Title
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Publication Date
1963-08-28

UCRL-10966

University of California

Ernest O. Lawrence Radiation Laboratory

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Research and Development

UCRL-10966
UC-4 Chemistry
TID-4500 (19th Ed.)

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

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Printed in USA. Price $2.00. Available from the
Office of Technical Services
U. S. Department of Commerce
Washington 25, D.C.

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ABSTRACT

A comprehensive survey of literature dealing with sulfate-reducing bacteria was made. Data were collected on metabolism, ecology, isolation, and cultivation of these bacteria. It was found that quantitative data on the bacterial reduction of sulfate are almost nonexistent.

Desulfovibrio aestaurii, a species of sulfate-reducing bacteria, was isolated from a mud sample obtained from a saltern. Isolation was complicated by an unknown straight rod bacteria, but was eventually achieved through the dilution method and through the "diffusion method."

The isolated bacteria were grown in batch experiments to measure bacterial counts and sulfide concentrations. From the experimental data the sulfate reduction rates in complex and simple lactate media were calculated. Correlations were set up expressing reduction rate in terms of growth rate and bacterial density. Reduction rates in a continuous cultivator were estimated.

With another set of experiments the effects of initial sulfate content on the sulfate reduction rate, and on per cent conversion were studied. Also investigated was the effect of sodium chloride concentration on the reduction rate. A gradual adaptation of bacteria to higher salinities was carried out to reduce their NaCl sensitivity.

I. INTRODUCTION

Sulfates in general, and calcium sulfate in particular, are a major impurity in the electrolytic decomposition of sodium chloride for the manufacture of sodium and chlorine, and in the sea-water conversion processes. In the electrolytic process, sulfates clog the cell membranes and corrode some types of electrodes. In sea-water conversion, they cause scaling in the evaporators.

Sulfates normally make up only from 0.1 to 0.3% of the salt brines, and can easily be removed by the classical method of precipitation with barium chloride. However, in most cases this chemical method is too costly to be employed, and a more economical process is desired.

Microorganisms exist in nature that can almost quantitatively deplete salt brines of sulfates by reducing them to hydrogen sulfide in the course of their normal metabolic activites. In the literature there is considerable information available on the physiology, morphology, and ecology of such sulfate-reducing bacteria, but there is little quantitative data on their reduction of sulfates.

In this work we obtain some basic quantitative data concerning the reduction of sulfates by bacteria; from this data an evaluation of the practicability of using sulfate-reducing bacteria for brine purification can be made.

According to the literature, bacteria of genus Desulfovibrio are best suited for the above purpose. Since the brines in electrolytic decomposition process are saturated, the bacteria should be able to grow in high salt concentrations. A pure strain of such a halophilic Desulfovibrio could not be procured, and therefore had to be isolated from nature.

To obtain the desired quantitative data the isolated strain was grown in batch cultivation, and the bacterial counts and sulfide concentrations were measured periodically. From these experimental data, rates of sulfate reduction under various conditions were calculated.

Then the effects of sodium chloride concentration and of initial sulfate concentration on the rate of reduction were studied. An attempt was also made to adapt the bacteria to high salinity.

II. LITERATURE SURVEY

Many bacteria reduce sulfates during the synthesis of sulfur-containing amino acids ("assimilatory" sulfate reduction). But by sulfate-reducing bacteria one usually means only those bacteria whose major energy-yielding metabolic reactions are linked to the reduction of the sulfate to the sulfide ion ("dissimilatory" sulfate reduction). Only a few specialized bacterial species are capable of this dissimilatory sulfate reduction.

The classical example of a sulfate-reducing bacteria, and the one with which most research work has been done is Desulfovibrio desulfuricans (Beijerick). Others have been reported, the most common among these being Desulfovibrio aestaurii (Van Delden) and Desulfovibrio rubentschickii (Baars). But there is still disagreement among bacteriologists whether these are separate species, adaptive strains or variants, or physiological artifacts. This confused taxonomic situation has been mainly caused by the technical difficulties of obtaining and maintaining pure cultures of sulfate-reducing bacteria, and the variability, complex life cycles, pleomorphism, adaptability, physiological versatility, and ecological capriciousness.

For the reasons of simplicity, and because taxonomic problems are of no particular interest in this study, the different strains and/or species will be simply and interchangeably referred to as sulfate-reducing bacteria or Desulfovibrio.

Desulfovibrios as a group are Gram-negative, obligately-anaerobic vibrio or spirilloid organisms, very often motile with polar flagellum. The are very versatile, and their natural habitats embrace a wide range of salinity, temperature, hydrostatic pressure, pH, E_h (redox potential), and other environmental conditions. For these reasons they occur extensively. They are found in soil, sewage, swamps and freshwater sediments, oil and sulfur deposits, drilling muds, some industrial waste and cooling waters, but most abundantly of all in marine bottom deposits, and around buried iron structures.

In the remainder of this survey the metabolism of sulfate-reducing bacteria will be considered first, and then their ecology, isolation, cultivation, and economic activities.

A. Metabolism

Ordinary aerobic metabolism can be divided into three main stages:[2] (a) the anaerobic breakdown of carbon components, (b) the transport of the generated electrons to a cytochrome oxidase system, and (c) the aerobic reduction of oxygen to hydroxyl ions. These processes take place somewhat independently, and the first two are coupled with the synthesis of energy-rich phosphate bonds.

The metabolism of Desulfovibrio shows a perfect analogy with aerobic metabolism (aerobic acetic acid bacteria metabolism in particular) the only difference being that in this case sulfate takes over the hydrogen acceptor function which is normally performed by molecular oxygen.[3]

Recently, it has been demonstrated that Desulfovibrio contains a cytochrome system,[2] which indicates that, as in aerobic metabolism, there exists in sulfate-reducers a physiological separation between the oxidative steps of carbon metabolism and the reduction of sulfate.

1. Carbon Metabolism

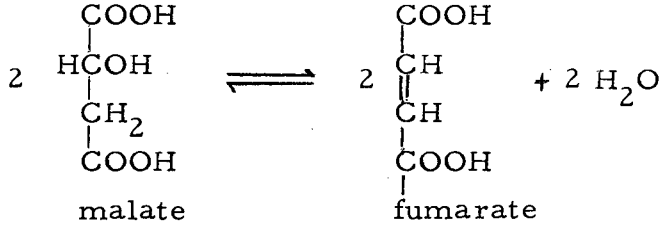
Strains or species differ somewhat as to the type of organic compounds they can utilize as the energy-yielding oxidizable substrates. However, as a class, sulfate reducers utilize a large variety of organic compounds, including lactate, malate, citrate, pyruvate, tartrate, fatty acids ranging from formic to stearic, amino acids, peptone, simple alcohols above methyl, glycerol, monosaccharides, certain disaccharides, possibly petroleum hydrocarbons, and some others.[1,8]

Studies of the carbon metabolisms of these bacteria indicate the possibility of a terminal carbon cycle similar to the tricarboxylic acid cycle or Krebs cycle of the aerobes, linked more or less remotely to the sulfate reducing system.

As an illustration of carbon metabolism, the metabolism of malate will be examined in some detail.

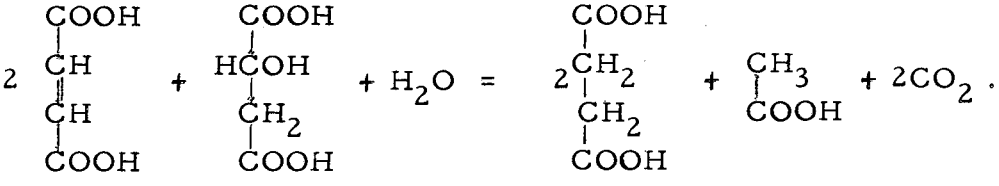
Malate (also fumarate succinate, lactate, and pyruvate) is oxidized quantitatively to acetate in cultures containing excess sulfate. Acetate is the usual end product of carbon compound oxidation by Desulfovibrio.

The first step in malate metabolism is a fumarase type of reaction leading to an equilibrium mixture of malate and fumarate. In the next step, two molecules of fumarate are reduced during the oxidation of one molecule of malate to yield two molecules of succinate.[4] Stoichiometrically, we have



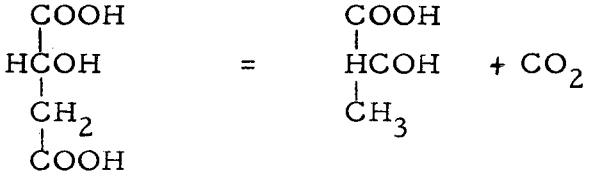
malate fumarate

followed by

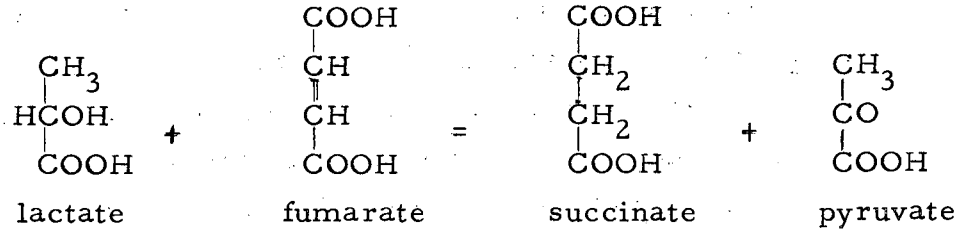


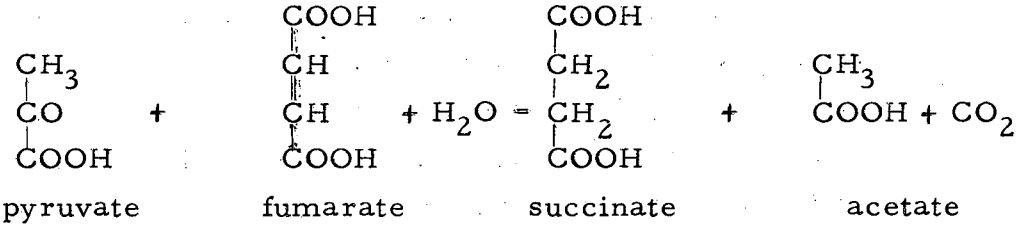
fumarate malate succinate acetate

The reduction of the two fumarate molecules, which in the absence of sulfate is coupled with the oxidation of malate to acetate, probably follows the course[4]

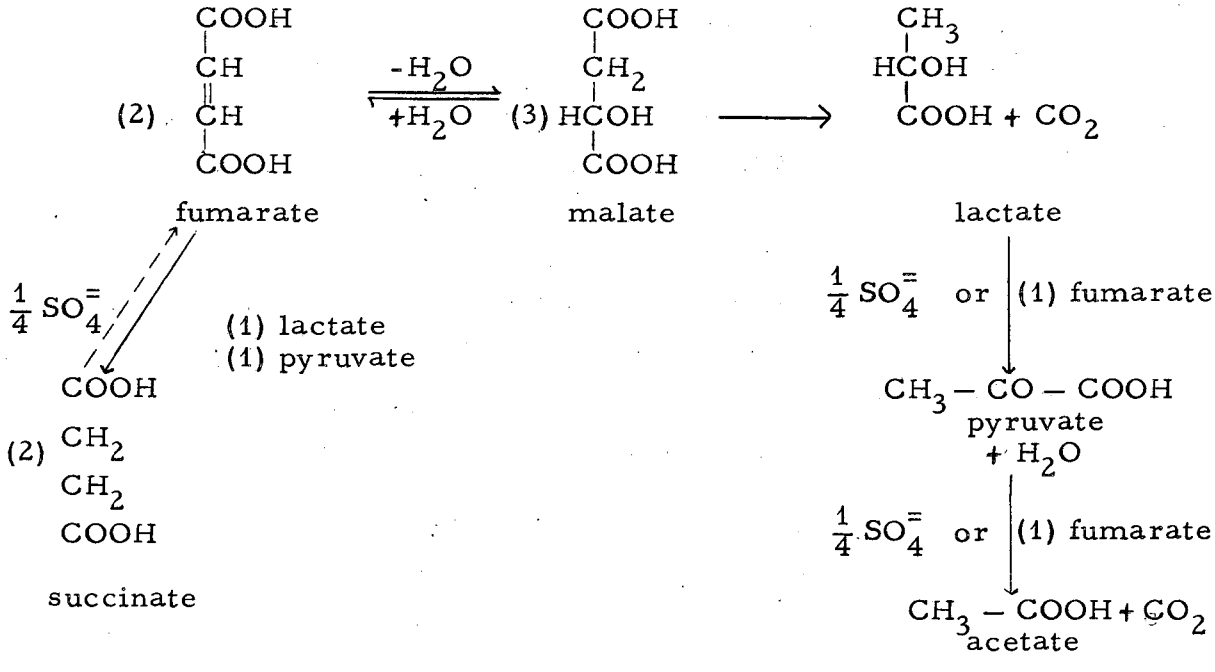


malate lactate





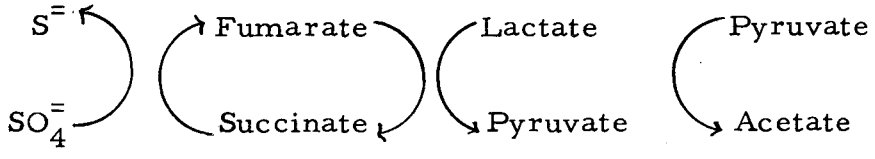
So far our discussion has been restricted to malate breakdown without sulfate, but there is strong evidence that similar reactions take place when sulfate is present. The following is the scheme for malate metabolism by Desulfovibrio:[4]



The foregoing scheme leads us to the conclusion that the oxidation of succinate to fumarate is the only reaction in the metabolism of malate for which sulfate is essential. Sulfate reduction can also be linked to lactate and pyruvate oxidation, but fumarate can act as an alternative hydrogen acceptor, and in fact it does so even when sulfate is present.

In its role of hydrogen acceptor the fumarate is reduced to succinate (which in the presence of sulfate is eventually converted to acetate), but the reaction is not restricted to malate metabolism. It has been observed in autotrophically grown cells, and is probably common to all hydrogenase containing strains of Desulfovibrio.[4]

If the foregoing scheme is assumed to be true, an overall process can be visualized in which the oxidation of succinate is linked to sulfate reduction, and the reduction of fumarate linked to the oxidation of either pyruvate or lactate. This fumarate-succinate cycle would thus be part of an electron-transport system between the oxidation of hydrogen donors and the reduction of sulfate.[4]



As mentioned, malate metabolism suggests the existence of a terminal cycle similar to the Krebs cycle. However, the evidence for the operation of the Krebs cycle in these organisms is lacking.

2. Autotrophic Metabolism

Many strains of Desulfovibrio are capable of autotrophic growth; that is, growth at the expense of exclusively inorganic substrates. Autotrophic strains contain enzyme hydrogenase which enables them to reduce sulfate with gaseous hydrogen:[6]

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

Some nonautotrophic cultures can be induced to develop the ability to utilize hydrogen by cultivating them in the presence of hydrogen for several generations. This suggests that for Desulfovibrio, hydrogenase may be an adaptive enzyme.[7] In our strain, however, the ability to utilize hydrogen could not be induced.

The carbon for the autotrophic growth is obtained by CO_2 (or bicarbonate) fixation, which is also catalyzed by enzyme hydrogenase. In "resting cells" the ratio of H_2 consumed to H_2S produced approximates four, which is the theoretical value for the reduction of sulfate. In cultures growing in an inorganic medium, however, this ratio exceeds four, and values ranging from five to ten were reported.[7] This indicates that H_2 is not only used for sulfate reduction, which provides energy, but also for the reduction of CO_2, which provides building material for growth.

The autotrophic fixation of CO_2 by Desulfovibrio is a very inefficient process. It was observed that ten to twenty molecules of sulfate are reduced for each molecule of CO_2 fixed.[9] This observation is in direct contradiction to the above reported values of five to ten for the H_2/H_2S ratio during the growth. High ratios of H_2/H_2S can be partially explained by the formation of cell material more reduced than CH_2O, but even if all CO_2 were converted to CH_4 the H_2/H_2S ratio could not exceed 4.4.

Aside from the above contradiction, the fact remains that the efficiency of autotrophic growth is low, and this explains poor growth in autotrophic media. It is interesting to note, however, that when these bacteria grow heterotrophically, if H_2 is avilable they use it in preference to an organic compound, even though the H_2 may contribute negligibly to their growth.[2]

The poor growth in inorganic media led some researchers to suspect that the apparently autotrophic growth of Desulfovibrio takes place at the expense of small amounts of organic materials in the media, and they put these bacteria in the nutritional category of metatrophs:[10] organisms capable of CO_2 reduction, but requiring small amounts of organic nutrients. A more reasonable hypothesis, however, would be

that growth factors must be supplied to permit Desulfovibrio to grow at the expense of H_2 and CO_2.

3. Hydrogen Acceptors

After considering the oxidizable substrates, let us now turn our attention to the reductive side of the metabolism. In aerobic metabolism the terminal reaction is the reduction of oxygen to hydroxyl ions:

$$O_2 + 4\,e + 2\,H_2O \longrightarrow 4\,OH^- .$$

In Desulfovibrio the oxygen comes from the sulfate ion:[2]

$$SO_4^= + 8\,e + 4\,H_2O \rightleftharpoons S^= + 8\,OH^- .$$

The sulfate ion is most probably reduced in steps, and there has been much speculation as to the type of intermediates. The natural place to look for these intermediates is among those sulfur compounds that can replace sulfate as hydrogen acceptors in different strains of Desulfovibrio. These compounds are: sulfite ($SO_3^=$), thiosulfate ($S_2O_3^=$), dithionate ($S_2O_4^=$), tetrathionate ($S_4O_6^=$), perdisulfate ($S_2O_8^=$), metabisulfite ($S_2O_5^=$), and free sulfur in colloidal form. However, the fact that these substances can be directly reduced by Desulfovibrio does not mean that they are necessarily intermediates in the normal reduction of sulfate.[3] In fact, according to some percent research[2, 11] on the above compounds, sulfite is the only one definitely in the direct pathway of sulfate reduction. This can simply be represented as follows:[12]

$$SO_4^= \xrightarrow{H_2} SO_3^= \longrightarrow \left\{\begin{array}{l}\text{unknown intermediates}\\ \text{containing one atom}\\ \text{of S per divalent ion}\end{array}\right\} \longrightarrow S^= .$$

At this point it should be mentioned that in sulfate-free media some strains can use certain nonsulfur compounds as hydrogen acceptors. Some actively growing cultures of autotrophic sulfate reducers can reportedly use carbon dioxide or bicarbonate as hydrogen acceptors:[7]

$$CO_2 + 2\,H_2 \longrightarrow (CH_2O) + H_2O .$$

The utilization of pyruvate, fumarate, and malate as hydrogen acceptors and/or "co-acceptors" has already been discussed.

Sulfate, the natural hydrogen acceptor, can be utilized over a wide range of concentrations. The optimum sulfate concentration (i.e., giving maximum growth rate) is in the range of 0.01 M[12] and 0.006 M,[13] and the maximum sulfate-reducing activity was obtained at concentrations higher than 0.014 M.[13] Results of the experiments, given later in this report, are in good agreement with the above values. The concentration required for the initiation of growth was reported to be only 0.00028 M.[13]

Desulfovibrio can also withstand very high sulfate concentrations. It was observed that cells of a fresh-water strain were not plasmolyzed by a 15% solution of Na_2SO_4.[14] Two possible explanations for this behavior are that in high sulfate concentrations the organisms either render themselves impermeable to water, or adjust their internal osmotic pressure without allowing sulfate to come to osmotic equilibrium with the cytoplasm.[2]

There is other evidence[14] that also suggests that cells are not freely permeable to sulfate ions, and it was speculated that the initial step of sulfate reduction either takes place at the surface of the cell, or else that penetration of the sulfate ion into the cell is regulated so that extremely small amounts of sulfate can enter.

4. Electron Transport System

Like aerobes the sulfate-reducing bacteria also link their oxidative and reductive reactions by an electron transport system involving cytochromes.

Desulfovibrio is among the very first obligate anaerobes that has been found to contain a cytochrome.[2,15] This cytochrome is known as cytochrome c_3, and is chemically similar to the muscle cytochrome c, except that it has twice as many prosthetic groups per molecule.[2] Cytochrome c_3 is a low potential cytochrome with $E_0 = -204$ mV.

So far the enzyme preparations able to reduce sulfate have not been obtained. However, despite the lack of direct enzymological evidence on the function of cytochrome c_3 in sulfate reduction, it is a

valid working hypothesis[5] to assume that cytochrome c_3 is one of several electron-transporting enzymes linking sulfate reduction to carbon and hydrogen oxidation in the metabolism of Desulfovibrio.

5. Nitrogen Metabolism

To fulfill the nitrogen requirements for the optimum growth of Desulfovibrio cultures, most recent media specify from 0.02 to 0.1% of either ammonium chloride or ammonium sulfate.

Neither nitrate nor nitrite are reduced and utilized as nitrogen sources by Desulfovibrio. However, some autotrophic cultures have been observed to fix gaseous nitrogen.[16]

Ability of Desulfovibrio to utilize amino acids similtaneously as carbon and nitrogen sources has not yet been definitely established.[5] Complex proteinaceous compounds supply neither available nitrogen nor energy for these bacteria; this fact suggests a general lack of proteolytic enzymes.[1]

B. Ecology

In this section the discussion is focused on some general properties of sulfate-reducing bacteria, on the effects of environmental conditions on these bacteria, and, conversely, on the effects of bacterial growth on the environment.

1. Temperature Range of Growth

Sulfate-reducing bacteria have been observed to grow throughout the temperature range of 0° to 100°C. However, the mesophilic strains which have been mostly investigated grow best between 25° and 45°C.[1,17] In our investigation the effects of temperature have not been studied; all the work was done at 30°C.

On the lower end of the temperature range, some marine bacteria have been reported to grow slowly at temperatures as low as -3° to -11°C. At the other extreme some strains from deep oil and sulfur wells reproduced at 104°C (at 1000 atm).

The maximum temperature at which thermophilic cultures are still active is increased by compression. Higher salinity also increases the high temperature tolerance of marine cultures. By gradual "training" many cultures can be brought to tolerate temperatures considerably higher or lower than the temperatures of their natural environments.

2. Hydrostatic Pressure

Bacteria isolated from soil, sewage, and shallow water fail to grow when compressed above 400 atm. In contrast, some barophilic strains from deep-sea deposits, and from oil and sulfur wells grow only when compressed by 700 to 1000 atm.[1] Not all the strains from deep wells are either barophilic or thermophilic, yet many tolerate pressures to 600 atm and temperatures to 85°C. The common, non-barophilic strains generally have higher pressure tolerance when held at temperatures from 5° to 20°C higher than their normal temperature optima.[19]

Pressure affects not only growth and reproduction, but also some other physiological processes. Some species grown at 1 atm for instance, produce H_2S three to four times faster at 1000 atm than at 1 to 300 atm. But in the range of 1400 to 1800 atm the production stops completely.[1]

The effect of pressure is not only a function of temperature, as already mentioned, but also a function of the chemical composition of the medium, as well as its osmotic pressure, redox potential, and pH.[20]

3. Osmotic Behavior and Salinity Requirements

The salts that are of main interest here are sulfates and sodium chloride. Sulfate-reducing bacteria are not freely permeable to either.[14] Effects of sulfate concentration have already been reported (Sec. II.A.3).

The bacteria's tolerance of NaCl depends greatly on the salinity in the natural habitat of the particular strain in question. Most marine cultures require media containing from 1.5% to 5% NaCl, while freshwater strains require salinity below 1%.

Strains of Desulfovibrio isolated from certain oil-well brines, salt lakes, and marine salterns were reported by different observers to develop in various salt concentrations up to 30%.[21, 22, 23] Even though some bacteria do develop in such high salt concentrations, a halophilic strain whose optimum salinity would exceed 10 to 12% has not yet been found. Confirming this is the fact that even though our strain was isolated from saturated salt solution, and is able to grow in at least 16% high salt concentrations, its optimum salinity is below 4% NaCl.

High salinity usually greatly lengthens the lag period of a culture, and especially so when the culture has been transferred from a lower to a higher salinity medium. Lag periods of two to six weeks have been recorded.[1] In our strain, for example, the lag period was lengthened from 20 hours to 2 weeks as the salinity was increased from 10% to 16% NaCl.

Even the salt tolerance of the same strain can vary considerably since it is influenced by temperature, pH, hydrostatic pressure, chemical composition of the medium, and other environmental factors.

Some investigators have stated that cultures of Desulfovibrio can be acclimatized to develop in salt concentrations much different from that of their natural habitats.[24, 17, 25] This is supported by our work in which a strain with optimum growth in the range of 1% to 4% NaCl has been acclimatized to develop in a 16% salt concentration. The possibility of acclimatization raised the question whether the halophilic, marine, and freshwater varieties represent only strains which are interconvertible by acclimatization and adaptation, or whether they are separate species. No definite conclusion on the matter has been reached yet.

4. Hydrogen Ion Concentration (pH), and Redox Potential (E_h)

Sulfate reducers are more exacting in their requirements for anaerobic conditions than are most anaerobes. They require a redox potential lower than -200 mV (European sign convention) for the initiation of growth; the lower is the initial redox potential of the media, the

greater is the rate of growth.[26,5] Neglect in providing Desulfovibrio with the proper E_h is without doubt primarily responsible for the difficulties encountered in isolating and cultivating.[27]

It is very important to ensure the proper E_h for the initiation of growth. After the growth is established, the bacteria themselves develop and maintain reducing conditions, primarily by the production of H_2S.

The pH tolerance of sulfate-reducing bacteria, both in their natural habitat and under controlled laboratory conditions, is affected by the redox potential and by the chemical composition of the media.[21,28] In milieu more reducing than $E_h = -150$ mV, sulfate reducers were found to be active up to pH = 9.5; in milieu at $E_h = 0$ they could tolerate pH as acidic as 4.2. Most of the cultures, however, grew best between pH 6.2 and 7.9, and E_h - 50 to 0150 mV.

Under aerobic conditions, growth is possible only in the presence of reducing agents, such as ascorbic acid, sodium sulfide, or cysteine.

5. Hydrogen Sulfide: Rate of Production, Yield, and Toxicity

Hydrogen sulfide is an indicator and a measure of the metabolic activities and growth of sulfate-reducing bacteria. It is of particular interest in this study because it is the product of the sulfate-reduction reaction with which we are primarily concerned.

Unfortunately, there are no true quantitative data available on the rates and yields of hydrogen sulfide production. Even the available qualitative and semiquantitative data and observations are for the most part nonreproducible and hard to compare because they were obtained under various conditions and with different organisms.

Rate and yield of hydrogen sulfide production vary greatly, not only with different strains and environments, but also with the same strain and some environment, depending on the physiological state and age of bacteria.

The maximum yields of H_2S reported vary anywhere from a few milligrams up to about 3000 milligrams per liter of medium; the time necessary to obtain these yields varies from a few days to several weeks.

The medium claimed by investigators to be best suited for the development of high concentrations of H_2S by both marine and non-marine strains has the following composition:[29]

NH_4Cl	1.00 g
$MgSO_4 \cdot 7H_2O$	2.00 g
Na_2SO_4	18.25 g
K_2HPO_4	0.50 g
$CaCl_2 \cdot 2H_2O$	0.10 g
Sodium lactate	21.0 g
NaCl	10.0 g
$CaCO_3$	1.00 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	trace
Distilled water to bring total to	1000 ml

Conditions encouraging high H_2S yields in the above medium are the ample quantities of sulfate and lactate, and the high initial pH brought about by the $CaCO_3$.

To obtain high yields and rates of H_2S production, it is necessary not only to use proper media, but to have bacteria in a vigorous condition. Such a vigorous condition is obtained by transfers at short intervals because the H_2S production in a given culture is influenced not only by the age of the culture from which it was transferred, but also by the frequency with which a number of the preceeding cultures had been transferred.[29] The H_2S forms most rapidly when the inoculum is from a culture about two days old. As the age of inoculum increases the rate of H_2S formation in the new culture decreases. Contrary to the conclusions of many researchers, it also appears that the production of high concentrations of H_2S is associated with its rapid rate of formation.[30]

Another way of increasing the rate of H_2S production is addition of peptone and yeast extract to the medium. Maximum response is obtained at the concentrations of 5 to 10 g/liter, but measurable effects were observed at concentrations less than 10 mg/liter.[30]

It was reported[30] that the addition of peptone and yeast extract is stimulatory only to the rate of H_2S formation, and does not increase the total quantity produced. This was interpreted as an indication that these additives merely supply a factor (or factors) which are formed slowly enough to be growth-rate limiting. This view is not entirely supported by our work from which it appears that yeast extract and peptone not only provide growth factors, but also some oxidizable organic substrates. The addition of 1 g/liter of peptone and yeast extract not only increased the rate of H_2S formation by a factor of ten, but also tripled the total quantity produced.

Besides stimulating growth, the peptone and yeast extract extend the range of temperature, pH, and salt concentration tolerated, and also increase the range of substrates attacked.[30, 27]

Since hydrogen sulfide is toxic to most forms of life, it can be expected to have some deleterious effect on sulfate-reducing bacteria also, even though it is a product of their metabolism. If during its formation some of the H_2S is removed by the use of zinc acetate above the medium, or by the use of ferrous sulfate or cadmium carbonate in the nutrient medium, considerably larger total amounts of H_2S are formed.[33] This indicates that large amounts of H_2S in the culture media are a deterrent to further development. There is no complete agreement on the extent to which the H_2S yield is determined by the bacterial sensitivity to H_2S,[33] but the fact remains that Desulfovibrio is sensitive to H_2S.

The exact tolerance of Desulfovibrio for H_2S is, however, hard to define because sulfide may occur only partly as H_2S; it occurs more often as insoluble sulfides and sulfide ions. Thus, the concentration of H_2S will not only be influenced by the total amount produced, but to a greater extent by the pH and chemical composition of the media, temperature, pressure, and other factors.[1]

C. Isolation and Cultivation

There are many strains, and perhaps several species of sulfate-reducing bacteria. By using a variety of media, crude cultures are readily obtained, but the isolation of absolutely pure cultures is usually difficult.[31]

Grossly mixed to almost pure cultures may be obtained by an enrichment culture method. Enrichment media should be as simple as possible: phosphate buffer, an ammonium salt, magnesium and/or sodium sulfate, sodium chloride in case of marine strains, and a carbon source, such as lactate or ethyl alcohol. At least a trace of ferrous ion is also required. No growth takes place in media rendered "biologically free" of iron. The ferrous ion also helps to maintain reducing conditions, and enhances bacterial resistance to inhibitors (selenate). Another function of the ferrous ion is to serve as an indicator of growth, since it reacts with H_2S to cause blackening of media (black, insoluble FeS). Any other elements required by sulfate-reducing bacteria are present in the medium in sufficient amounts as impurities.

Addition of Na_2SO_3 to the media is sometimes very effective in eliminating most of the contaminant organisms in crude cultures; it also facilitates subsequent purification.[31]

To obtain pure strains, solid media are usually employed (plates, or preferably deep agar "shake" cultures). Composition of solid media is similar to that of liquid ones, except that while the addition of a reducing agent is only helpful in liquid media, it is essential in solid media. In solid media containing a trace of a ferrous salt, the sulfate-reducers form black colonies which can easily be picked off. However, it is difficult to obtain colonies free from contaminats, and a tedious succession of "shake" and plate cultures is required before a really pure culture is obtained.

In our work, even a succession of "shake" cultures failed to eliminate a persistent contaminating bacteria. The dilution method, and the "diffusion" method (based on difference in bacterial motility) were much more effective in obtaining a pure culture of Desulfovibrio.

For autotrophic cultivation, media are used which are different from heterotrophic media in three important respects: organic carbon compound is substituted by bicarbonate of carbon dioxide; the medium is placed in intimate contact with molecular hydrogen, which should be free of oxygen contamination; and any organic contaminants are excluded if the growth is to be completely autotrophic.

Importance of maintaining strictly anaerobic conditions have been pointed out in a previous section. To ensure reducing conditions necessary for the initiation of growth, it is often effective to add to the media a reducing agent such as sodium sulfide, cystein, ferrous ion, and ascorbate.[4,27] Of these compounds, sodium sulfide was found to be most effective, and it was used throughout our work. The same effect may also be obtained by using a large inoculum.

D. Economic Activities

Postgate states that sulfate-reducing bacteria have been implicated in a wide variety of phenomena which can be broadly classed as of economic importance.[5] These activities will not be discussed here in any detail, but just to give an idea of their wide-spread occurrence, we present a list of the main phenomena:

(a) anaerobic corrosion of iron and steel,[34]
(b) acid corrosion of metal and stone,[35]
(c) formation of sulfur deposits,[36,37]
(d) formation of soda deposits,[38]
(e) formation of metal sulfide deposits,[34]
(f) formation and release of natural oil deposits,[39]
(g) plugging of systems for the secondary recovery of oil,
(h) spoilage of oily materials,
(i) pollution of waters,[40]
(j) blackening of paper pulp,[41]
(k) contamination of town gas by H_2S,
(l) separation of sulfur isotopes.[42]

III. ISOLATION OF SULFATE-REDUCING BACTERIA

A. Enrichment Cultures

It was desired to obtain a strain of sulfate-reducing bacteria that would be able to tolerate as high salt concentrations as possible. The best place to look for such a salt-tolerant strain is in oil-well brines, marine salterns of salt lakes.

Mud samples from which Desulfovibrio was eventually isolated were obtained from salterns of the Leslie Salt Co. at Newark, California. The black mud was found about 3 in. under the bottom of a saltern containing 1.5 ft of almost saturated brine.

Samples of much weighing approximately 2 grams each were used to inoculate the lactate media, and the resulting cultures were incubated at 30°C. Several types of lactate media were used (see Appendix B, Sec. 1, 2, and 3). Most of the media contained 10% NaCl, but some had 3% or 13% NaCl. To some cultures small amounts of Na_2SO_3 or Na_2S were added. Before inoculation, the media were adjusted to have the pH in the range of 7.0 to 7.5. Best results were obtained with Medium C (in which we added 0.15% Na_2SO_3 and 10% NaCl).

The initial enrichment cultures developed quite slowly. Some enrichment cultures showed good development in three to five days, but the incubation period was usually ten days or more. Growth of bacteria was indicated by development of turbidity, and in some cultures by gas formation. The reduction of sulfate to hydrogen sulfide was indicated by the formation of iron sulfide which imparted a black color to the medium, and in some cultures caused thin black deposits on the bottles.

After various times of incubation, ranging from 10 days to 5 months, the original enrichment cultures were transferred into fresh media. Differences in media, times of incubation, and numbers of subsequent transfers of each original enrichment culture will not be discussed. Only the path of the culture that finally yielded the pure culture is described (Sec. III.5).

Most of the final enrichment cultures contained only two or three distinct species. A few cultures, which contained more than three species, were discarded. All of the cultures contained a curved rod or vibrio type organism that was suspected to be Desulfovibrio, and a straight rod. Some cultures also contained a much longer (10 to 15 μ) thin rod. Separation of the first two organisms by further application of the enrichment culture method did not appear probable, and several other techniques were tried.

B. Solid Media

The use of solid media, being often the most simple and effective means of obtaining pure cultures, was tried first. These solid media had the composition of Medium A or B (in Appendix B) to which 2.5% agar has been added. As pointed out (Sec. II.C), a reducing agent must be added to the solid media to make growth of Desulfovibrio possible. Cystein, sodium sulfide, and sodium sulfite were tried for this purpose. Of these the sodium sulfide gave by far the best results.

Streak plates and pour plates were made with various enrichment cultures, and incubated in a Brewer's jar under a hydrogen atmosphere. But black colonies, which would indicate growth of Desulfovibrio, did not develop on any of the plates. Failure of sulfate-reducers to develop could have been caused by some oxygen impurity in the hydrogen, or by the exposure to air during the preparation of the plates.

Next, the dilution shake cultures were tried. A large number of such cultures were prepared as follows. Sterile 12 ml test tubes covered with rubber caps were first filled with sterilized water with the aid of a hypodermic needle. Water was then displaced with hydrogen. The reducing agent and the melted agar medium were then injected to completely fill the tube. Before solidification inoculum was injected and the tubes well sealed by applying parafin over the rubber caps. The above procedure was followed to insure strictly anaerobic condition of the culture. All work was done with sterile technique.

Many small light-gray colonies, and a few even smaller (1 to 2 mm diam) dark gray or black colonies developed. The dark colonies were cut out of the agar medium and transferred to liquid media. In each case a mixed culture developed, and even after several sequences of solid and liquid cultures no enrichment of Desulfovibrio occured.

It appears that in the type of solid media used Desulfovibrio grew preferentially in mixed cultures, or in the near vicinity of a culture of the contaminating organism. The contaminant, which was suspected to be a facultative anaerobe, might have consumed the last traces of oxygen and made conditions more favorable for the growth of Desulfovibrio. Some evidence will be presented later than in liquid media of high salinity Desulfovibrio also grows better in mixed cultures than in pure ones.

C. Micromanipulator

Only a few attempts were made to achieve isolation with the use of a de Fonbrune Pneumatic Micromanipulator. From the beginning the method appeared tedious and unpromising. A few bacteria that were separated were incubated in 2 ml flasks, but no growth developed. Apparently exposure to the air was much too long even though the drops of culture were kept under mineral oil during most of the manipulation.

D. Inhibitors

1. Crystal Violet

Crystal violet (hexamethylpararosaniline) is known to be a much stronger inhibitor of facultative anaerobes than of strict anaerobes. Since Desulfovibrio is a strict anaerobe, and the contaminating rod-like organism is a facultative anaerobe, the possibility of isolation with the help of crystal violet was explored.

Crystal violet dye used was of the composition that is normally used in Gram stain reactions (2-g crystal violet, 0.8-g ammonium oxalate, 20 ml of 95% ethyl alcohol, 80 ml of water). This dye was added to the inocula and the media in different amounts. At the concentration

of 0.2 ml dye per 100 ml of medium, growth of both bacteria was completely inhibited. At lower concentrations of crystal violet, the relative inhibition of the contaminant was much stronger, but strangely enough as the concentration of crystal violet was increased complete inhibition of Desulfovibrio was reached sooner than the complete inhibition of the contaminant. Best results were obtained with 0.02 ml of the dye in 100 ml of medium. By making an early transfer (2 to 3 days after inoculation), the percentage of Desulfovibrio increased from 20% to 80%.

Transfers had to be made with very young cultures because the enrichment of Desulfovibrio was found to decrease with the time of incubation. The typical crystal violet color also faded with time. Both these effects can be explained by the reduction and discoloration of crystal violet caused by H_2S, which is produced from the growing Desulfovibrio.

Complete isolation was not obtained with the use of crystal violet, but considerable enrichment was achieved, and the success of the "diffusion method" described later was also made possible by crystal violet.

2. Sulfide and Sulfite

Sulfide and sulfite are both common biological inhibitors. However, in the sulfate reduction by Desulfovibrio, sulfite is an intermediate and sulfide the end product of the normal metabolism. Desulfovibrio was for this reason expected to be less sensitive to these compounds than the nonsulfate-reducing bacteria.

Additions to the media of different concentrations of sulfide (0.002 to 0.015 mole per liter) and sulfite (0.03 to 0.25 mole per liter) were tried, but absolutely no enrichment of Desulfovibrio was noticed.

E. Dilution Method

It was by means of the dilution method that the isolation was finally accomplished.

The original enrichment culture used in this work showed very little turbidity and blackening, but when it was opened after five months

of incubation it contained 0.021 mole per liter of hydrogen sulfide. (The sulfide exists in the form of free hydrogen sulfide, sulfide ions, and ferrous sulfide. The analytical results include all these forms but are expressed throughout the paper under the general term of hydrogen sulfide.) This indicates the reduction of 78% of total sulfate and sulfite initially present in Medium C. The bacteria in the culture were not distinguishable under the microscope. A first subculture was made by inoculating 2 ml of the original enrichment culture into Medium D (pH = 7.9, E_h = - 80 mV, 0.0003 mole per liter H_2S). After eight days of incubation at 30°C 0.011 mole/liter H_2S was produced (55% conversion). Microscopic examination revealed an apparently pure culture of vibrios of the type shown in Fig. 2(A). However, the second subculture in the same medium contained besides vibrios also some Gram-negative straight rods shown in Figs. 1(C) and 1(D). In the third subculture, the rods outnumbered the vibrios 3 to 1. It should be mentioned here that several other investigators also experienced considerable difficulties with the isolation because of a similar Gram-negative organism which they identified as a facultative coliform bacteria.

A 10^{-5} dilution of the third subculture in sterile Medium D was made. With the help of a micropipette, fifty 2 ml bottles, filled with sterile Medium D, were inoculated each with a 0.002 ml drop of the above dilution. Each drop was estimated to contain one or less bacterium. After four days of incubation the small bottles were microscopically examined. Some bottles had no growth, a few contained mixed cultures of rods and vibrios, but most contained only rods. There were no pure cultures of vibrios.

Dilution of a mixed culture was made again in the same way as described above. But instead of inoculating the small drops into Medium D, they were inoculated into a 50:50 mixture of Medium D and a filtered culture. The filtered culture was obtained by growing a mixed culture of vibrios and rods in Medium D, and when turbidity appeared after two days filtering it through a milipore filter. This sterile filtrate was added to the regular medium in order to approach as closely as possible the state of a culture medium in which the growth of Desulfovibrio is taking place.

It is the opinion of some bacteriologists that if Desulfovibrio is to develop, the ratio of medium to inoculum size should not be too large. According to this theory, a single Desulfovibrio cell will grow only if inoculated into a very small drop. However, to maintain such small drops strictly anaerobic would be very difficult. It was believed that this problem could be bypassed by placing a single Desulfovibrio cell into a filtrate of a growing culture which could reasonably be expected to possess all the conditions required for growth.

Of the fifty 2-ml bottles inoculated in the above described manner at least three were found to contain pure cultures of what was believed to be a strain of Desulfovibrio.

F. Diffusion Method

At the same time, and independently of the isolation by the dilution method, a pure culture of Desulfovibrio was also obtained by another technique. In this "diffusion" method, advantage was taken of the difference in motility of the two organisms in the presence of crystal violet.

Under normal conditions there appeared to be no appreciable difference in the degree of motility of the two organisms. However, 20 minutes or longer after the addition of crystal violet dye to a mixed culture, there was a very marked difference in the motility of the two. While the motility of vibrios remained practically unchanged, the rods appeared almost immobile. One loop-full of a crystal-violet solution of the type used in Gram-stain reactions was normally added to 2 ml of culture. The motility of the vibrios, estimated by observing them under a microscope, was approximately 40 to 100 μ/sec.

A 0.1-mm-i.d. melting-point capillary tube was sealed at one end and then filled up with the crystal violet-treated culture. A similar capillary tube was filled with a sterile culture medium. The open ends of the two capillaries were then placed in contact and fixed in position with melted paraffin wax. After a measured time interval, the capillary filled with the sterile medium was broken off at a point 6 cm from the

open end, and the sealed-end section was incubated in a 2-ml bottle of sterile medium. In a series of such capillaries the ends were broken off at different time intervals after the joining of the two capillaries to obtain one in which only a few, most vigorously motile bacteria have "diffused" through the 6 cm of medium into the broken-off end. Then after the incubation, a bottle with growth was selected that contained the capillary with the shortest "diffusion time."

In this manner, several cultures were obtained that, after repeated transfers and microscopic examinations, still appeared to be pure. However, for the later experiments a pure culture obtained by the dilution method was selected because this culture most probably originated from a single cell. The same cannot be stated about the culture isolated by the diffusion method.

G. Identification and Brief Description of the Isolated Species

The isolated bacterium is a Gram-negative curved rod or vibrio of varying morphology. It is very actively motile, and from its appearance the motility seems to be caused by a polar flagellum, or polar flagella.

The organism reduces sulfates almost quantitatively to hydrogen sulfide. It is capable of growth in simple media containing only one organic compound (Medium A). Growth is greatly accelerated by the addition of yeast extract and peptone. There is no growth in media free of sulfates, or other oxidized sulfur compounds (sulfite, thiosulfate, etc.).

There is no growth under aerobic conditions. Sensitivity toward oxygen seems to be great; for the growth to start, the conditions must be strictly anaerobic and reducing (negative redox potential). The most favorable condition for the initiation of growth is obtained by adding 0.002 to 0.004 moles per liter of hydrogen sulfide to the medium.

Growth rate is reduced, and the lag period lengthened with the increase of sodium chloride concentration in the medium, but in the absence of sodium chloride there is no growth. The organism is capable of acclimatization to higher salt concentrations.

From the above description it can be concluded that the isolated sulfate-reducing bacteria is of the Desulfovibrio aestaurii species.

The isolated strain of Desulfovibrio a. was found to be heterotrophic. Several attempts to grow it on media free of organic compounds, but which contained carbon dioxide and molecular hydrogen, were unsuccessful.

H. Morphology

A variety of forms and sizes of the organism were observed, and several factors are found to have direct effect on the morphology.

Sodium chloride concentration has a marked effect on the size of bacteria. In a culture medium containing three per cent of sodium chloride the bacteria appear as small curved rods, 1 to 2μ in length, and about 0.5μ in diameter [Figs. 1(A) and 1(B)].

With increasing salt concentrations, size also increases. In 10% NaCl media (most of the experiments were done in this concentration) the bacteria are 3 to 6μ long. Most cells are lightly curved in the shape of an S, but there are some which are almost straight, and others which have more than two turns [Fig. 1(A)].

Length further increases with salinity. At 16% NaCl, which is the highest concentration in which an appreciable growth has been obtained, the bacterial size is 5 to 15μ, and even longer in old cultures.

Size of bacteria increases also increases with the age of the culture. Ratio of straight bacteria to curved bacteria is also higher in older cultures.

In the complex media (media containing yeast extract and peptone) the increase in size with age is moderate, but in simple media it can be considerable [Fig. 2(C)]. This may be due to the inability of bacteria to synthesize at a sufficient rate some growth factor which is necessary for cell division and is not present in simple media.

The type of the energy-yielding organic compound in the medium also has an effect on the size. For example, in the complex sodium lactate medium (10% NaCl), cells are 3 to 6 μ long, but in a similar

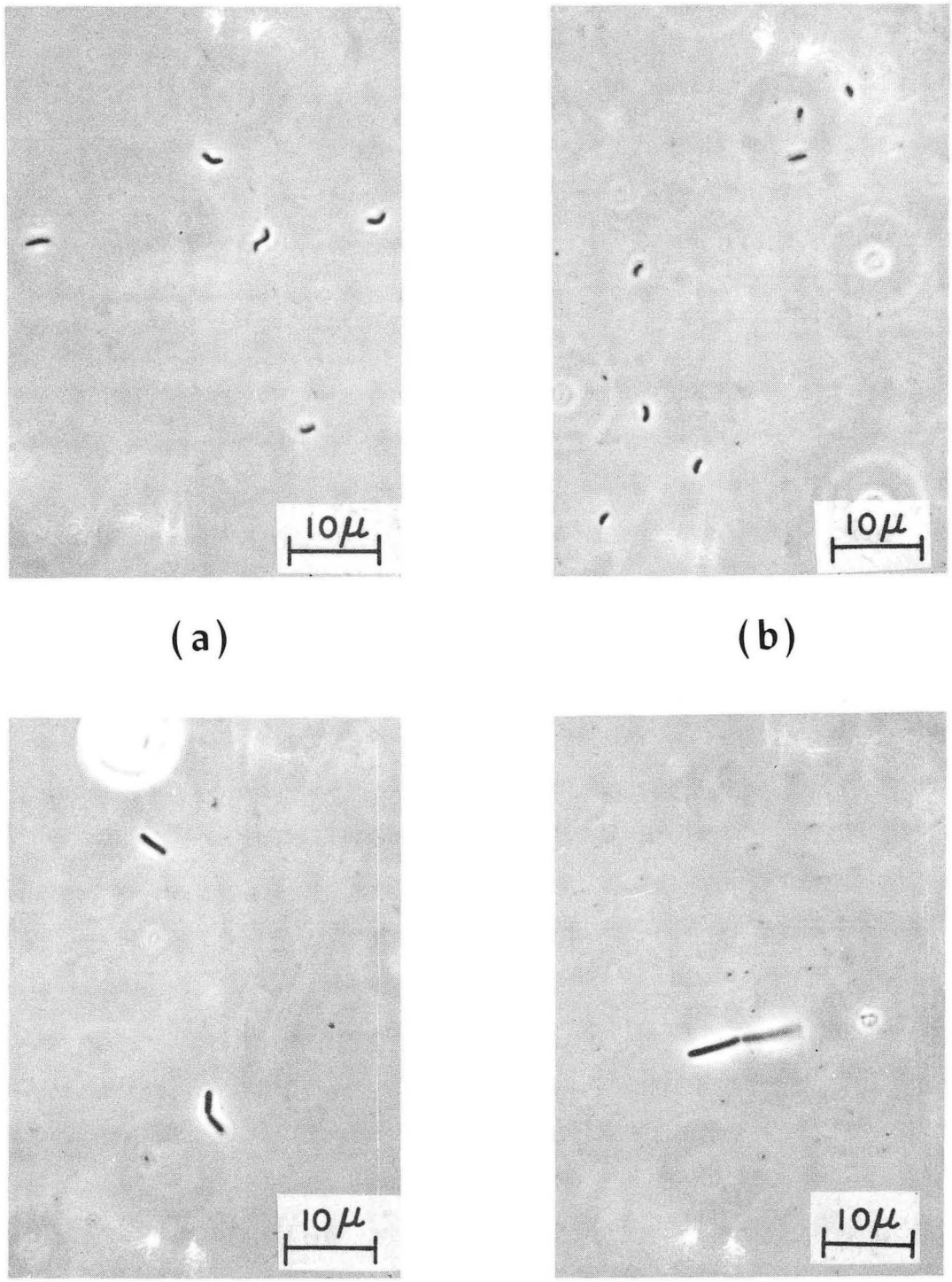


Fig. 1. Desulfovibrio and the unknown contaminating organism. (A) and (B), Desulfovibrio grown on complex lactate medium containing 3% sodium chloride. (C) and (D), the unknown bacteria. Phase contrast (x970).

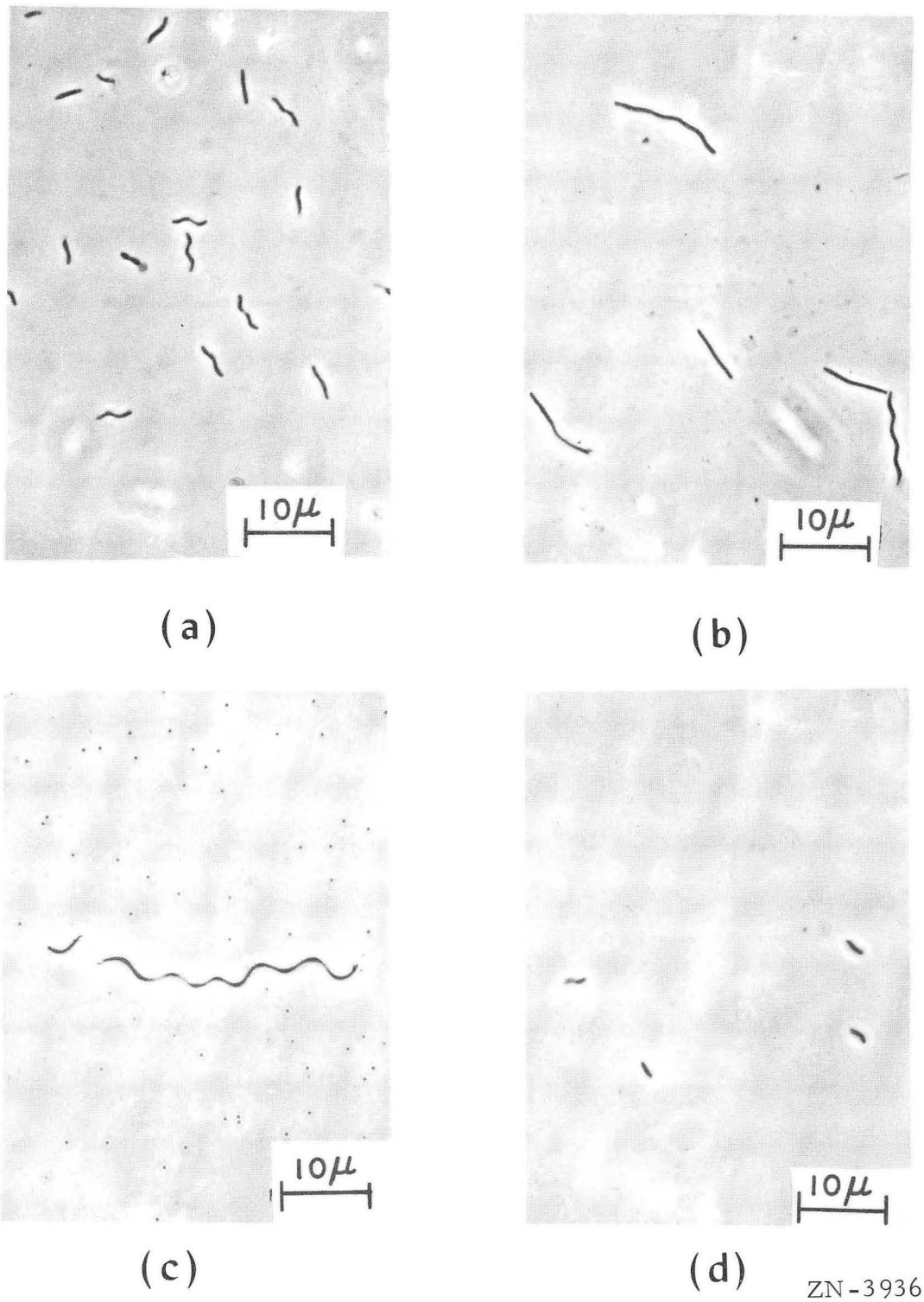


Fig. 2. Morphological variations of Desulfovibrio. (A) young cells grown on complex lactate medium containing 10% NaCl. (B) cells grown on complex lactate medium containing 16% NaCl. (C) old cells grown on simple lactate medium (10% NaCl). (D) cells grown on complex ethyl alcohol medium (10% NaCl). Phase contrast (x970).

medium in which sodium lactate was substituted with ethyl alcohol the length is only 1 to 3 μ.

In each case it can be observed that the diameter of the bacterial cells remains fairly constant despite the manyfold increase in the length. This could be interpreted as indicating that the long forms are not single cells, but several bacteria clinging together end to end. Supporting this view is also the observation that in long curved forms there is normally one turn per each section 2-μ long. Bacteria in their natural environment (3% NaCl) are normally about 2μ long, and have one curve. On the other hand the long bacteria appear very uniform under the microscope, and there are no distinct transverse breaks visible, as is the case for instance with the coliform rod [Fig. 1(C) and 1(D)].

Motility of bacteria also decreases with their length, and with the age of the culture. Motility of young, small cells can exceed 100μ per second, while old and long cells move very slowly, if at all.

IV. KINETICS OF BACTERIAL SULFATE REDUCTION

We now examine some aspects of bacterial sulfate reduction from a quantitative viewpoint. This work is done to obtain basic rate data on which to base future evaluations of the usefulness of sulfate-reducing bacteria for brine purification.

Growth curves and sulfide formation curves for two different media were obtained experimentally. Calculated from these experimental data were growth rates, sulfate concentrations, sulfate reduction rates per volume of culture, sulfate reduction rates per bacteria, and a generalized expression for sulfate reduction.

In the next experiment the effect of initial sulfate concentration on the rate of sulfate reduction and on the efficiency of sulfate utilization were examined.

Finally, the effects of salinity on bacterial growth and sulfate reduction were studied. A process of adaptation of Desulfovibrio to higher salt concentrations was started, and its practicality evaluated.

A. Sulfate Reduction Rates

1. Growth Curves and Sulfide Formation Curves

The growth and sulfide formation curves afford the basic experimental data on which most of our studies of bacterial sulfate reduction were based.

The experimental procedure used in obtaining these curves was as follows: A glass-stoppered flask, filled with a sterile medium of desired composition, pH, and redox potential, was inoculated with a pure culture of Desulfovibrio. The normal inoculum size was from one to two ml of a young culture (about 5×10^7 bacteria per ml) for 100 ml of medium. Additional medium was added, if necessary, to have the bottle completely filled and so minimize contact with the air. Inoculated medium was then throughly mixed, and distributed into sterile 60 ml glass-stoppered reagent bottles which were then incubated at 30°C. The entire batch of medium was inoculated together instead of inoculating each 60 ml bottle separately in order to have the initial conditions in all the small bottles as uniform as possible. Bottles were

then opened at different time intervals after inoculation. Their sulfide content was determined by titration, as described in the Appendices, and a bacterial count was made with a Petroff-Hausser counting chamber. A cumulative amont of sulfide produced in any incubation period was obtained as the difference between the sulfide concentration measured after that period and the concentration of sulfide in the sample taken immediately after inoculation.

a. Complex medium. Throughout this report a medium to which yeast extract andpeptone were added will be referred to as complex, and a medium without these additives as simple.

Bacterial count and sulfide production as functions of incubation time for the complex lactate medium, designated as Medium E in the Appendices, are tabulated in Table I, and plotted in Fig. 3, Fig. 4, and Fig. 5. Three separate experiments, each one on a smaller time scale, were done with the same type of medium in order to obtain a more detailed picture of the break in the exponential growth region. By making compensations for the small differences in inoculum size, the results of the three experiments are very consistent. In the subsequent calculations they were combined by using each one in the region where it gives the most data.

From the growth curves in the complex medium we can see that after a lag period of 18 hours there are two distinct phases of exponential growth. The first one lasts for 22 hours and has the growth rate k equal to 3.27/day, and a generation time t_G equal to 5.1 hours. The first exponential phase passes directly into the seond one, which lasts for 40 hours and has k = 1.157/day and t_G = 14.4 hours. The stationary phase is reached after an additional 4 days of growth, a total of 8 days after inoculation.

The presence of two distinct and different exponential growth phases is caused by the complexity of the nutrient medium. The second exponential phase is due to growth on lactate alone. This is confirmed by the fact that an identical exponential growth phase is present in the growth curve obtained with a simple lactate medium containing only a

Table I. Experimental data on the growth of Desulfovibrio in complex lactate medium (Medium E) at 30°C.

Time of incubation (days)	Cumulative H_2S produced (mole/liter)	Bacterial count(No. of cells/ml)	Logarithm of No. of cells/ml	Sulfate concentration (mole/liter)
		First Experiment		
0.04	0	1.74×10^6	6.240	0.0162
0.23	0	-	-	0.0162
0.53	0	-	-	0.0162
0.90	0.00025	-	-	0.01595
1.20	0.00025	1.05×10^7	7.021	0.01595
1.51	0.00075	1.77×10^7	7.248	0.01545
1.92	0.0010	2.20×10^7	7.342	0.0152
2.17	0.0010	2.46×10^7	7.391	0.0152
2.51	0.00125	4.54×10^7	7.657	0.01495
2.92	0.00185	5.62×10^7	7.750	0.01435
3.17	0.0025	1.10×10^8	8.041	0.0137
3.50	0.0029	1.32×10^8	8.121	0.0133
3.92	0.00345	1.56×10^8	8.193	0.01275
4.47	0.0043	1.76×10^8	8.246	0.0119
5.51	0.0060	2.12×10^8	8.326	0.0102
6.04	0.0075	2.22×10^8	8.346	0.0087
7.10	0.0090	2.22×10^8	8.346	0.0072
8.23	0.0110	2.66×10^8	8.425	0.0052
10.15	0.0135	2.54×10^8	8.405	0.0027
13.19	0.0153	2.58×10^8	8.412	0.0009
18.17	0.0160	2.62×10^8	8.418	0.0002
21.12	0.0163	2.40×10^8	8.380	0.0
25.00	0.0166	2.44×10^8	8.87	0.0
		Second Experiment		
0.04	0	1.375×10^6	6.139	0.0162
0.22	0.00005	1.75×10^6	6.243	0.01615
0.44	0.00005	1.875×10^6	6.273	0.01615
0.92	0.00015	4.07×10^6	6.610	0.01605
1.38	0.00035	2.00×10^7	7.301	0.01585
1.60	0.0004	3.60×10^7	7.556	0.0158
1.98	0.0005	4.86×10^7	7.687	0.0157
2.36	0.00135	8.30×10^7	7.919	0.01485
2.56	0.0014	9.44×10^7	7.975	0.0148
2.94	0.0028	1.69×10^8	8.228	0.00139
3.23	0.0030	2.38×10^8	8.377	0.0132
3.53	0.0035	2.54×10^8	8.405	0.0127
3.96	0.0040	2.56×10^8	8.408	0.0122
4.25	0.00525	2.60×10^8	8.415	0.01095
5.92	0.0069	2.56×10^8	8.408	0.0093

Table I. (continued)

Time of incubation (days)	Cumulative H_2S produced (mole/liter)	Bacterial count(No. of cells/ml)	Logarithm of No. of cells/ml	Sulfate concentration (mole/liter)
		Third Experiment		
0	0	1.25×10^6	6.097	0.0162
0.375	0	1.25×10^6	6.097	0.0162
0.562	0.00025	1.25×10^6	6.097	0.01595
0.75	0.00035	1.25×10^6	6.097	0.01585
1.02	0.0004	2.50×10^6	6.398	0.0158
1.31	0.0004	6.44×10^6	6.809	0.0158
1.60	0.0005	1.68×10^7	7.227	0.0157
2.02	0.0010	2.90×10^7	7.462	0.0152
2.40	0.0010	4.21×10^7	7.615	0.0152

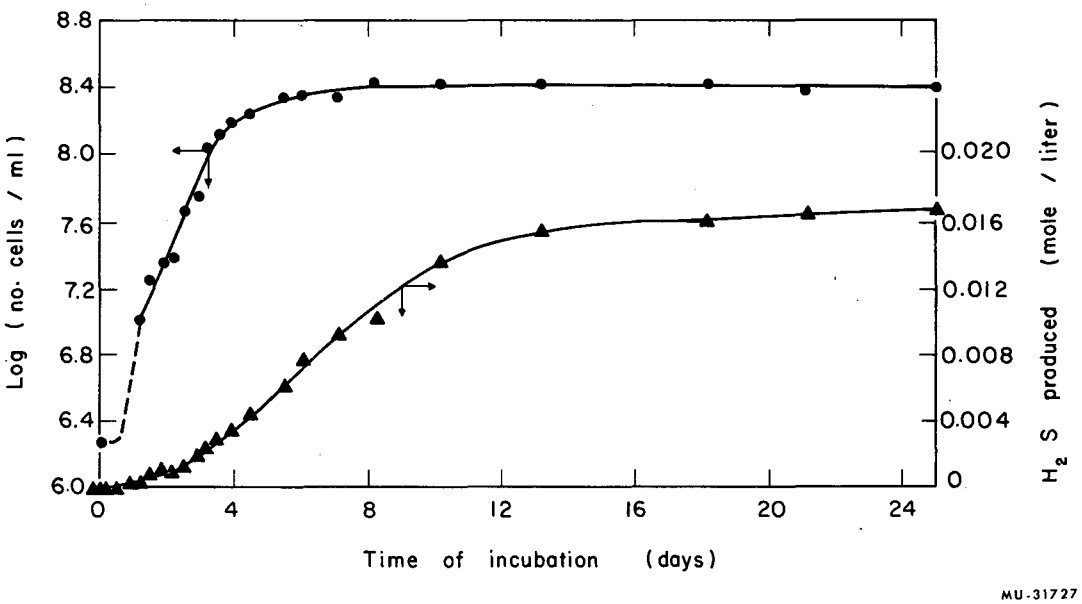


Fig. 3. Growth curve of Desulfovibrio in complex lactate medium at 30°C. Experiment No. 1.

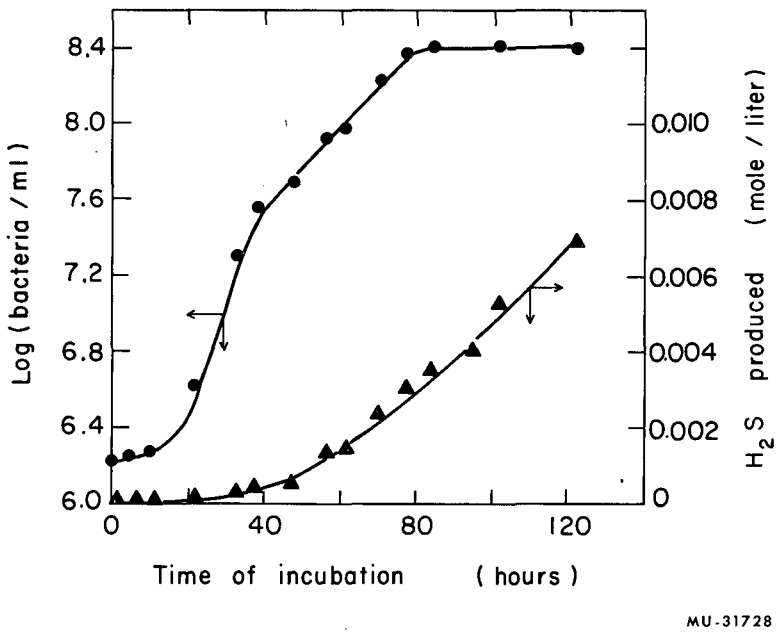


Fig. 4. Growth curve of Desulfovibrio in complex lactate medium at 30°C. Experiment No. 2.

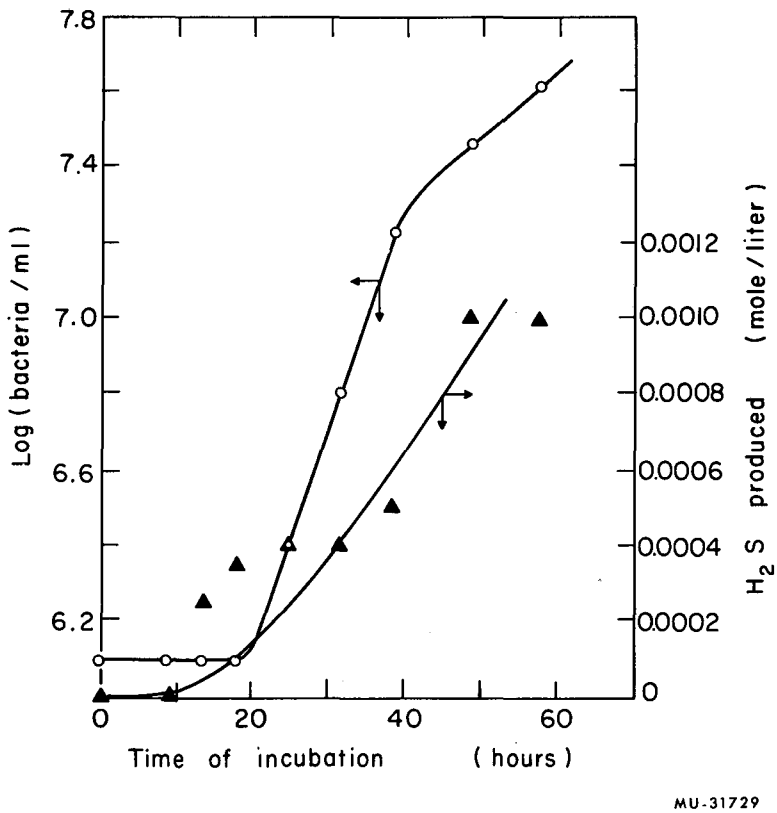


Fig. 5. Growth curve of Desulfovibrio in complex lactate medium at 30°C. Experiment No. 3.

trace of yeast extract and peptone. The first exponential phase was probably caused by the presence in yeast extract or peptone of a component, or components that promote faster growth of Desulfovibrio than does the lactate. Such a component could be a free amino acid. After this component was consumed, growth continued exclusively on lactate as the oxidizable substrate.

The maximum bacterial density reached in the stationary phase was 2.52×10^8 cells per ml, and the total sulfide production was 0.0166 moles per liter.

b. Simple medium. Only one experiment on simple lactate medium (Medium F) has been conducted. The data are shown in Table II, and plotted in Figs. 6 and 7 using two different scales.

The sulfide-production curve is similar, but the growth curve is considerably different from the one obtained with the complex medium. Lag phase is of comparable duration (20 hours), and the first exponential phase is of almost exactly the same duration and rate as the second exponential phase in the complex medium (length = 42 hours, $k = 1.136$/day, $t_G = 14.65$ hours). Then there is another lag phase lasting one day, followed by much slower exponential growth (duration = 3 days, $k = 0.144$ per day, $t_G = 115$ hours = 4.8 days). After a five-day deceleration phase, a total of 12 days after inoculation, the stationary phase is reached. Maximum cell density and maximum sulfide concentration are only 2×10^7 cells per ml and 0.0058 moles per liter respectively.

The behavior in the simple medium can be explained as follows. An equivalent of the first exponential phase in the complex medium is absent because in a simple medium, lactate is the only organic substrate. However, by using an inoculum from the complex medium there were added to the simple medium approximately 5 mg/liter each of yeast extract and peptone. It had been observed by Miller[30] that 10 mg/liter of peptone or 4 mg/liter of yeast extract are sufficient to produce an appreciable stimulation of the growth. This explains the identity of the second exponential phase in the complex medium and the

Table II. Experimental data on the growth of Desulfovibrio on simple lactate medium (Medium F) at 30°C.

Time of incubation (days)	Cumulative H_2S produced (mole/liter)	Bacterial count(No. of cells/ml)	Logarithm of No. of cells/ml	Sulfate concentration (mole/liter)
0.17	0	1.25×10^6	6.097	0.0151
0.56	0	1.25×10^6	6.097	0.0151
1.0	0	1.50×10^6	6.176	0.0151
1.5	0	2.88×10^6	6.460	0.0151
1.94	0.00025	4.0×10^6	6.602	0.01485
2.23	0	6.75×10^6	6.829	0.0151
2.54	0	8.75×10^6	6.942	0.0151
2.96	0.00025	1.025×10^7	7.011	0.01485
3.25	0	1.03×10^7	7.013	0.0151
3.52	0.00025	1.05×10^7	7.021	0.01485
4.0	0.0005	1.025×10^7	7.011	0.0146
4.46	0.0005	1.075×10^7	7.031	0.0146
4.97	0.00065	1.225×10^7	7.088	0.01445
5.47	0.0006	1.262×10^7	7.101	0.0145
5.97	0.0008	1.375×10^7	7.139	0.0143
6.53	0.0010	1.50×10^7	7.176	0.0141
7.0	0.0010	1.575×10^7	7.197	0.0141
8.04	0.0012	1.75×10^7	7.243	0.0139
9.15	0.0016	1.89×10^7	7.276	0.0135
10.21	0.0019	1.96×10^7	7.292	0.0132
12.5	0.0025	2.10×10^7	7.303	0.0126
15.17	0.0031	1.94×10^7	7.288	0.0120
17.12	0.0037	1.99×10^7	7.299	0.0114
19.35	0.0044	1.81×10^7	7.258	0.0107

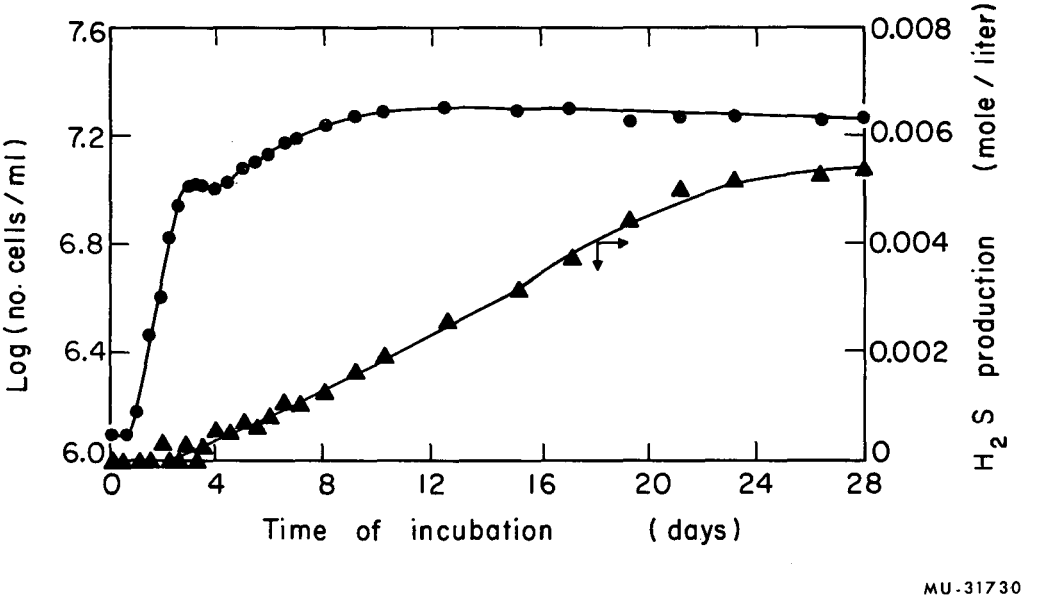


Fig. 6. Growth curve of Desulfovibrio in simple lactate medium at 30°C.

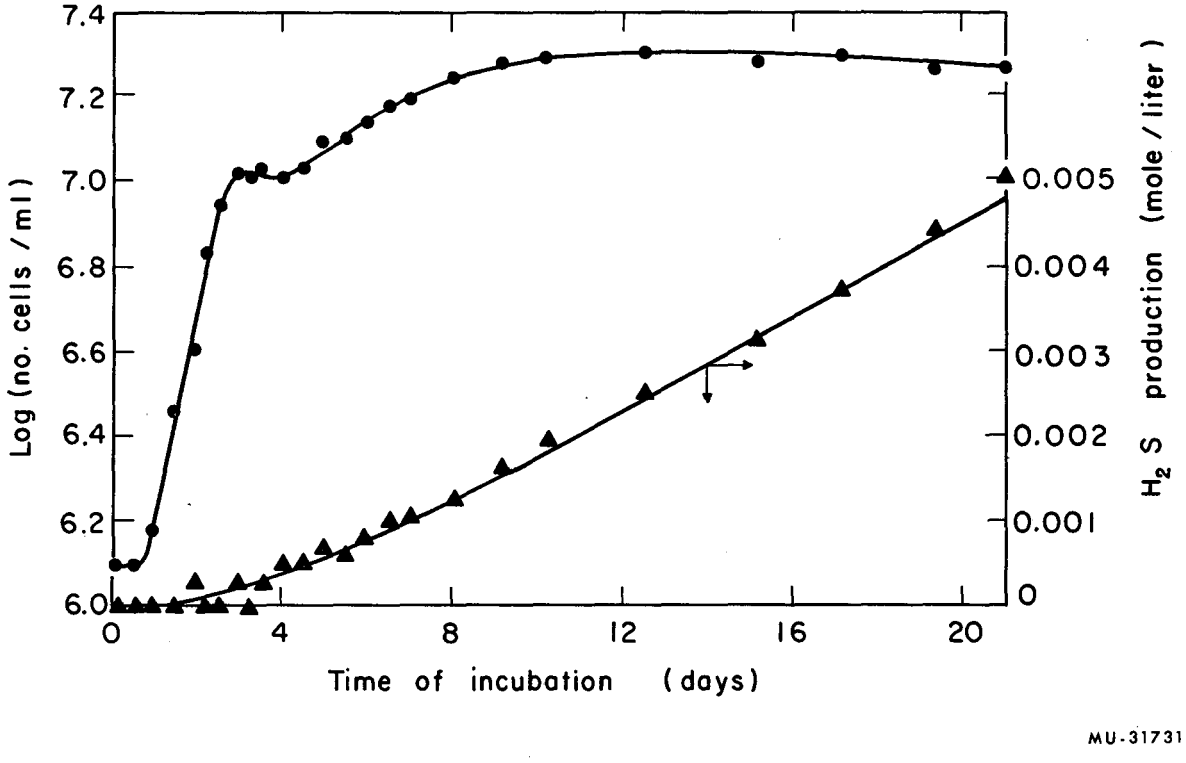


Fig. 7. Growth curve of Desulfovibrio in simple lactate medium at 30° C (same as Fig. 6, but larger scale).

first exponential phase in the simple medium: both represent exponential growth on lactate as the sole substrate, with yeast extract and peptone supplying only the growth factors. The large difference in the amounts of growth factors available (ratio 200:1) has no apparent effect on the growth rate. But as the small amount of growth factors in the simple medium is exhausted growth stops completely; hence the second lag period appears. During this period Desulfovibrio reacquires the ability to synthesize its own growth factors, and the growth proceeds exponentially again, however, at a much slower rate. The medium has become a truly "simple medium."

The lower final concentration of bacteria and sulfide in the simple medium, as compared to the complex one, is mainly caused by the lower concentration of organic nutrients in the simple medium resulting from the absence of yeast extract and peptone.

2. Growth Rates

Growth rate, or what is sometimes called specific growth rate k, is given by

$$k = \frac{1}{n} \frac{dn}{dt} ,$$

where n is the number of bacteria. This expression can be written as

$$k = \frac{d \ln n}{dt} ,$$

which is the expression for the slope of a growth curve with the log number of cells plotted versus time. Thus the growth rates for the entire life of the culture can be obtained by measuring the slope of the growth curve at various incubation times. The results are tabulated in Table III, and plotted in Fig. 8. In these curves, the characteristic growth behavior in the two media plainly stands out (see Sec. IV. A.1).

3. Rate of Sulfate Reduction Per Volume of Culture

The sulfate concentration at any time was obtained by subtracting sulfide produced up to that time from the sulfate initially present in the medium. This indirect method was used because experimental sulfide determination is much simpler and more accurate than sulfate determination. The method of sulfide determination is described in Appendix C.

Table III. Growth rates of Desulfovibrio at 30°C in complex and simple lactate media.

Time of incubation (days)	Growth curve phases	Growth rate k (day^{-1})
	Complex medium	
0	lag phase	0
0.75		0
0.75	first exponential	3.27
1.67	phase	3.27
1.67	second exponential	1.157
3.33	phase	1.157
3.5	deceleration	0.65
3.7	phase	0.612
3.9		0.378
4.1		0.32
4.3		0.248
4.8		0.179
5.3		0.135
6		0.0966
7		0.0635
8		0.0248
9		0.0038
13	stationary and	0
17	death phase	0
20		0.005
	Simple medium	
0	first lag	0
0.83	phase	0
0.83	first exponential	1.136
2.6	phase	1.136
2.7		0.415
3	second lag	0
4	phase	0
4	second exponential	0.1444
7	phase	0.1444
7.5	deceleration	0.102
8	phase	0.08
9		0.055
10		0.0279
11		0.0138
12.5	stationary phase	0

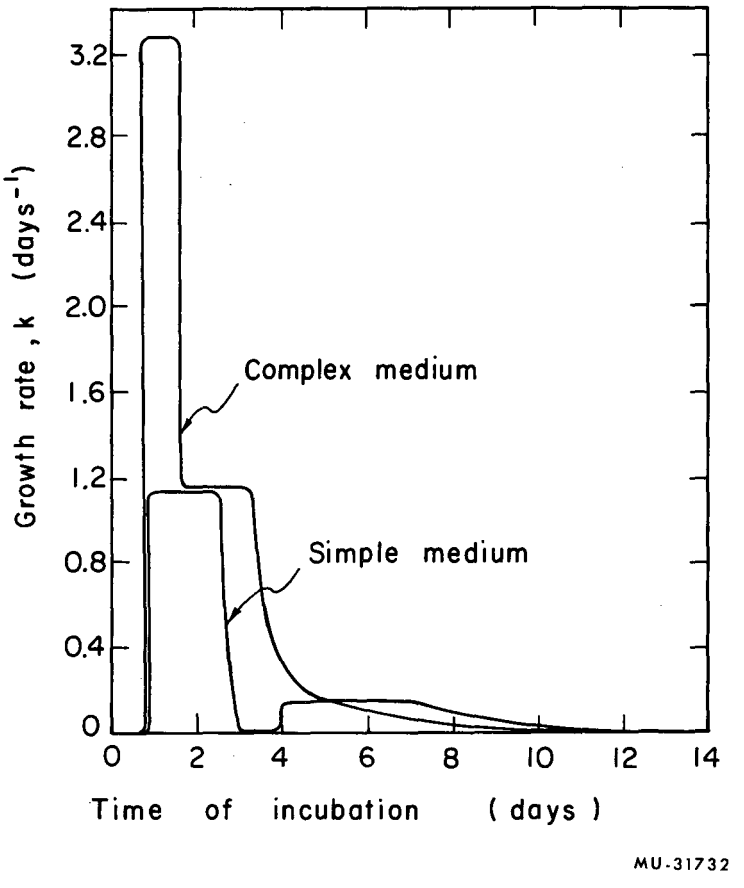


Fig. 8. Growth rate of Desulfovibrio in complex and simple lactate medium at 30°C.

A test was conducted to determine the error involved in using the indirect instead of a direct method of sulfate determination. Two Desulfovibrio cultures were analyzed for H_2S, and the sulfate concentrations were then calculated in the above manner. Sulfate concentrations of the same two cultures were also determined by the direct gravimetric method of sulfate precipitation with barium chloride. A discrepancy of less than one per cent was found between the sulfate concentrations obtained by the two methods.

In both types of media, 0.0151 mole per liter of sulfates were added to the media in the form of sodium and magnesium salts. Some sulfates were added to the complex media with the yeast extract and peptone. Other chemicals making up the media also contain some sulfates as impurities. This additional sulfate was determined to amount to 0.0011 moles/liter.

These sulfate concentrations were plotted versus time of incubation in Fig. 9 and Fig. 10, and tabulated in Table I and Table II. From the slopes of these curves, the sulfate utilization or reduction rates $d[SO_4^{--}]/dt$ for different incubation times were obtained. The sulfate reduction rates in terms of moles of sulfate per day per liter are tabulated in Table IV, and plotted in Fig. 9 and Fig. 10.

These curves show that sulfate reduction rate per volume of culture in both media increases rapidly after the initiation of growth, until it reaches its maximum at the end of the second exponential growth period. The rate then remains at this maximum for a considerable time period: in a complex medium the rate is maximum until the stationary phase is reached after four days, and in simple media long after reaching the stationary phase, for a total of 12 days. In contrast to its duration the value of the maximum reduction rate in the complex medium is much higher (0.0017 moles/liter/day) than in the simple medium (0.000275 moles/liter/day) because of the larger bacterial population in the complex medium.

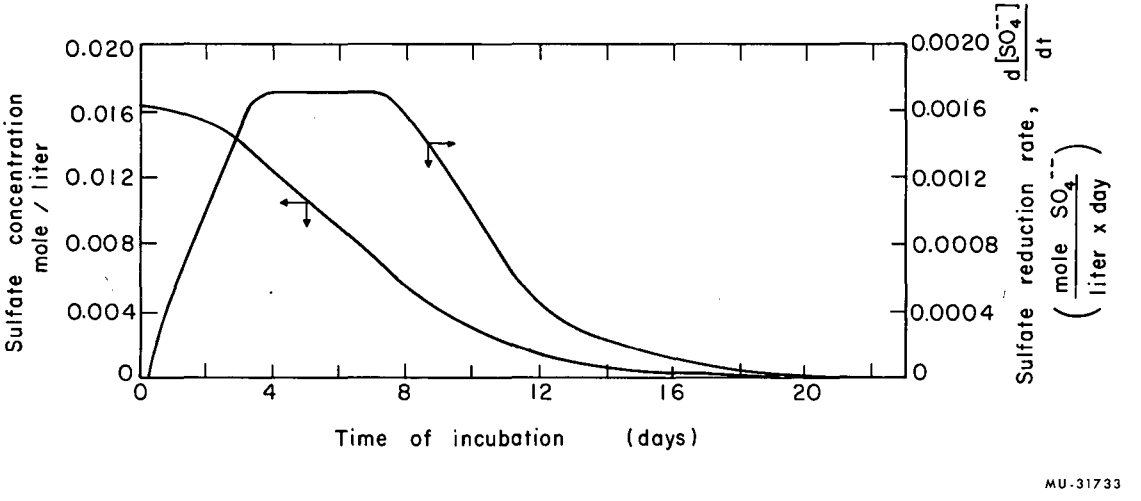


Fig. 9. Sulfate reduction rate in complex medium.

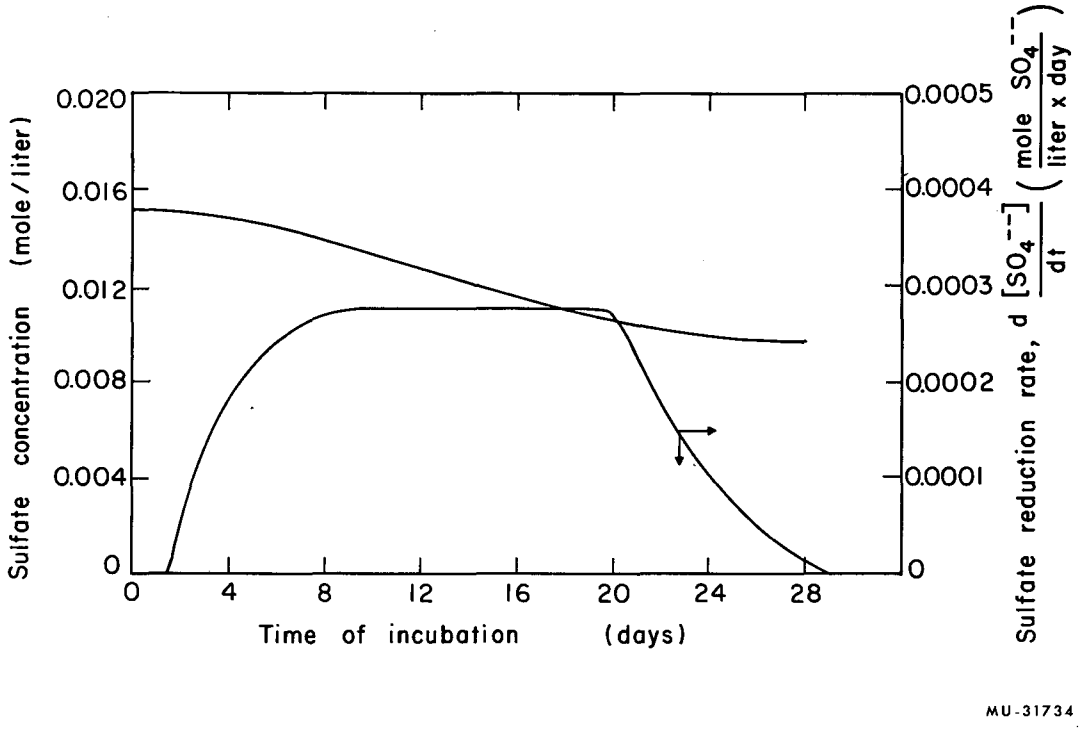


Fig. 10. Sulfate reduction rate in simple lactate medium.

Table IV. Rate of sulfate utilization data for Desulfovibrio at 30°C, in complex and simple lactate media.

Time of incubation (days)	Growth rate k (day^{-1})	Rate of sulfate reduction per volume (mole/day/liter)	Rate of sulfate reduction per bacterium (mole/day/cell)
		Complex medium	
0	0	0	0
0.3	0	0	0
0.5	0	0.00018	1.43×10^{-13}
0.75	3.27	0.000354	2.81×10^{-13}
1.0	3.27	0.00050	2.19×10^{-13}
1.25	3.27	0.00062	1.195×10^{-13}
1.5	3.27	0.00076	6.47×10^{-14}
2.0	1.157	0.00098	4.1×10^{-14}
2.5	1.157	0.00125	3.0×10^{-14}
3.0	1.157	0.00148	1.96×10^{-14}
3.5	0.24	0.00167	1.36×10^{-14}
4	0.11	0.00171	1.08×10^{-14}
5	0.05	0.00171	8.55×10^{-15}
6	0.04	0.00171	7.63×10^{-15}
7	0.02	0.00171	7.13×10^{-15}
8	—	0.00156	6.2×10^{-15}
9	0.007	0.00135	5.36×10^{-15}
10		0.000955	3.76×10^{-15}
11		0.000666	2.5×10^{-15}
13		0.000373	1.4×10^{-15}
15		0.000154	6.0×10^{-16}
18		0.000057	2.22×10^{-16}
22		0	0×10^{-16}
		Simple medium	
0	0	0	0
0.83	0→ 1.136	0	0
1.5	1.136	0	0
1.7	1.136	0.000020	5.9×10^{-15}
1.8	1.136	0.000054	8.42×10^{-15}
2.0	1.136	0.000054	1.13×10^{-14}
2.2	1.136	0.00072	1.21×10^{-14}
2.4	1.136	0.000090	1.203×10^{-14}
2.6	1.136	0.000105	1.137×10^{-14}
2.8	0.415	0.000118	1.18×10^{-14}
3.0	0	0.000132	1.29×10^{-14}
3.5	0	0.000160	1.56×10^{-14}

Table IV. (continued)

Time of incubation (days)	Growth rate k (day^{-1})	Rate of sulfate reduction per volume (mole/day/liter)	Rate of sulfate reduction per bacterium (mole/day/cell)
4.0	0 → 0.1444	0.000183	1.785×10^{-14}
4.3	0.1444	0.000195	1.82×10^{-14}
4.6	0.1444	0.000206	1.89×10^{-14}
5.0	0.1444	0.000218	1.83×10^{-14}
5.5	0.1444	0.000232	1.83×10^{-14}
6.0	0.1444	0.000243	1.76×10^{-14}
6.5	0.1444	0.000250	1.69×10^{-14}
7.0	0.1444	0.000260	1.65×10^{-14}
8	0.08	0.000273	1.57×10^{-14}
9	0.055	0.000276	1.484×10^{-14}
10	0.0279	0.000276	1.416×10^{-14}
11	0.0138	0.000276	1.38×10^{-14}
12	0	0.000276	1.37×10^{-14}
14	0	0.000276	1.37×10^{-14}
15		0.000276	1.37×10^{-14}
16		0.000276	1.384×10^{-14}
18		0.000276	1.40×10^{-14}
20		0.000276	1.416×10^{-14}
21		0.000223	1.155×10^{-14}
22		0.000180	9.47×10^{-15}
24		0.000105	5.57×10^{-15}
26		0.000048	2.59×10^{-15}
28		0.000012	6.6×10^{-16}
30		0	0

4 Rate of Sulfate Reduction Per Bacterial Cell

By dividing the sulfate reduction rates per volume of culture, shown in Figs 9 and 10, by the bacterial counts obtained from the growth curves, sulfate reduction rates per single bacterial cell are obtained. These rates are tabulated in Table IV, and plotted in Figs 11 and 12.

Again there is considerable difference between the two media In the complex medium the maximum sulfate-reducing activity of bacteria is reached immediately after the initiation of growth. This is to be expected since in the acceleration phase the metabolic activites are normally at the maximum. The reduction rate then starts to drop off with the exponential growth, and continues to drop. The dropping of the reduction rate is somewhat retarded only in the deceleration phase.

In the simple medium, the maximum reducing activity is not reached till the middle of the second exponential phase, then drops off in the deceleration phase, but remains constant throughout the stationary phase. This behavior could be partially explained by the growth of bacterial size during the stationary phase. To compensate for this increase in bacterial size, long bacteria were counted as more than one cell, according to their length. The small increase in cell diameter, however, was not compensated for.

5. Fitting Sulfate-Reduction-Rate Data Into a Generalized Correlation

A commonly used expression for fermentation relates the product formation in the simplest possible manner to the growth rate and to bacterial density;[43] this expression is

$$\frac{dP}{dt} = \alpha \frac{dn}{dt} + \beta n , \qquad (1)$$

where P is the concentration of fermentation product, and α and β are fermentation constants fixed by the organism, substrate, temperature, etc.

The form of Eq. (1) was suggested by the fact that the energy derived from the product formation is consumed in two main processes: growth, and basic metabolic activities Growth rate per volume of

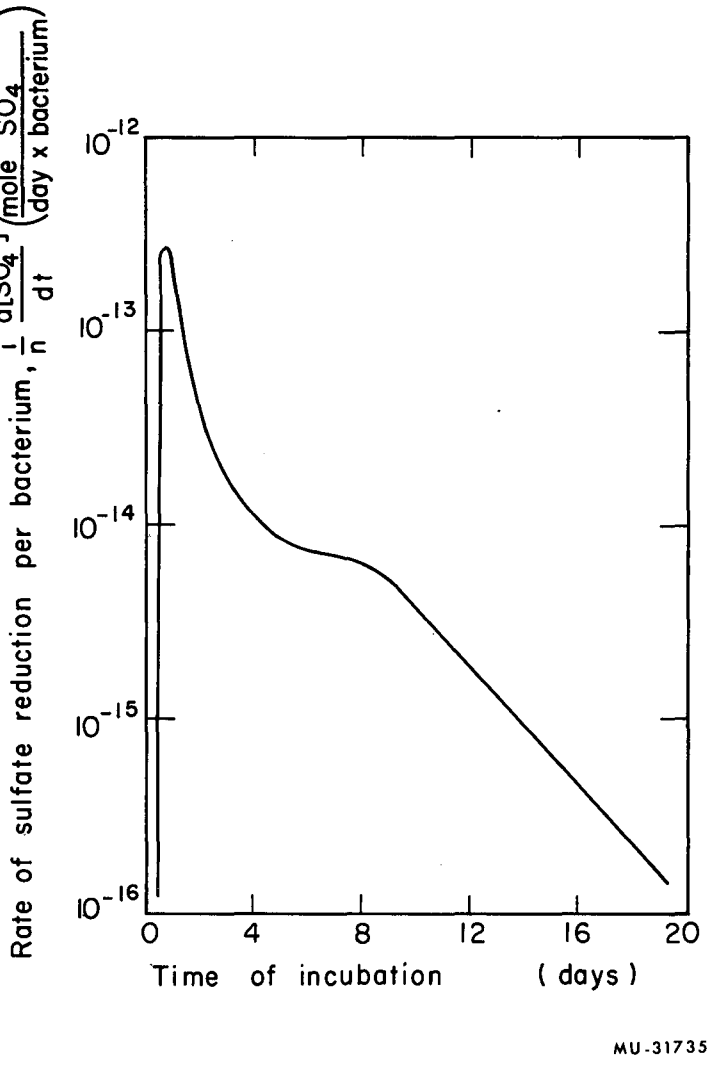


Fig. 11. Sulfate reduction rate per bacterium in complex lactate medium.

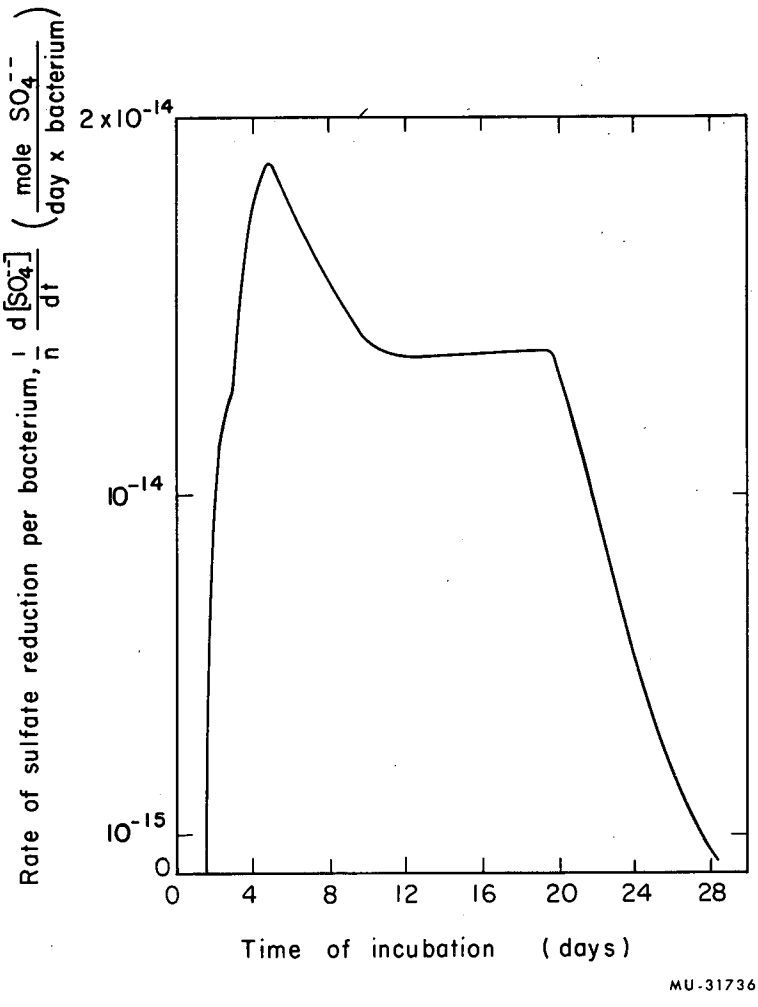


Fig. 12. Sulfate reduction rate per bacterium in simple lactate medium.

culture is given by $\frac{dn}{dt}$, and the basic metabolic activites are proportional to the quantity of bacteria present, n.

In the case of lactic acid fermentation at constant pH, Eq. (1) was found to apply over the entire batch fermentation, except possibly in the lag phase and at the very end of the fermentation.[44] Similarity of the sulfate reduction process to the lactic acid fermentation lies in the formation of a single end product which is also toxic. In sulfate reduction the pH is not controlled, but its variation is not as great as in the lactic acid for fermentation.

In the case of sulfate reduction, Eq. (1) can be written

$$\frac{d[H_2S]}{dt} = \alpha \frac{dn}{dt} + \beta n \, . \tag{2}$$

Since the rate of product formation is proportional to the rate of substrate utilization, we have

$$\frac{d[H_2S]}{dt} = - E \frac{d[SO_4^{=}]}{dt} \, , \tag{3}$$

where E is the efficiency of a fermentation process expressed as the ratio of product formed to substrate consumed, and is, for the present, assumed to be unity.

As already shown, the expression for the growth rate is

$$k = \frac{1}{n} \frac{dn}{dt} \, . \tag{4}$$

By substituting Eq. (4) and Eq. (3) into Eq. (2) and rearranging one obtains

$$\frac{1}{n} \frac{d[SO_4^{=}]}{dt} = \alpha k + \beta \, , \tag{5}$$

where $\frac{1}{n} \frac{d[SO_4^{=}]}{dt}$ is the already familar rate of sulfate reduction per bacterium, compiled in Table IV.

By plotting sulfate reduction rate per bacterium versus growth rate, and by fitting the data with a line, the constants α and β

can be obtained as the slope and the intercept of the line. The resulting plots are shown in Figs. 13, 14, and 15.

For the complex medium (Fig. 13), the line drawn has a slope $a = 2.2\times10^{-14}$ and an ordinate intercept $\beta = 7\times10^{-15}$. The equation of the line is therefore

$$\frac{1}{n}\frac{d[SO_4^{=}]}{dt} = 2.2\times10^{-14}k + 7\times10^{-15} . \qquad (6)$$

From the plot in Fig. 13 it can be seen that Eq. (6) is a fair approximation of the reduction rate over most of the batch process. It does not apply in the acceleration period, the first half of the first exponential period, and in the very last part of the stationary period. However, these periods comprise only about 15% of the duration of this particular batch cultivation. As it will be shown in the next section, a possible continuous cultivation would operate entirely in the region where Eq. (6) applies.

In the case of a simple medium, Eq. (5) could not be applied over the entire batch process, as seen in Fig. 14. However, for the period starting with the second exponential growth, Eq. (5) fits the data very well, as seen in the Fig. 15. The equation of the line is

$$\frac{1}{n}\frac{d[SO_4^{=}]}{dt} = 3\times10^{-14}k + 1.34\times10^{-14} . \qquad (7)$$

Actually, Eq. (7) applies over the entire range of batch growth in a real simple medium. Growth prior to the second exponential period, where the equation cannot be used, is not growth in simple, but in complex medium, as has been pointed out earlier.

6. Estimation of Sulfate Reduction in a Continuous Process

Because of the many advantages of a continuous over a batch operation, the possibility of a continuous process of brine purification by sulfate-reducing bacteria should be considered.

Because a turbidistat type continuous cultivator usually operates in the exponential growth phase, there is no dependence of reduction rate on the growth rate. The growth rate is constant. Bacterial population is also constant, and to have a more efficient process the population should be maintained at the highest possible level still compatible with the exponential growth.

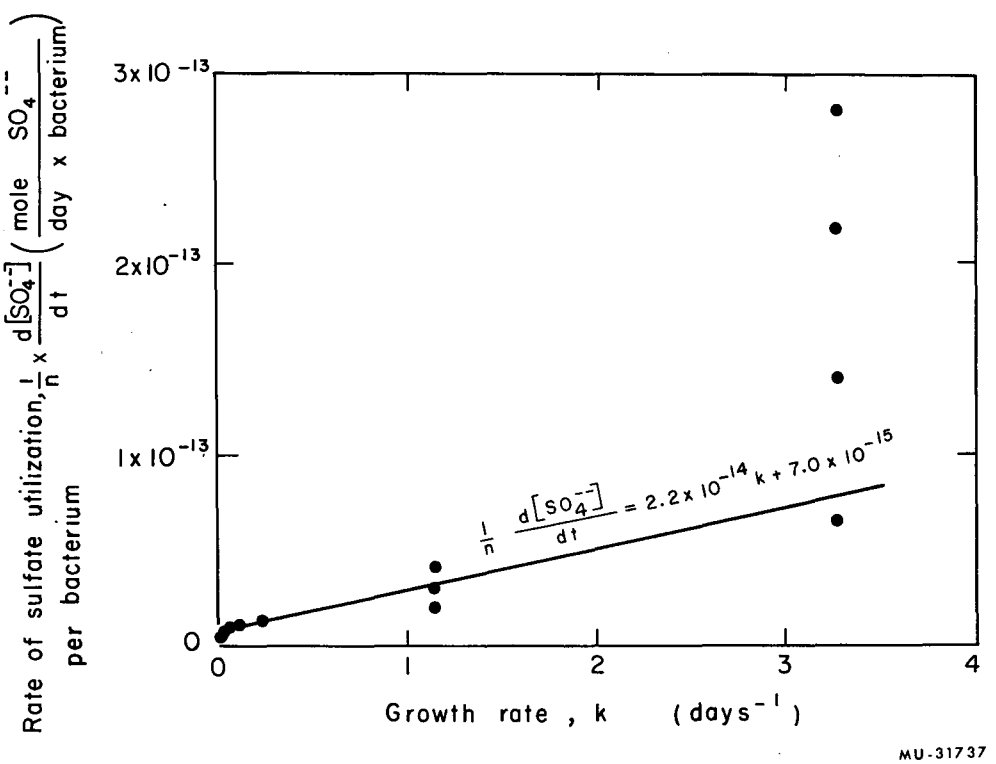


Fig. 13. Correlation for sulfate reduction in complex medium.

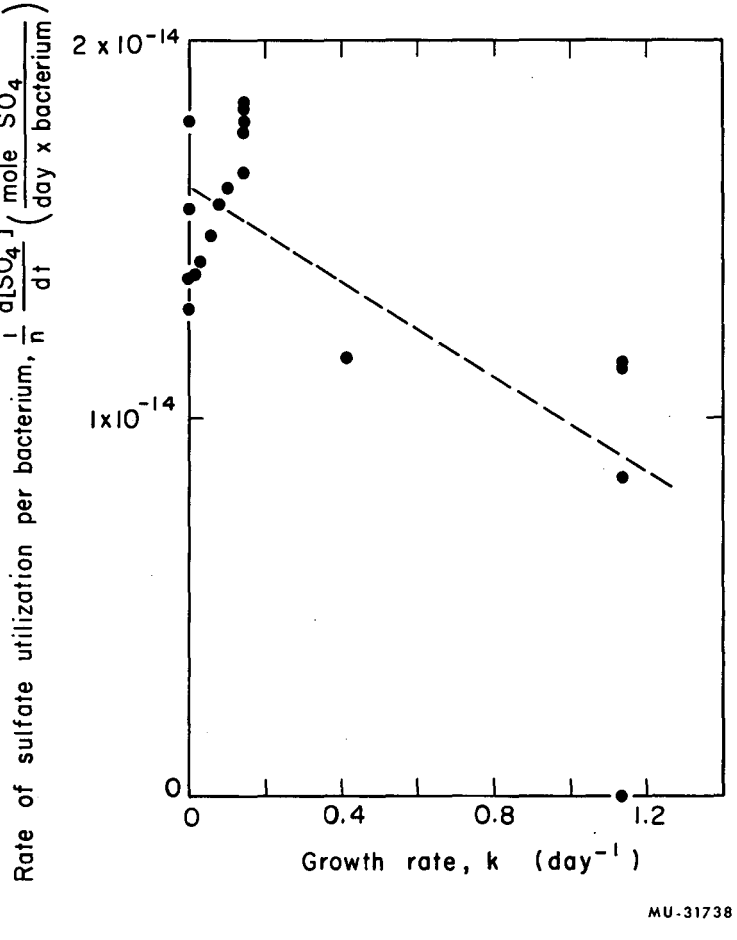


Fig. 14. Correlation for sulfate reduction in complex-simple lactate medium.

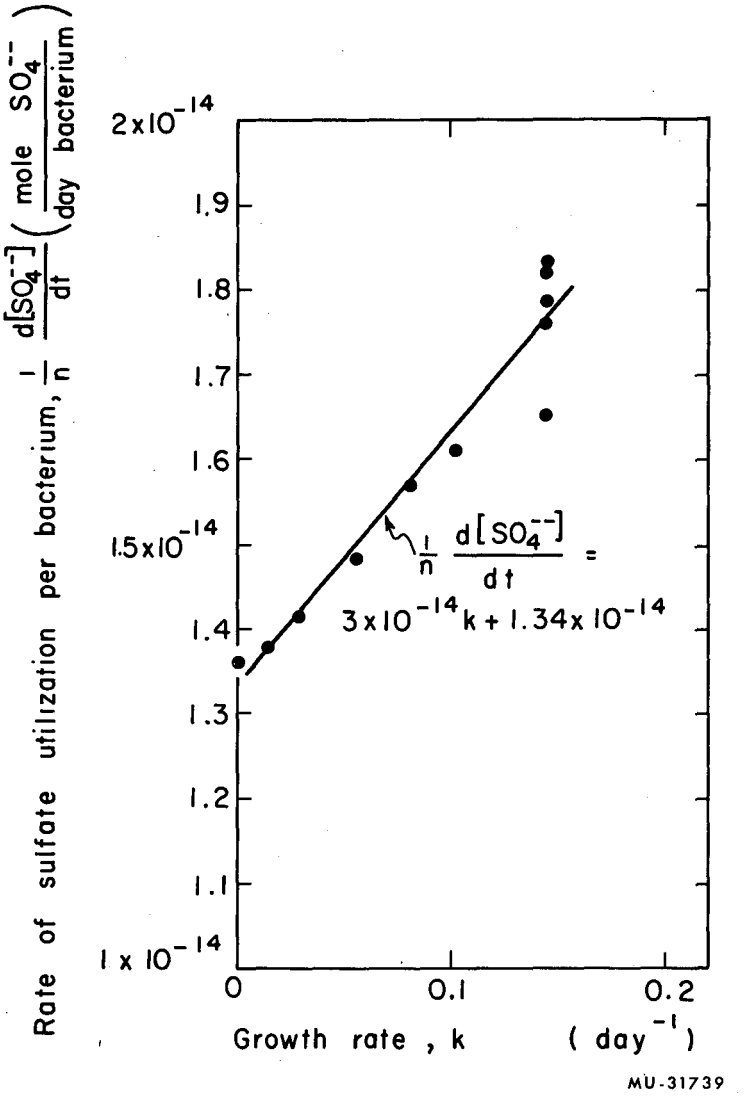


Fig. 15. Correlation for sulfate reduction in real simple lactate medium.

In such a continuous cultivator, the sulfate reduction rate can then be approximated as follows: Using the values derived during the second exponential phase in Medium E, where lactate may be the only energy source (k is 1.157 per day, and n is approximately 7.5×10^{10} cells per liter), we find that the sulfate reduction rate according to Eq. (6) is

$$\frac{d[SO_4^{=}]}{dt} = 7.5\times10^{10}\,[(2.2\times10^{-14})(1.157)+(7\times10^{-15})]$$

$$= 0.00244 \text{ moles/liter/day} .$$

For the growth in a simple lactate medium, in which we use the second exponential phase where no growth factors are present in the medium, k is 0.1444 per day and n is approximately 1.5×10^{10} cells per liter. Substituting these values in the Eq. (7) one gets

$$\frac{d[SO_4^{=}]}{dt} = 1.5\times10^{10}\,[(3\times10^{-14})(0.1444)+(1.34\times10^{-14})]$$

$$= 0.000266 \text{ moles/liter/day} .$$

B. Effect of Initial Sulfate Concentration on Sulfate Reduction

To determine how the sulfate reduction is affected by the initial sulfate concentration, an experiment was conducted in which a batch from a complex lactate medium was inoculated and then distributed into bottles containing different amounts of sterile sulfate.

Four concentration levels of sulfate were added: zero, 0.001, 0.01, and 0.1 moles per liter. The medium to which no sulfate was added had been analyzed for sulfate by the standard gravimetric method of $BaSO_4$ precipitation, and 0.0011 moles per liter sulfate were determined. So the actual sulfate concentrations prepared were 0.0011, 0.0021, 0.0111 and 0.1011 moles per liter.

If the effect of sulfate is to be studied, the organic substrate should be added in a sufficient amount to make sulfate the growth

limiting factor. Theoretically, two moles of lactate are oxidized to acetate for each mole of sulfate reduced, so that at least 0.202 moles per liter of lactate should be available. Actually 0.214 moles per liter were added to the medium (Medium G in the Appendices), and from subsequent evidence it is suspected that the lactate was never present in limiting amounts.

As in the previous experiments, the sulfate concentrations were obtained from the experimental hydrogen sulfide determinations. The results are shown in Table V and Fig. 16. The ratios of sulfate to the initial sulfate concentraion, which are indicative of the relative rate of sulfate utilization, are plotted in Fig. 17.

Reduction rates obtained by measuring slopes of the curves in Fig. 16 are shown in Table VI. Also shown are the average reduction rates, which were obtained by dividing total sulfate reduced by the time period during which the reduction was accomplished. In Case C, for instance, the average reduction rate is

$$\frac{(0.0111 - 0.0001)\text{moles } SO_4^{=}/\text{liter}}{6 \text{ days}} = 0.00183 \text{ mole/day/liter.}$$

The average rates were calculated because of the insufficient number of experimental points to permit a good plot of reduction rates versus time. The average sulfate reduction rates, and the final per cent conversions of initial sulfate, are plotted in Fig. 18.

From the above plots it can be observed that the reduction rate is greatly affected by the initial sulfate concentration. The highest rate was obtained with 0.0111 moles/liter initial sulfate (Case C). This finding is in good agreement with the results of previous work.[12, 13] The rates with lower sulfate concentrations (Cases A and B) are understandably lower because of low bacterial count. A high-density culture could not develop because of lack of sulfate. In the case of the highest sulfate concentration (Case D), a dense culture did develop, and the reduction rates during the first few days of growth are higher than in Case C. As the growth proceeds, however, the rate drops off and causes the average rate to be lower than in Case C. The slowing down

Table V. Experimental data on the reduction of sulfate by Desulfovibrio in complex lactate media of various initial sulfate concentrations.

Time of incubation (days)	Sulfate concentration (mole/liter)	Sulfate concentration / Initial sulfate concentration	Reduction of initial sulfate (%)
	Case A: Initial sulfate conc. = 0.0011 moles/liter		
0.21	0.0011	1.0	0
3.25	0.00085	0.772	33
6.0	0.00045	0.41	59
9.25	0.00035	0.318	68
13.0	0.00025	0.227	77
16.17	0.00015	0.136	86
	Case B: Initial sulfate conc. = 0.00213 moles/liter		
0.21	0.00213	1.0	0
3.25	0.00063	0.403	60
6.0	0.00033	0.211	79
9.25	0.00023	0.147	85
13.0	0.00013	0.061	94
16.17	0.00013	0.061	94
	Case C: Initial sulfate conc. = 0.0111 moles/liter		
0.21	0.0111	1.0	0
3.25	0.0056	0.051	95
6.0	0.0001	0.009	99
9.25	0.0001	0.009	99
13.0	0.0001	0.009	99
16.17	0.0001	0.009	99
	Case D: Initial sulfate conc. = 0.1011 moles/liter		
0.21	0.1011	1.0	0
3.25	0.0971	0.962	4
6.0	0.0900	0.89	11
9.25	0.0851	0.842	16
13.0	0.0821	0.812	19
16.17	0.0789	0.78	22
22.5	0.0753	0.745	26

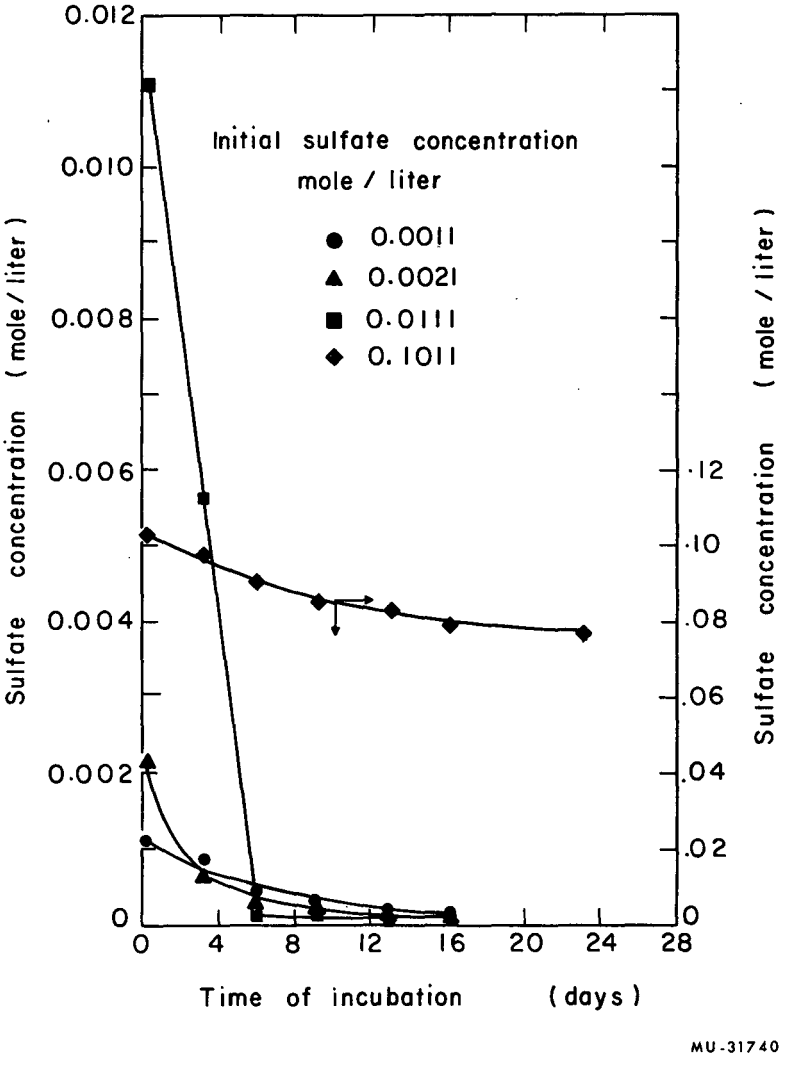


Fig. 16. Sulfate reduction at various initial sulfate concentrations.

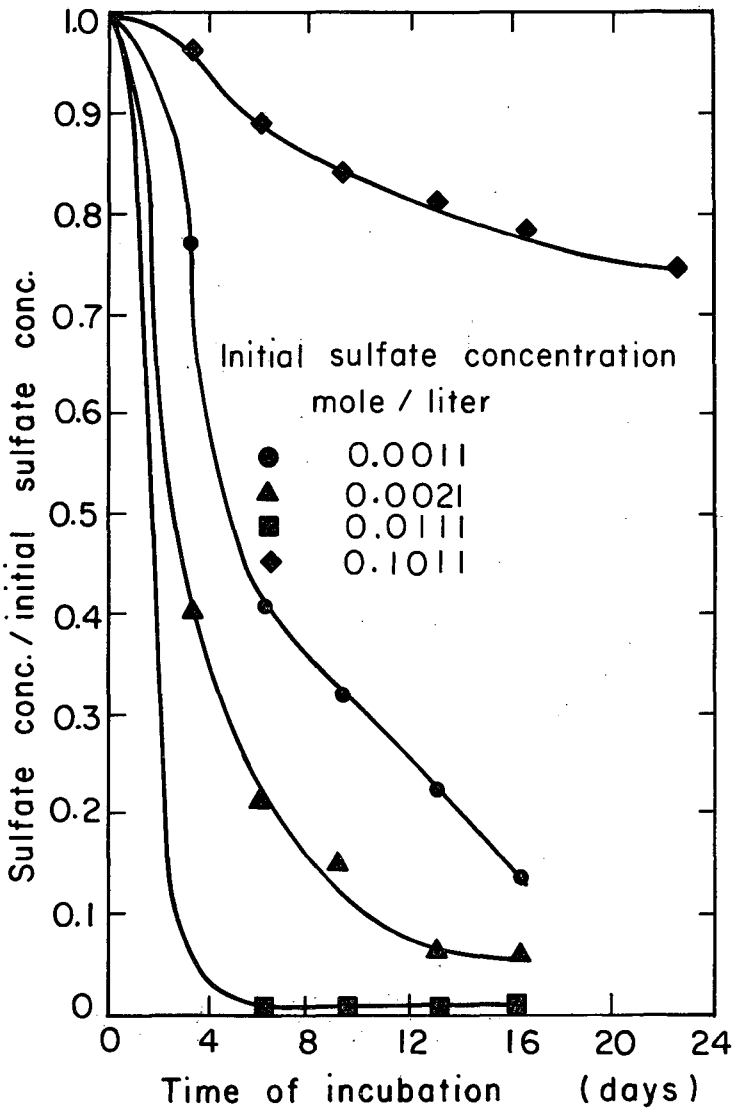


Fig. 17. Fraction of sulfate reduced at various initial sulfate concentrations.

Table VI. Sulfate reduction rates at different initial sulfate concentrations.

Time of incubation (days)	Rate of sulfate reduction (moles/day/liter)	Average rate of sulfate reduction (moles/day/liter)
	Case A: Initial sulfate conc. = 0.0011 moles/liter	
1	1.25×10^{-4}	
2	1.07×10^{-4}	
4	0.833×10^{-4}	5.86×10^{-5}
7	0.585×10^{-4}	
12	0.30×10^{-4}	
	Case B: Initial sulfate conc. = 0.00213 moles/liter	
1	5.88×10^{-4}	
2	3.33×10^{-4}	
4	1.18×10^{-4}	1.54×10^{-4}
6	0.67×10^{-4}	
8	0.41×10^{-4}	
12	0.20×10^{-4}	
	Case C: Initial sulfate conc. = 0.0111 moles/liter	
1	1.87×10^{-3}	
2	1.87×10^{-3}	
4	1.87×10^{-3}	
5	1.87×10^{-3}	1.83×10^{-3}
6.8	1.87×10^{-3}	
7	0	
	Case D: Initial sulfate conc. = 0.1011 moles/liter	
1	2.32×10^{-3}	
4	1.89×10^{-3}	
7	1.46×10^{-3}	1.15×10^{-3}
12	1.06×10^{-3}	

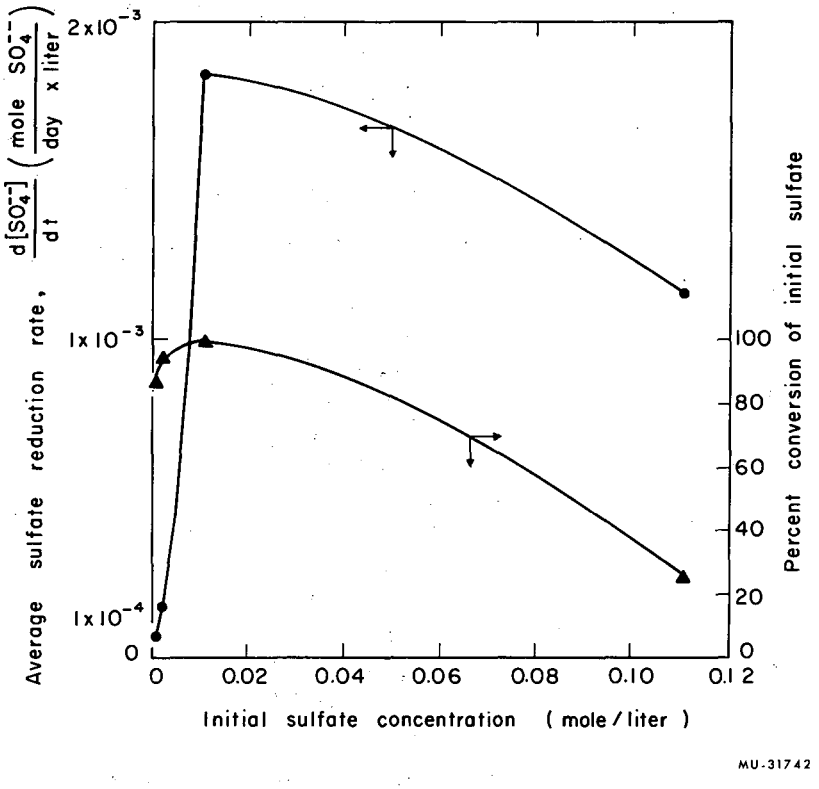


Fig. 18. Rates and efficiencies of sulfate reduction as a function of initial sulfate concentration.

of metabolic activites in Case D was certainly due to the inhibition by H_2S, which reached a concentration of 0.0258 moles/liter, compared to only 0.011 moles/liter in Case C. From this discussion, it can be concluded that the initial sulfate concentration has a strong effect on the reduction rates, but only in an indirect way. It is also obvious that if high reduction rates and per cent conversions are to be obtained with high initial sulfate concentrations, some hydrogen sulfide should be removed from the culture medium during the course of reduction. This could be done either by venting the excess H_2S, or in a closed system by adding ferrous sulfate or cadmium carbonate to the nutrient medium.[33]

The overall reduction of sulfate, or the per cent conversion (Fig. 18) is also highest in Case C, and lowest in Case D, due again to H_2S inhibition. At lower initial concentration (Cases A and B), the total conversion is somewhat lower than in Case C, but this observation is not very conclusive because of the large relative error involved in measuring very low concentrations.

C. Salinity Considerations

A necessary condition for the possible use of Desulfovibrio for purification of salt brines prior to their electrolytic decomposition is that the bacteria should be able to reduce sulfate at an appreciable rate in almost saturated NaCl solutions. For this reason the isolated bacteria were obtained from a natural habitat of very high salinity. In the following section the degree of NaCl tolerance of the isolated strain of Desulfovibrio aestaurii is examined.

1. Effect of Salinity on Sulfate Reduction

To determine the effect of salinity on sulfate reduction by the isolated strain of Desulfovibrio, several experiments were conducted in which the bacteria were grown in media of varying NaCl content, but of otherwise identical composition. Sulfide production, measured in the usual way, was then compared at the different salinities.

Three separate experiments were performed in a similar manner, but using different cultures for inoculum. The first experiment was done before the final isolation of Desulfovibrio and the inoculum contained some of the unknown rod-shaped bacteria. The results are shown in Table VII and Fig. 19. The second experiment was done with a pure culture of Desulfovibrio (Table VIII, Fig. 20), and the third one with an "adapted culture" discussed in the next section; its results are shown in Table IX and Fig. 21. The curves plotted are not for the entire growth range, and sulfide production is shown directly instead of the usual sulfate concentration. This is sufficient, however, since only the relative effects are significant when a comparison is made between different experiments. Growth in 10% NaCl media is used as the basis for comparison.

In the range from 1% to 10%, the effect of NaCl on the growth is very small, as seen in Fig. 19. Sulfide-production curves for different salinities within this range are not plotted because they all fall in the small area between the 1% and the 10% curve.

The effect of salinity above 10%, however, is very pronounced in all three experiments. Sulfide production and the total yield of sulfide

Table VII. Experimental data on sulfate reduction by an impure culture of Desulfovibrio in media of various salinity.

NaCl concentration weight (%)	Time of incubation (days)	H_2S production (mole/liter)
1	5	0.0148
	9	0.0158
	13	0.0158
	30	0.0158
	40	0.0160
2	5	0.0143
	16	0.0156
	40	0.0160
4	5	0.0153
	16	0.0158
	40	0.0155
6	5	0.0138
	9	0.0158
	13	0.0155
	16	0.0158
	30	0.0158
8	5	0.0143
	16	0.0153
	40	0.0155
10	2	0.0110
	5	0.0136
	9	0.0148
	13	0.0153
	20	0.0158
	30	0.0153
	40	0.0150
12	5	0.0073
	16	0.0133
	40	0.0140
14	5	0.0023
	16	0.0100
	40	0.0128

Table VII. (continued)

NaCl concentration weight(%)	Time of incubation (days)	H_2S production (mole/liter)
16	5	0.0003
	9	0.0033
	13	0.0078
	30	0.0110
	40	0.0118
18	5	0
	16	0.0018
	40	0.0038
20	5	0
	16	0
	40	0.002
22	5	0
	33	0
	40	0.0002

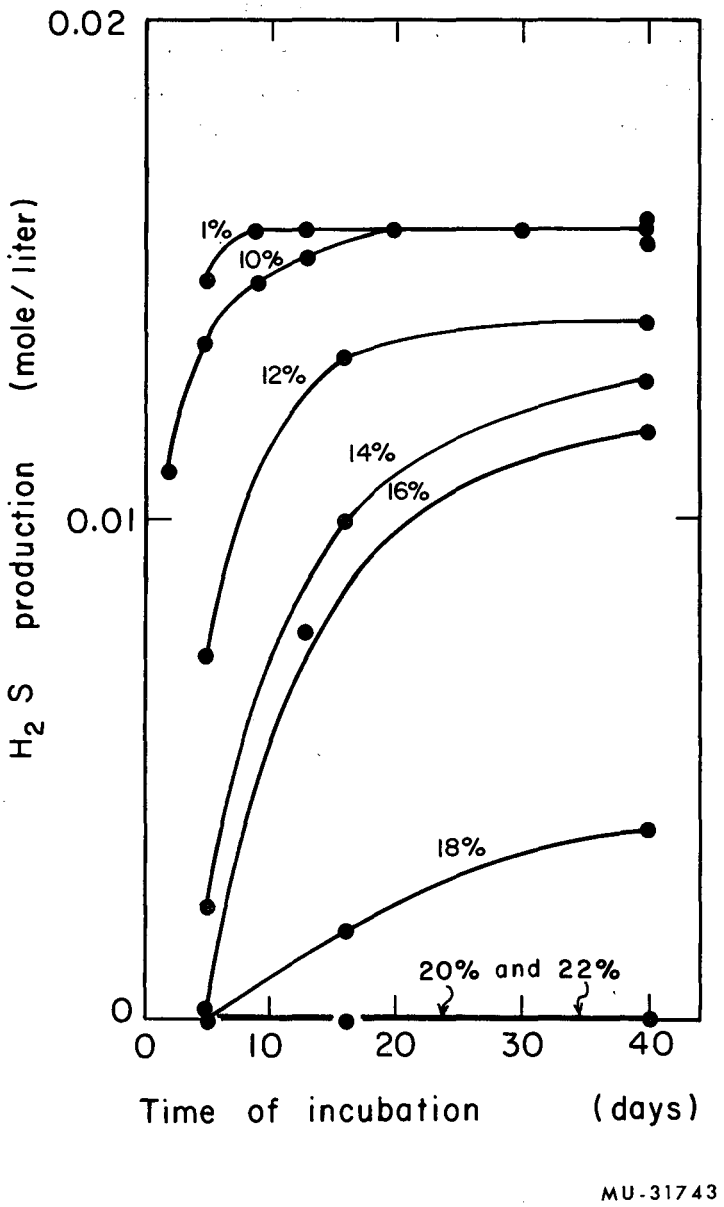


Fig. 19. Sulfide production by an impure culture of Desulfovibrio at different salinities.

Table VIII. Experimental data on sulfate reduction by a pure culture of Desulfovibrio in media of various salinity.

NaCl concentration weight (%)	Time of incubation (days)	H_2S production (mole/liter)
0	2	0
	6	0
	21	0
4	1	0
	2	0.0020
	4	0.0080
	7	0.0155
	15	0.0158
10	2	0
	6	0.004
	13	0.013
13	3	0
	7	0.0005
	17	0.0065
16	4	0
	7	0
	13	0
	21	0.0004
	31	0.0005
18	7	0
	21	0
	31	0

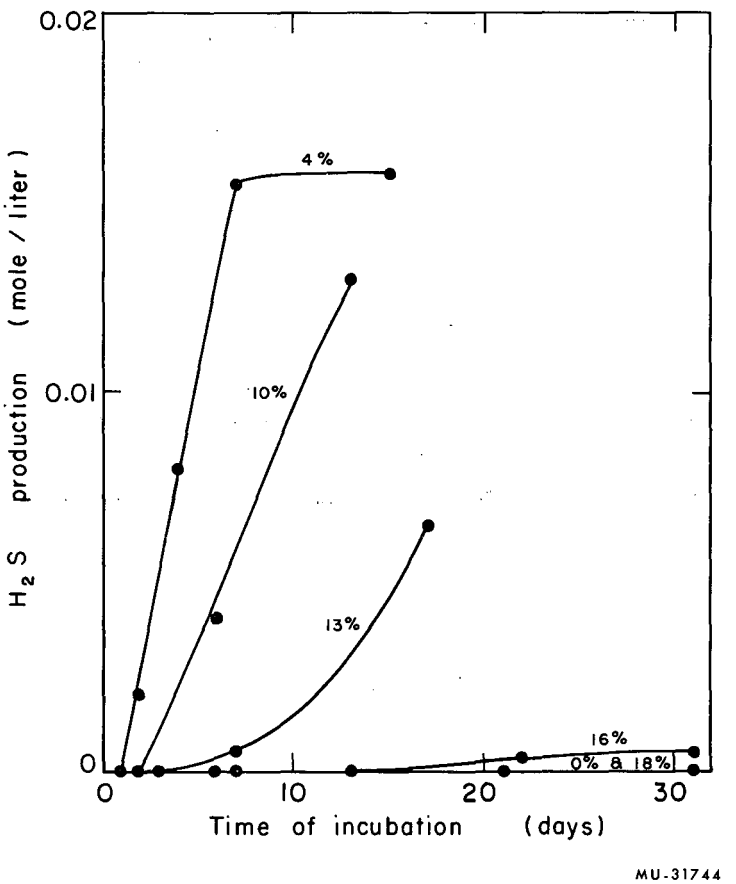


Fig. 20. Sulfide production by a pure culture of Desulfovibrio at different salinities.

Table IX. Experimental data on sulfate reduction by an adapted strain of Desulfovibrio in 10% and 16% NaCl media.

NaCl concentration weight(%)	Time of incubation (days)	H_2S production (mole/liter)
10	0.1	0
	2.0	0.00065
	3.3	0.0020
	4.9	0.00327
	6.9	0.0055
	11.9	0.0105
	15.9	0.0130
16	0.1	0
	4.9	0.0005
	6.9	0.0013
	9.0	0.00225
	11.9	0.0039
	15.9	0.00625

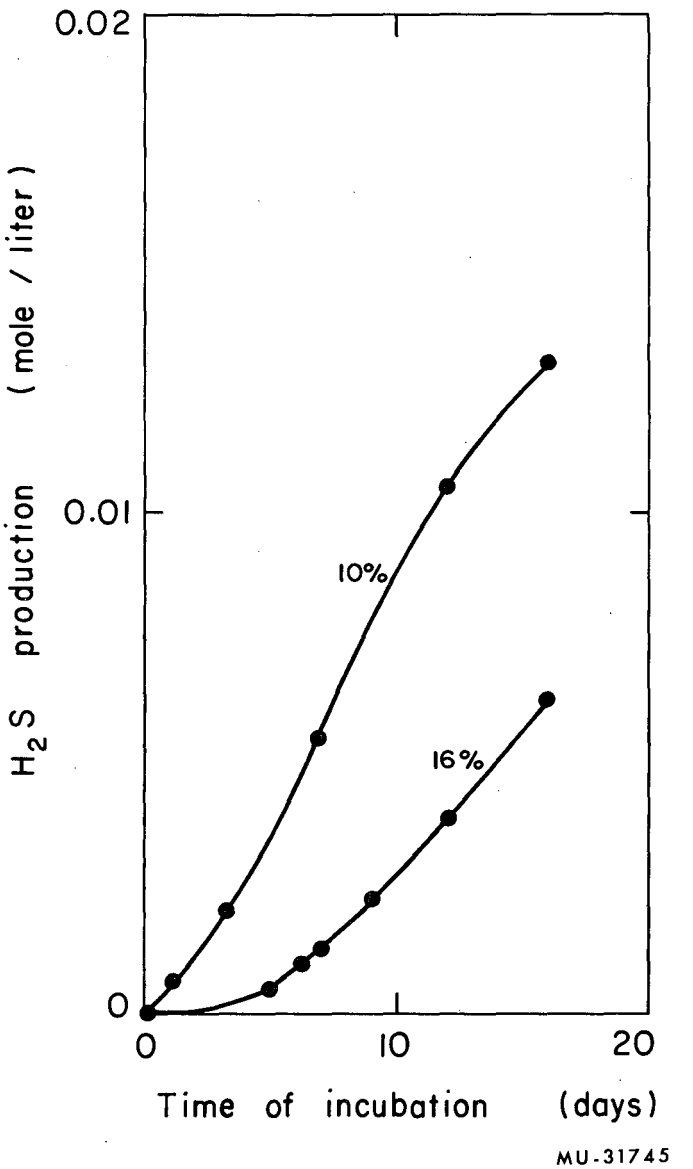


Fig. 21. Sulfide production by an adapted culture of Desulfovibrio in 10% and 16% salinity.

drop off rapidly as the NaCl concentration is increased above 10%. It is of interest to observe that the NaCl tolerance of Desulfovibrio is considerably greater in the mixed culture than in the pure one. While with the pure culture in 16% NaCl there is only a trace of H_2S produced (0.0005 mole/liter), with the mixed culture in 18% NaCl the H_2S production is still considerable (total yield in 16% equal $\frac{1}{4}$ of total yield in 10%).

Despite the fact that the bacterial strain used has been grown for many generations in 10% NaCl media the rate of sulfate reduction increases slightly as the NaCl concentration decreases from 10% to 1%, as is observed in Fig. 19. This indicates that the isolated strain is not a halophile, but that it is merely able to tolerate high salt concentrations. Some NaCl is, however, required for the growth. No growth has been obtained on NaCl-free media (Fig. 20).

2. Adaptation to Higher Salinity

Adaptability of Desulfovibrio to salt concentrations higher than those of the natural habitats has been reported in the literature.[17, 24, 25] The possibility of adaptation is also indicated by the fact that the NaCl level at which our strain was grown for a long time is also the limit below which the effect of salinity is very small. (Our strain was grown at the 10% level.)

The adaptation of Desulfovibrio was attempted by gradually raising the salt content of the inoculated media. A normal culture grown in a 10% NaCl medium (Medium E) was inoculated into a similar medium containing 11% NaCl. After the growth that indicated cloudiness appeared, part of the 11% culture was inoculated into a 12% NaCl medium. This procedure was continued up to 16% NaCl. With the increase in salinity, the lag period also lengthened. Upon transfer from 10% to 11% it took only 3 days for some growth to develop, but in the 15% to 16% transfer it took 18 days.

The effectiveness of the adaptation process was studied by measuring sulfate reduction by the "adapted strain" in both a 10% NaCl and

a 16% NaCl medium (Table IX, Fig. 21), and by comparing the results with those obtained previously with the original strain grown in 10% NaCl medium.

Comparing the curves obtained from growth at 16% NaCl of the normal pure culture and the adapted culture (Fig. 22), it is apparent that the NaCl sensitivity of Desulfovibrio was considerably lowered by the adaptation process. In 16% NaCl the sulfate reduction rate of the adapted strain is similar to that of the mixed culture, but much higher than the rate of the normal pure strain. With the adapted strain, and with the mixed culture the lag period in the 16% NaCl medium lasted approximately four days. The total sulfate reduced after ten days of incubation was equal to one third, and after fifteen days to one half of the total sulfate reduced in the 10% NaCl medium. This indicates an average reduction rate of 3.8×10^{-4} mole/day/liter in the 16% medium, as compared to 8.4×10^{-4} mole/day/liter in the 10% medium. In the case of the normal pure strain, the lag period lasted about fourteen days, and after twenty days of incubation only 0.0004 mole/liter of H_2S was produced, giving the average reduction rate of 2.7×10^{-5} mole/day/liter.

It should be pointed out here that the adaptation to higher salinity is not an irreversible process. No adverse effects were noticed upon inoculation of a culture grown in 10% NaCl into a medium containing only 1% NaCl. On the contrary, the lag period in the 1% medium was shorter than in the normal 10% medium.

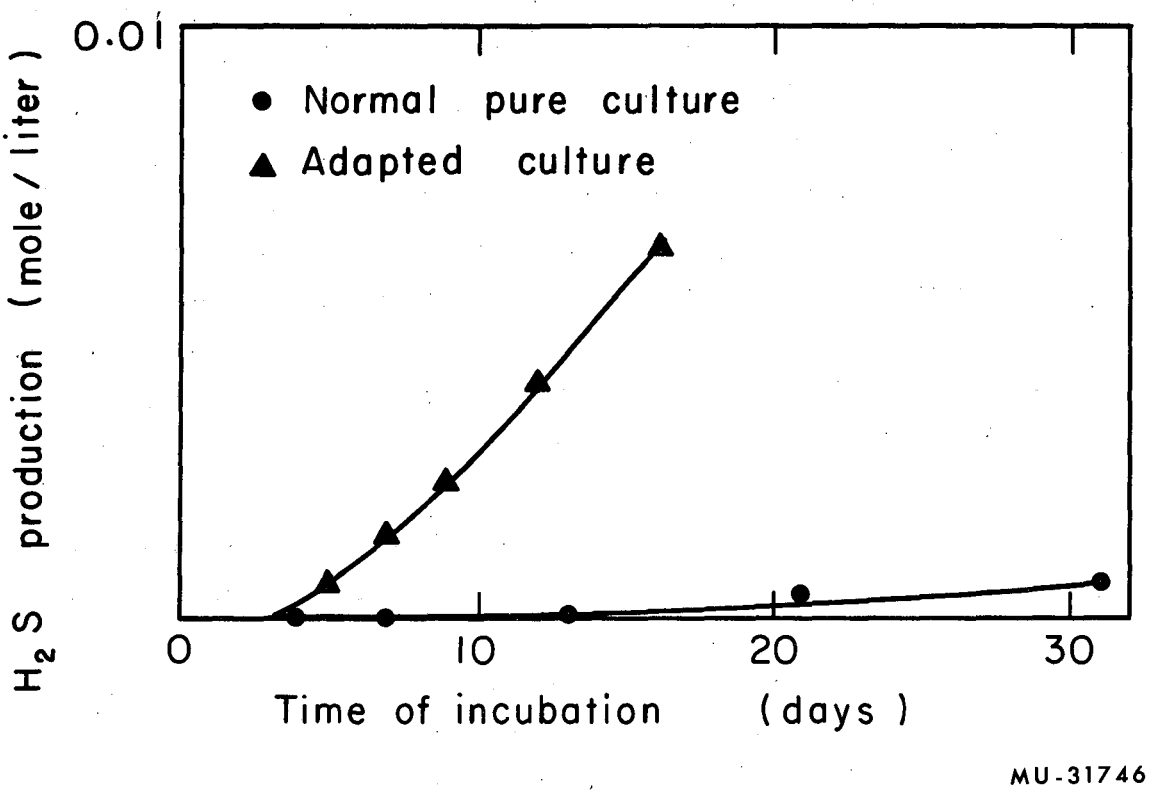


Fig. 22. Sulfide production in 16% salinity by the normal pure culture and by the adapted culture of Desulfovibrio.

V. SUMMARY AND CONCLUSIONS

The purpose of this research was to determine some of the growth characteristics of sulfate-reducing bacteria. The work is part of a general program to study possible utilization of sulfate-reducing bacteria to effect desired chemical transformations including the removal of sulfate impurities from salt brines. This latter problem has never been considered before.

Before the study of sulfate reduction could be started, a suitable microorganism had to be obtained. It was believed that a species of genus Desulfovibrio would be most appropriate because some species have been reported that not only reduce sulfate, but are either halophilic or tolerant of high salt concentrations. The latter property is desirable because some brines might have to be purified in an almost saturated state.

Considerable difficulties and delay were experienced with the isolation of the desired bacteria because of a very persistent rod-shaped contaminant. Several techniques were tried, and the isolation was finally achieved by the dilution method, and by another method utilizing the difference in motility of the two bacterial species when treated with crystal violet. The isolated sulfate-reducing bacteria were of species Desulfovibrio aestaurii, not a halophile, but tolerant of salt concentrations up to 16%, and probably higher.

The desired kinetic data on sulfate reduction were obtained from experiments in which accurate measurements of bacterial population and sulfide production were made over extended time periods. Experiments were done in complex and in simple, or synthetic lactate media. Experimental growth curves, and sulfate-reduction curves were used in calculating growth rates, sulfate reduction rates per volume of culture, and per single bacterium. Expressions relating sulfate reduction to growth rate and bacterial density were obtained that apply over most of a batch process. The expressions for complex and simple media are respectively

$$\frac{1}{n} \frac{d[SO_4^{=}]}{dt} = 2.2\times10^{-14} k + 7\times10^{-15} ,$$

and

$$\frac{1}{n} \frac{d[SO_4^{=}]}{dt} = 3\times10^{-14} k + 1.34\times10^{-14} .$$

In the application of these expressions to a continuous process, the sulfate reduction rates were found to be independent of growth rate and bacterial population for a well defined medium. The rates are 0.00244 and 0.000266 moles sulfate reduced per day per liter for complex and simple bactate media, respectively. Because the rate in the complex medium is ten times the rate in the simple medium, it is very advantageous to use complex media, especially since only 10 mg/liter of yeast extract or peptone are necessary to make a medium complex.

The effects of the initial sulfate concentration on its reduction were examined. The highest per cent reduction of sulfate (99%) was obtained with 0.011 mole/liter initial sulfate. By raising the initial sulfate concentration, the total reduction was diminished (26% reduction with 0.1 mole/liter initial sulfate), and by lowering it the rate of reduction was diminished (0.00183 moles reduced/liter with 0.011 mole/liter initial sulfate, and only 0.000154 moles reduced/liter with 0.0021 mole/liter initial sulfate).

Sodium chloride has the effect of slowing down the rate of reduction, although some NaCl is required for growth. The effect of NaCl concentration up to 10% was small, but above 10% it becomes very strong, with only very limited growth taking place in 16% NaCl. It should be noted that the isolation took place at 10% salinity.

It was observed that the effect of salinity on Desulfovibrio was reduced in impure cultures containing the unknown rod-shaped bacteria. Another way of reducing the effect of salinity was by gradual adaptation to higher NaCl concentrations. By such a method a culture was obtained that could grow in 16% at a rate only 50% lower than the rate in 10% NaCl.

A tentative answer to the feasibility of industrially applying bacterial sulfate reduction can be obtained by performing an economic evaluation on the basis of the given kinetic data. To make a definite answer, however, more experimental work is needed. Some areas to be investigated are: effect of pH and E_h and their control; upper limit of the bacteria's adaptability to NaCl; reduction rates of the adapted cultures; use of impure cultures to reduce the effect of salinity and sterilization requirements; use of cheaper organic nutrients; separation of bacteria, acetate, and excess nutrients from the "purified" brines; and autotrophic growth on hydrogen and carbon dioxide.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Professor Alvin J. Clark.

This work was done under the auspices of the U. S. Atomic Energy Commission.

APPENDICES

A. Preparation of the Media

All the ingredients of a particular culture medium, except $FeCl_3$ and $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, were dissolved in an appropriate amount of distilled water. The solution was then sterilized in a steam-autoclave at 17 psig for twenty to thirty minutes. After cooling, which was normally done in the sterilizer itself, the pH and redox potential of the solution were measured with a Beckman Zeromatic pH Meter. From 0.5 to 0.8 g/liter of $Na_2S \cdot 9H_2O$ were then added to obtain a pH of 8.0 to 8.2 and a redox potential of -100 to -120 mV. Sometimes it was necessary to add from 0.05 to 0.2 g/liter of ascorbic acid. The ascorbic acid lowered the pH without raising the redox potential. Such a medium contained from 0.001 to 0.003 moles per liter of H_2S. The medium was then filtered to remove any precipitate formed during sterilization, and a trace of $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ or $FeCl_3$ was added. All the work described was done under sterile conditions.

The prepared medium was stored in sterile, completely full, glass-stoppered, Pyrex reagent bottles of various sizes. All of the growth experiments were done in 60 ml bottles.

B. Composition of the Media

The compositions of all the media mentioned in the report, in which they are referred to only by their letters, are given below:

The values given are for one liter of medium after dilution; distilled water was used in each case.

1. Medium A	Weight (grams)
K_2HPO_4	0.5
NH_4Cl	1.0
$CaSO_4$	1.0
$MgSO_4 \cdot 7H_2O$	2.0
Sodium lactate (60% syrup)	6.0
$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$	trace
NaCl	100.0

Some variations of Medium A used as enrichment media also contained 3% NaCl and 13% NaCl.

2. Medium B	Weight (grams)
K_2HPO_4	0.5
NH_4Cl	1.0
$MgSO_4 \cdot 7H_2O$	2.0
Na_2SO_4	18.25
$CaCl_2 \cdot 2H_2O$	0.1
Sodium lactate (60% syrup)	21.0
$CaCO_3$	1.0
$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$	trace
NaCl	10.0

3. Medium C	Weight (grams)
K_2HPO_4	0.5
NH_4Cl	1.0
$CaSO_4$	1.0
$MgSO_4 \cdot 7H_2O$	2.0
Sodium lactate (60% syrup)	6.0
$FeCl_3 4H_2O$	trace
Na_2SO_4	1.5
NaCl	100.0

4. Medium D	Weight (grams)
K_2HPO_4	0.5
NH_4Cl	1.0
$CaSO_4 \cdot 2H_2O$	1.4
$MgSO_4 \cdot 7H_2O$	2.0
$FeCl_2 \cdot 4H_2O$	trace
$FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$	trace
Sodium lactate (60% syrup)	6.0
Peptone	1.0
Yeast extract	1.0
NaCl	100.0

5. Medium E	Weight (grams)
K_2HPO_4	0.5
NH_4Cl	1.0
$MgSO_4 \cdot 7H_2O$	2.0
Na_2SO_4	1.0
$CaCl_2 \cdot 2H_2O$	0.1
$FeCl_2 \cdot 4H_4O$	trace
Sodium lactate (60% syrup)	6.0
Peptone	1.0
Yeast extract	1.0
NaCl	100.0

6. Medium F

Same composition as Medium E, except no peptone and yeast extract.

7. Medium G

Same composition as Medium E, but no $MgSO_4$ and Na_2SO_4, and 40 grams sodium lactate instead of 6 grams.

C. Sulfide Determination

A measured quantity of culture medium, usually 10 ml, was added to 5 ml of 0.1 normal iodine solution in a 50 ml Erlenmeyer flask. We added 5 ml of 10% acetic acid and the mixture was allowed to stand for about two minutes. The excess iodine was then back titrated with 0.1 normal thiosulfate solution, using starch as an indicator.

If the solution to be analyzed contained substances other than sulfide capable of reacting with iodine, two samples were analyzed. The first sample was analyzed in the manner described above. The second sample was first boiled for a few minutes with 5 ml of one normal sulfuric acid, cooled, then reacted with iodine and back titrated with thiosulfate in the same way as the first sample. Boiling the sample with sulfuric acid drove out sulfide before the addition of iodine. By subtracting the iodine consumed in the second sample from the iodine consumed in the first one, the iodine that reacted with sulfide only was obtained.

D. Nomenclature

Symbols are listed in the order of their appearance in the report.

k = growth rate of bacteria, day^{-1}

$t_G = \frac{\log 2\ (t_2-t_1)}{\log n_2 - \log n_1}$ = generation time, hours or days

n = number of bacteria

t = time, hours or days

$[SO_4^{=}]$ = concentration of sulfate, mole per liter

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