The proportions of labeled CO_2 incorporated during putatively autotrophic growth never exceeded 15 to 25% of the cell carbon and usually only differed slightly from the amounts of CO_2 assimilated

during heterotrophic growth. This conflict with earlier findings might be dismissed as due to strain differences, but the strain used in this work was in fact "Hildenborough," one of those found by Butlin and Adams (42) to establish autotrophy originally. The explanation seems to depend on the fact that even the purest laboratory reagents contain traces of organic matter; for example, 0.067 M phosphate buffer can support growth of about 10^6 coliform bacteria per milliliter (61, 187). These impurities support growth of *Desulfovibrio* and the illusion of autotrophy occurs, because, when incubated under hydrogen, the bacteria utilize the impurities more efficiently because they obtain energy from the H_2-sulfate reaction. Consequently, better growth on impurities appears under H_2 than under N_2, under A, or in vacuo. Mechalas and Rittenberg showed that H_2 is an inorganic representative of several substrates that are energy, but not carbon, sources for *Desulfovibrio*, and they studied particularly the case of *iso*-butanol. Growth with this substrate required yeast extract, and neither labeled CO_2 nor *iso*-butanol carbon was incorporated into the cells appreciably, though *iso*-butanol was oxidized stoichiometrically to *iso*-butyric acid. Postgate (142) isolated a strain uniquely able to utilize oxamate and showed that the function of oxamate was probably similar to that of H_2: it was an energy, but not a carbon, source. Oxamidase, oxalic decarboxylase, and formic hydrogenlyase activities were demonstrated, indicating that oxamate was formally equivalent to hydrogen in the metabolism of this strain. Assimilation reactions coupled with oxidation of energy sources such as H_2, *iso*-butanol, or oxamate recall the function of glucose in the metabolism of *Streptococcus faecalis* (see 18) and are probably of wide importance in the ecology of *Desulfovibrio*; their use of hydrogen as an energy source for such reactions is at present a unique example of "litho-heterotrophy" among microorganisms. Comparable assimilatory reactions should be sought among other chemotrophic bacteria, particularly those that give poor growth yields in "autotrophic" conditions.

ESTIMATION AND CULTIVATION

Abd-el-Malek and Rizk (1) published evidence that the presence of contaminants was not necessary for the success of their counting procedure involving the use of an iron nail. Bufton (36) examined several media and showed that none was quantitatively satisfactory for the thermophile *D. nigrificans*. Postgate (143) published a procedure permitting colony counts on impure cultures and natural samples for which only most probable number (MPN) determinations were hitherto reliable. Genovese, Rigano, and La Cava (see 62*j*) had success with a comparable thioglycolate medium. Postgate (138, 146) described procedures for obtaining enrichment cultures and isolating pure cultures of sulfate-reducing bacteria.

Salt Tolerance

Ochynski and Postgate (123) showed that salt-water strains of *Desulfovibrio* differed from fresh-water strains in having lower osmotic fragility, a different amino nitrogen pool, and in forming a viscous mucin. Some, but not all, of the characters studied by Ochynski and Postgate were acquired or lost on training a salt- or fresh-water strain to grow in the opposite environment. The strains compared by these authors are now known to differ according to the DNA composition and chlorhexidine resistance tests mentioned above.

Dostalek and Kvet (57) observed that sulfate-reducing bacteria can be placed in three different groups described by their ability to grow in media of various salinities; Kutznetzova and Pantskhava (107) and Kutznetzova, Li, and Tiforova (108) used a similar principle in ecological studies to be discussed later; Hata (71) surveyed the salt tolerance of various strains of marine sulfate-reducing bacteria.

INHIBITION

A comprehensive survey of inhibitors tested with sulfate-reducing bacteria was published, with full quantitative details, by Saleh, Macpherson, and Miller (158). It is a valuable summary of an otherwise scattered literature to which the interested reader is referred. Notable points concerning inhibitor studies include the finding of Bennett and Bauerle (21) that mixed populations of *Pseudomonas aeruginosa* and *D. desulfuricans* show a different pattern of sensitivity to inhibitors from those shown by either species alone, and Saleh's (157) discovery that various strains of sulfate-reducing bacteria can be categorized according to their resistance to certain inhibitors. *Desulfotomaculum* species show greater general sensitivity to inhibitors than do the nonsporulating types. The latter show three classes of sensitivity to the microbicides chlorhexidine and cetyltrimethylammonium bromide. The taxonomic importance of this categorization was mentioned earlier; in the present context emphasis should be placed on the remarkably high resistance to these microbicides shown by Saleh's "group 3" strains of *Desulfovibrio*.

Of more academic importance is the finding of Ghose and Wiken (63) that short chain fatty

acids exert an inhibitory effect by lowering the growth rates of cultures. Acetate is an end product of growth in most conventional media, but the concentrations recorded by Ghose and Wiken as inhibitory are above those normally to be expected before the stationary phase of growth is reached.

HYDROGEN METABOLISM

Though Stephenson and Stickland (186) regarded hydrogenase as exceptional when they first discovered it in sulfate-reducing bacteria, it is now known to be widespread. Sisler and ZoBell (174) observed that 33 of 39 cultures absorbed hydrogen over nine weeks at 28 C. Twenty-four pure strains of *Desulfovibrio*, tested as nongrowing suspensions by the author, showed hydrogenase activity towards sulfate or dyestuffs. Adams et al. (7) reported a hydrogenase-deficient strain of *Desulfovibrio*, but the culture was lost. *D. ruminis* showed hydrogenase activity (50); *D. orientis* did not (5). Only four of seven strains of *D. nigrificans* examined by the author showed hydrogenase activity to dyestuffs. The substrates that act as hydrogen acceptors for sulfate-reducing bacteria are discussed briefly below.

Sulfur-containing anions such as sulfate, sulfite, thiosulfate, tetrathionate, etc. are reduced in hydrogen by intact cell suspensions of *Desulfovibrio*. *D. ruminis* reduced sulfate, sulfite, thiosulfate, or, apparently, persulfate (50), provided yeast extract was present in the reaction vessel. *D. nigrificans* does not reduce sulfate in hydrogen when tested as a washed suspension in phosphate buffer.

Dye reductions are generally faster than substrate reductions. Methylene blue can inhibit hydrogenase activity of *Desulfovibrio*, a phenomenon augmented by NaCl (101, 115), and methyl violet or a viologen dye seem less risky as substrates for tests with this genus. Methyl violet is unsuitable for *D. nigrificans* (9). Table 1 illustrates that the rate of dye reduction by *Desulfovibrio* may depend on a permeability factor: organisms made permeable to small molecules by treatment with a detergent reduced certain dyes more rapidly than did undamaged cells.

The "Knallgass" reaction occurs in *Desulfovibrio* as a consequence of the autoxidizability of cytochrome c_3 and has no known physiological importance (*see* 137).

Nitrate is not reduced by pure cultures of *Desulfovibrio* (170). Nitrite or hydroxylamine become reduced as a consequence of the autoxidizability of cytochrome c_3 (149, 167, 168). Hydroxylamine is reduced by hydrogenase from *D. nigrificans* (195).

Sorokin (176) obtained CO_2 fixation by resting *Desulfovibrio* utilizing hydrogen to reduce sulfate. CO_2 uptake could be separated from hydrogen absorption and involved no direct coupling of the hydrogenase and CO_2.

Certain *Desulfovibrio* strains reduce fumarate faster than sulfate in hydrogen (67, 174) to yield succinate. Fumarate and other carbon compounds accelerate the reduction of sulfate in hydrogen by resting suspensions (67, 163).

Selenite is reduced by extracts of *D. desulfuricans* (120) but not by intact cells (133) and is thus an extreme case of the permeability barrier referred to above. Cytochrome c_3 and flavins are reduced readily by enzyme preparations but slowly, if at all, by intact cells. Enzyme preparations from *Desulfovibrio* reduce ferricyanide (152), nitroprusside (104), and azo-pyridine but not several other azo-compounds (45). The deuterium exchange reaction and the ortho-para hydrogen conversion, as well as "reversed" evolution of H_2 from dithionite, have been demonstrated (103, 131, 149).

TABLE 1. *Influence of permeability on rate of dye reduction in hydrogen**

Acceptor dye	Q value†	
	Intact bacteria	Bacteria + CTAB
Janus green	690	3,100
Sodium indigodisulfonate	300	960
Phenol-indo-dichlorophenol	210	940
Methylene blue	2,730	2,730

* *Desulfovibrio desulfuricans* (Hildenborough) from a continuous culture was washed and tested for dye reduction under hydrogen by conventional Warburg manometry. The osmotic barrier was destroyed by adding cetyltrimethylammonium bromide (CTAB) at 0.1 mg/mg (dry weight) of equivalent organisms, and in some cases an augmented reduction rate was observed.

† Rates (Q) expressed as microliters of H_2 absorbed per milligram (dry weight) of organisms per hour.

Hydrogenase may be extracted from cells of *Desulfovibrio* by many conventional procedures; what proportion of the hydrogenase is truly soluble apparently depends on the strain of bacteria, the manner in which it is grown, and the extraction procedure. Rittenberg and Krasna (149) obtained exclusively particulate preparations, but Sadana and Jagannathan (154) obtained a soluble preparation using ostensibly the same strain. Sadana and his colleagues have obtained the purest hydrogenase preparation on

record from *Desulfovibrio* (153, 154, 155), including one that is free from cytochrome c_3. Riklis and Rittenberg (149*a*) later obtained a soluble hydrogenase from *Desulfovibrio* and estimated its molecular weight as between 9,000 and 15,000 from diffusion and ultracentrifugal studies. Krasna, Rickles, and Rittenberg (105) studied the inhibition pattern of hydrogenase from *Desulfovibrio* and showed that it resembled that of *Proteus vulgaris* though it conducted a more rapid exchange reaction between H_2 and D_2O. Sadana's group decided from inhibition studies that iron was a functional element in the prosthetic group of the enzyme, a view also favored by Rittenberg's school. Sadana's group claimed activation of purified enzyme preparations by $FeCl_2$ or $FeCl_3$, but Rittenberg's school, using less highly purified preparations, has not confirmed this report. Sadana and Rittenberg (156) provided added support for the view that iron is a cofactor by demonstrating light-reversible inhibition by carbon monoxide of the D_2-exchange reaction. Since contamination of preparations by iron-containing materials such as cytochrome c_3 or ferredoxin is probable, analytical data are not of great value in considering whether iron is involved. Postgate (136) showed that iron-deficient strains possess about one-fifth of their normal hydrogenase content, but his media may well have been deficient in trace metals other than iron as well.

A cell-free hydrogenase from *D. nigrificans* was particulate and, unlike the enzyme of *Desulfovibrio*, it did not reduce methyl violet: benzyl viologen was the least inhibitory substrate. Loss of activity after dialysis and its restoration by adding inorganic iron were demonstrated; inhibition by iron-chelating agents was also reported (9). A very similar particulate enzyme from *D. ruminis* also showed activation by iron (37). A curious finding, that cytochrome c_3 is reduced by hydrogenase from *D. nigrificans*, was mentioned by Valentine and Wolfe (195).

CARBON METABOLISM

The important observations of Mechalas and Rittenberg concerning the assimilation of nutrients by *Desulfovibrio*, together with their reflection on the autotrophic status of this group, were discussed earlier.

Stüven (188) studied an obligate salt-requiring strain of *Desulfovibrio* and mentioned that mucin formation was maximal in media based on pyruvate. Studies with carbon-labeled substrates indicated that heterotrophic and "autotrophic" assimilation took place and also that acetate, the normal end product of carbon dissimilation, was assimilated (189). CO_2 assimilation by *Desulfovibrio* during heterotrophic growth is more pronounced than by other heterotrophs: Postgate (140) quoted figures about three times those obtained with *Aerobacter aerogenes*, and Sorokin's (177, 178) strain achieved the impressive figure of 30% of its cell carbon fixed from CO_2 compared with 3 to 8% fixed by other heterotrophs. With lactate as growth substrate, Sorokin's strain fixed 30% of its cell carbon from the carboxyl group (179).

Though acetate is formed from substrates such as ethyl alcohol, succinate, malate, and lactate, Mechalas and Rittenberg (117) reported formation of homologues of acetate from homologues of ethyl alcohol (e.g., propionic acid from propanol). Formate does not yield acetate during growth and is probably formally equivalent to hydrogen and not a true substrate; certainly it gives very low yields of *Desulfovibrio*. Yagi (200, 201, 202) reported reversible oxidation of CO by sulfite in cell-free extracts, stimulated by formate. The reaction was essentially a generation of hydrogen from the reaction, $CO + H_2O \rightarrow CO_2 + H_2$, followed by the normal sulfite-hydrogenase interaction.

Postgate's (142) proposal of the pathway oxamate $\rightarrow$ oxalate $\rightarrow$ formate $\rightarrow H_2 + CO_2$ for oxamate metabolism by a special strain was mentioned earlier. Hayward and Stadtman (74) described an organism, later classified as *Desulfovibrio*, which was able to grow in sulfate-free media with choline as growth substrate. Sonic extracts decomposed choline to ethyl alcohol, acetate, and trimethylamine; the reaction was coupled with ATP formation and iron was involved (75). Acetaldehyde was formed in the dismutation reaction, and glutathione, nicotinamide adenine dinucleotide phosphate (NADP), and coenzyme A were needed in addition (73). Choline metabolism is fairly common among *Desulfovibrio* species (13, 166). Sulfate-free growth in pyruvate media has been reported in *D. nigrificans* (144) to yield acetate, hydrogen, and CO_2; Akagi (8) extracted its phosphoclastic system, which functions at 50 C and requires coenzyme A, thiamine diphosphate, and certain cations. *D. ruminis*, unlike *D. orientis*, also shows sulfate-free growth with pyruvate (147).

Kaplan and Rittenberg (98) have shown that fractionation of carbon isotopes takes place during growth of *Desulfovibrio* on lactate and, if sulfite is the electron acceptor, is greater the slower the culture grows. C^{12} is enriched in both cell carbon and CO_2.

SULFUR METABOLISM

The major advance in this field during the last 5 years has been the resolution of the sulfate

reductase system of *Desulfovibrio* by Peck (124) and by Ishimoto (81). Cell-free extracts of *Desulfovibrio* species were found to reduce sulfate provided stoichiometric amounts of ATP were present, and the primary activation step proved to be formation of adenosine-5-phosphosulfate (APS), not the phospho-adenosine phosphosulfate (PAPS) of sulfate esterification (82, 124, 126, 128, 129, 130). APS is formed from ATP and sulfate by the enzyme ATP-sulfurylase. This enzyme has been extracted from *Desulfovibrio* and considerably purified (10, 14). The ATP-sulfurylase of *D. nigrificans* has an increased heat stability over that from *Desulfovibrio* (10). APS is reduced by the enzyme APS-reductase which has also been extracted and partly purified (83, 129), and which yields sulfite, pyrophosphate (PP), and adenosine monophosphate (AMP) as the reduction products. Peck (128) showed that APS-reductase was also present in *Thiobacillus* species and that it is characteristic of organisms whose metabolism is based on a gross turnover of inorganic sulfur. Assimilatory sulfur metabolism is characterized by PAPS reduction (see also 77, 85). The APS-reductase of *Desulfovibrio* gives some reverse action when tested in the conditions in which thiobacilli form sulfate from sulfite (127). The reduction of APS is "pulled" in sulfate-reducing bacteria by a pyrophosphatase (124) which has been extracted and purified (11, 83, 130). Thus, the initial steps of sulfate reduction can be expressed as:

$$SO_4^{2-} + ATP \rightleftharpoons APS + PP$$
$$PP + H_2O \rightarrow 2\ P$$
$$APS + 2\ e \rightleftharpoons AMP + SO_3^{2-}$$

Peck (130) ably reviewed knowledge of these and associated reactions; details of their enzymology and enzyme chemistry will not be reviewed here.

During sulfate reduction these reactions lead to a net loss of one ATP molecule per sulfate ion reduced. Considering the reaction

$$2\ Lactate^- + SO_4^{2-} \rightarrow 2\ Acetate^- + 2H_2O + 2CO_2 + S^{2-}$$

which is known to pass through pyruvate and acetyl phosphate, it is obvious that substrate level phosphorylation is equivalent to the regeneration of one ATP molecule (equivalent to two acetyl phosphates) per sulfate ion reduced. Thus, respiratory chain phosphorylation must occur for the organisms to grow at all, and Peck (125) presented evidence for such reactions based on inhibitor studies. The energy balances in sulfate-reducing bacteria, particularly when growing with a relatively oxidized carbon source such as succinate, provide fascinating problems in bacterial energetics; it is unfortunate that the special growth characteristics of this group, discussed earlier, render studies of these problems by means of growth yields valueless at present.

Growth yield studies misled Senez (164) into proposing that sulfite required activation just as does sulfate, because cell yields were similar in sulfite and sulfate media. In fact, cell-free sulfite reduction takes place readily without ATP; Postgate's report (141) that ATP or acetyl phosphate augmented sulfite reduction can be attributed to the presences of sulfate in the substrate and of the sulfate reductase system in the particulate enzyme preparation. Though particulate preparations are active, soluble sulfite reductase preparations can be obtained and, by depletion with ion-exchange resins, the involvement of cytochrome c_3 claimed earlier has been demonstrated unequivocally by Ishimoto and Yagi (84). At least one, and possibly as many as three, electron-transporting cofactors seem to be involved in addition to c_3 during sulfite reduction (141). The later steps en route to sulfide are unknown and may not, in fact, be represented by stable intermediates.

Furusaka (59) criticized earlier work eliminating gross permeability of *Desulfovibrio* to sulfate and, using labeled sulfate, presented evidence that a pool of sulfate exists within the cell when sulfate is being metabolized; accumulation of sulfate in the pool was antagonized by selenate. His technique did not unequivocally identify the "pool" material as sulfate: any intermediate sulfur derivative would have been recovered as sulfate, but it provided evidence that radioactive sulfur accumulated in the cells at about one-eleventh of the rate of its reduction. A 10-fold difference in rate roughly distinguishes dissimilatory from assimilatory sulfate reduction; since dissimilatory sulfate reducers must also conduct assimilatory reactions the possibility remains that Furusaka's accumulated sulfur compounds were unrelated to the respiratory reduction of sulfate to sulfide. Kaplan and Rittenberg (97) considered the relevance of isotope fractionation data to the question of the existence of a sulfate pool and concluded that no useful deduction would be made, because an improbably strong pool (2 M with respect to sulfate) would be required to interpret the considerable fractionations performed by these bacteria. The fractionation of sulfur isotopes conducted by sulfate-reducing bacteria is of considerable importance in geochemistry and ecology (see below); it appears at its maximal extent when the bacteria are growing slowly at a relatively low temperature (91, 92, 96, 97, 121) and is generally more pronounced in nature than in the laboratory (121).

NITROGEN METABOLISM

Sisler and ZoBell's (174) early claim that certain strains of *Desulfovibrio* fix atmospheric nitrogen was confirmed by LeGall, Senez, and Pichinoty (111), who isolated new strains (the "Berre" strains) and deposited them with a recognized collection. LeGall and Senez (110) reported that growth was slow and yields were low when these strains grew with gaseous nitrogen. Most strains of *Desulfovibrio* do not fix nitrogen. Other aspects of the nitrogen metabolism of this group have shown little advance apart from Cattaneo and Senez's (47) 330-fold purification of a β-aspartate decarboxylase from *Desulfovibrio*.

CHEMISTRY AND FUNCTION OF CERTAIN CELL CONSTITUENTS

Subba Rao (190) found most of the common amino acids in hydrolysates of a salt-water *Desulfovibrio*. Work and Dewey (199) identified diaminopimelic acid. Pools of free amino material exist in *Desulfovibrio*, the character of which depends on the salt habit of the strain examined; sporeforming sulfate-reducing bacteria have little pool material (123). Qualitative analysis of cell walls of *Desulfovibrio* showed the usual amino acids, including diaminopimelic, together with glucosamine and a pentose resembling ribose (123).

Many workers have recorded the presence of mucoid material, particularly in old culture of *Desulfovibrio*. In the Hildenborough strain it is a muco-polymannoside (123, 183); its synthesis is related to the salt habit of the strain for reasons that are not clear.

Cytochrome c_3 was reviewed by the author (141). Takahashi, Titani, and Minakami (193) published its amino acid composition, Horio and Kamen (79) crystallized it and reported on various physical properties, and Corval, Horio, and Kamen (52) described its amino acid composition, noting its high content of cysteine and lysine. Slight differences between the findings of Takahashi et al. and those of Corval et al. may be attributed to strain differences between the organisms used. Unqualified involvement of cytochrome c_3 in sulfate reduction has been shown and was discussed in the section on sulfur metabolism. Cytochromes of the *b* type have been reported in both mesophilic *Desulfotomaculum* species (5, 147), but the evidence for cytochromes in the thermophiles remains oblique. Pyridine hemochromes may be found spectroscopically after suitably destructive treatment of the organisms.

The function of the porphyroprotein desulfoviridin has become more mysterious with the report of Miller and Saleh (118) of strain Norway 4, a putative mutant of *Desulfovibrio* that is devoid of this pigment. Though morphologically unusual, strain Norway 4 seems to be biochemically normal.

Ferredoxin has been extracted from *Desulfovibrio* (192), but its function is obscure. It will replace the natural nucleotide reductase of spinach leaves. Valentine and Wolfe (195) showed that ferredoxin from *Clostridium pasteurianum* would act as cofactor for pyruvic phosphoclastic enzymes extracted from *Desulfovibrio*, but they did not use the native ferredoxin from *Desulfovibrio*. Akagi (8) found that *C. pasteurianum* ferredoxin is active in the phosphoclastic system of *D. nigrificans*, though he could extract no native ferredoxin from *D. nigrificans*. This finding partly conflicts with Valentine and Wolfe's (195) earlier report that *C. pasteurianum* ferredoxin was not active for the phosphoclastic system of *D. nigrificans*; it was also inactive in hydroxylamine reduction by hydrogenase from *D. nigrificans* and in the sulfite-hydrogenase system of *Desulfovibrio*.

Coenzyme Q is absent from *Desulfovibrio* (112); a statement to the contrary (51) was mistaken (T. Cook, *personal communication*).

Data on the DNA base composition of various strains are available; serological data have also been obtained and used in taxonomic studies. Both subjects were discussed in the first section of this review in which recent literature citations may be found.

ECONOMIC ACTIVITIES

This section brings up to date my earlier review (139) of this subject.

Waters and Soils

Furusaka and Hattori (60) used the hydrogenase activity of paddy field soil samples towards sulfate, sulfite, or thiosulfate as a measure of their content of sulfate-reducing bacteria. Silica gel may be used as a soil substitute in laboratory experiments designed to mimic the natural environment (72). Desrochers and Fredette (55) showed that the population of sulfate-reducing bacteria in the Ottawa River increased dramatically downstream from a paper mill. Lighthart (114) showed that their growth in a California reservoir was associated with a marked drop in redox potential; he recorded bacterial numbers. Studies by Kadota's group on the ecology of sulfate-reducing bacteria in certain Japanese waters were summarized in English by Kadota and Miyoshi (95).

General experience with polluted waters seems

to indicate that counts of 10^4 to 10^6 sulfate-reducing bacteria per milliliter are associated with serious pollution; sewage may have counts as high as 10^7 per milliliter. Data for populations of sulfate-reducing bacteria at various times and locations in the river Thames over a period of 12 months are available in a report by Booth, Cooper, and Tiller (33) on corrosion experiments. Their report did not specify that a counting medium of poised E_h was, in fact, used (G. Booth, *personal communication*). Sorokin (180) used SO_4^{35} measurements to show that sulfate-reducing bacteria were most active in the near-shore sediments of the Black Sea. He has published several papers on the use of isotopes for determining microbial activity in natural ecosystems, normally including sulfate-reducing bacteria among the types investigated. His findings emphasize the importance of chemotrophic CO_2 fixation in the carbon cycle and give some measure of the contribution of sulfate-reducing bacteria to this cycle. This and related work is reviewed in two recent articles (181, 182); the former has an almost complete bibliography of Sorokin's work in this field. Kaplan, Emery, and Rittenberg (99) obtained the plausible count of 3.5×10^7 sulfate-reducing bacteria per milliliter of marine sediment off Santa Barbara by what is possibly the most back-handed count on bacteriological record: back-calculation from isotope fractionation data. Isotope fractionation is of great importance in studying the ecological and geological activities of these bacteria and has been the subject of a symposium (88); extreme isotope fractionation may occur in certain natural environments (54). Genovese, in a sequence of papers (62, 62*a* to *k*), has continued his studies of Lake Faro in Messina, recorded a new outbreak of red water (due to Thiorhodaceae), and recorded counts, sulfide concentrations, and E_h values at various levels over a long period. A paradoxical situation exists in the deeper mud, where the H_2S concentration is high but the bacterial count is low. Wood (198) reported an unusual type of water pollution due to these bacteria: they increased the Fe content of water in an iron main by corrosion, and the black FeS suspension subsequently yielded "rusty" water from domestic taps. Unaerated deep well water is likely to cause this problem; it can be solved by aeration (197).

Adams and Postgate (6) recorded that the heat-resistant sulfate-reducing bacteria in soils were not *Desulfovibrio* but were of the mesophilic *Desulfotomaculum* type. Among the limited numbers of soils examined they were the more ubiquitous. Ochynski and Postgate (123) mentioned the curious absence of reports of sporeformers from marine environments, despite the readiness with which fresh water strains of *Desulfotomaculum* acclimatize to seawater. Senez (165) reviewed the ecology of sulfate-reducing bacteria with particular emphasis on their activities in the sea.

Effluent Treatment

The use in India of crude cultures of sulfate-reducing bacteria for the treatment of distillery wastes has been reported (17, 64, 65). Comparable work has continued in Czechoslovakia (15, 16) where the bacteria were used to treat distillery and citric acid wastes. In both instances assignment of the effective organism to the species *Desulfovibrio rubentschickii* is probably incorrect. An important advantage, in general sewage works practice, of a sulfate-reducing fermentation as against a methane fermentation for the treatment of sewage sludge lies in the decreased water content of the digested product (39, 102).

Mineral Formation

The British project for the production of sulfide by bacterial fermentation was discontinued It had reached the stage of economic feasibility (44) and had shown the economic bonus of improved dewatering if sewage sludge were used as reducing agent. Accounts of pilot plant experiments in a sewage works were given (39, 40, 41). Pipes (132) studied a laboratory model of the sulfide fermentation of sewage. Details of yields as well as extraction, disposal, and corrosion problems associated with the process are now available; it may be expected to be revived as the world's resources of native sulfur become depleted once more. That the formation of natural sulfur from gypsum involves sulfate-reducing bacteria in the reductive steps is confirmed by quantitative counts (86, 151). Ivanov (see 106) attributed 20% of the oxidative step to thiobacilli and 80% to chemical action in the Or-Shu sulfur beds of the U.S.S.R.

Mineral sulfide and other reduced sulfur deposits occur where *sulfureta* are active, though Gunkel and Oppenheimer's (68) data indicate that much of the reduced sulfur in marine sediments originates from organic sulfur. Ault and Kulp (12), from a study of sulfur isotope ratios in very many deposits, have questioned the suggestion of Thode's school that the earliest *sulfureta* occurred some 8×10^8 years ago, and have detected biological isotope ratios in rocks as old as 2×10^9 years. Jensen (88) edited a symposium on the biogeochemistry of sulfur isotopes.

Berner (22) implicated sulfate-reducing bacteria in the formation of hydrotroilite, a precursor of iron pyrites. A remarkable observation,

that in special circumstances these bacteria gave rise to a magnetic iron sulfide, was reported by Freke and Tate (58). Sukow and Schwartz (191) added iron and copper salts to laboratory *sulfureta* and noted that their behavior was consistent with a probable function in the natural formation of copper sulfide ores. Davidson (53) criticized the idea of a biogenic origin of copper sulfide on the grounds that *Desulfovibrio* was excessively sensitive to copper ion toxicity, a criticism supported by Booth and Mercer (27). Cheney and Jensen (48) rightly objected to Davidson's argument on the grounds that much soil copper is nonionic and presumably nontoxic; Booth and Mercer's argument took no account of the detoxifying effect of H_2S towards copper and thus ignored the point that an actively growing population of sulfate-reducing bacteria could precipitate and therefore detoxify copper far in excess of the concentration required to prevent the initiation of growth. Studies on the sulfur isotope distribution in copper sulfide deposits might be valuable in this context. Abd-el-Malek and Rizk (2, 3, 4) confirmed the hypothetical role of sulfate-reducing bacteria in the formation of natural soda deposits by a chemical and bacteriological examination of Wadi Natrun in Egypt. Jensen and Nakai (89), from isotope fractionation data, attributed a biogenic origin to most of the atmospheric sulfur outside industrial areas.

Corrosion

Reviews of bacterial corrosion were published by Postgate (145) and Booth (26). Booth and his colleagues have made impressive progress in establishing that cathodic depolarization is a major mechanism of anaerobic corrosion by sulfate-reducing bacteria, correlating corrosion rates and polarization curves with the hydrogenase contents of the strain and species used (30, 31, 32, 194). Anodic processes may influence the corrosion rate with certain strains, however. Hoar and Farrer's (78) evidence against cathodic depolarization was insubstantial and has been properly criticized (25). The view that tannates inhibit growth may now be dismissed and so may the claim that they protect against anaerobic corrosion: careful experiments show that mimosa tannin may somewhat augment such corrosion (24, 29). The preservation of ancient iron objects in certain environments, once attributed to tannates, seems due to a particularly impenetrable phosphate coating (34, 35). Tannates lower the hydrogenase contents of *Desulfovibrio* strains (28); so, as mentioned earlier, does iron deficiency, and it is likely that the effects of tannates in this direction depend on their chelating effects on iron. Solti and Horvath (175) and Horvath (80) studied polarization curves of iron in the presence of sulfate-reducing bacteria and also supported the cathodic depolarization mechanism; Blanton and Oppenheimer (23) published an elementary demonstration of a function of sulfate-reducing bacteria in marine corrosion. Booth, Cooper, and Tiller (33) studied corrosion rates of steel samples in the river Thames and associated these findings with counts of sulfate-reducing bacteria in the water. Their experiments are being continued and should ultimately give an estimate of the importance of aerobic versus anaerobic corrosion in this environment.

Pitting of aluminum by *Desulfovibrio* has been claimed by Hedrick et al. (76), whose publication includes the impressive self-contradiction that the bacteria were cultured aerobically.

Oil Technology

Baumgartner (20) has reviewed the role of sulfate-reducing bacteria in corrosion and the blockage of injection waters. Dostalek (56) has provided more details of enhanced secondary recovery after injection of sulfate-reducing bacteria into oil wells. Some progress has been claimed on ZoBell's (203) provoking question whether the sulfate-reducing bacteria in oil wells are truly indigenous. Dostalek and Kvet (57), as well as Kutznetzova and her colleagues (107, 108), maintained that the salt tolerance of indigenous sulfate-reducing bacteria should match the salinity of their environment; Kutznetzova and her colleagues argued that, since the sulfate-reducing bacteria in brines from the Romaskhina oil field grew better in more dilute media they could not be indigenous. The logic of this argument eludes the present reviewer: inability to grow rapidly in its habitual environment may equally have survival value for an organism in nature.

Miscellaneous

Starkey (185) reviewed the behavior of these bacteria with particular emphasis on their role in the paper industry. Russell (150) uncovered a complex situation whereby growth of *Desulfovibrio* in waterlogged pulp wood led to the thiolignin formation. This material neutralized mercurical fungicides added at a later stage in paper manufacture and permitted rotting. Levin et al. (113) incriminated *Desulfovibrio* in the spoilage of brines used for preserving olives. In a publication inadvertently omitted from my earlier reviews, Johnson, Postlethwaite, and Rittenberg (90) proved that *Desulfovibrio* is not concerned in the maturing of pottery clays. Sisler (172) has

rediscovered the fact that an oxidation-reduction cell yields current, and has given a cell with a sulfate-reducing bacterial culture as half-cell the name "biochemical fuel cell." It seems that the aqueous environments, particularly the sea, are fraught with such cells which, according to Sisler and Senftle (173), will not behave in predictable fashions because of interference by the earth's magnetic field.

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