Title
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Publication Date
1965-10-01

UCRL-16420
Preliminary

University of California

Ernest O. Lawrence Radiation Laboratory

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Prepared for presentation at the U.S.-Japan Seminar on Dynamics of Microbial Populations, Kyoto, Japan

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AEC Contract No. W-7405-eng-48

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Mark Leban, V. H. Edwards, and C. R. Wilke

October 1965

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INTRODUCTION

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. Spore-forming clostridia that reduce sulfate are also known.

Desulfovibrios as a group are Gram-negative, obligately-anaerobic vibrio or spirilloid organisms, very often motile with polar flagellum. They are very versatile, and their natural habitats embrace a wide range of

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

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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.

The metabolism of Desulfovibrio shows an 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.[1]

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.

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.[3,4]

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.

A more complete review of the metabolism and ecology of Desulfovibrio has been presented previously by the authors.[5]

The present paper is concerned primarily with growth rate studies of a salt tolerant strain of Desulfovibrio isolated from San Francisco Bay. Details of the isolation procedures and description of the organism are given in Appendix I. Batch culture studies were made over a range of media compositions and other variables. Continuous culture studies of limited scope were made in an effort to relate the kinetics of the batch and continuous systems. The experiments and resulting conclusions are summarized briefly. More detailed descriptions of the work and tabulations of data may be obtained from the original reports.[5,6]

A. Effect of Peptone and Yeast Extract

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 thoroughly 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, and a bacterial count was made with a Petroff-Hausser counting chamber, as described in Appendix II. A cumulative amount 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 paper, a medium to which yeast extract and peptone 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 plotted in Figs. 1, 2, and 3. 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.

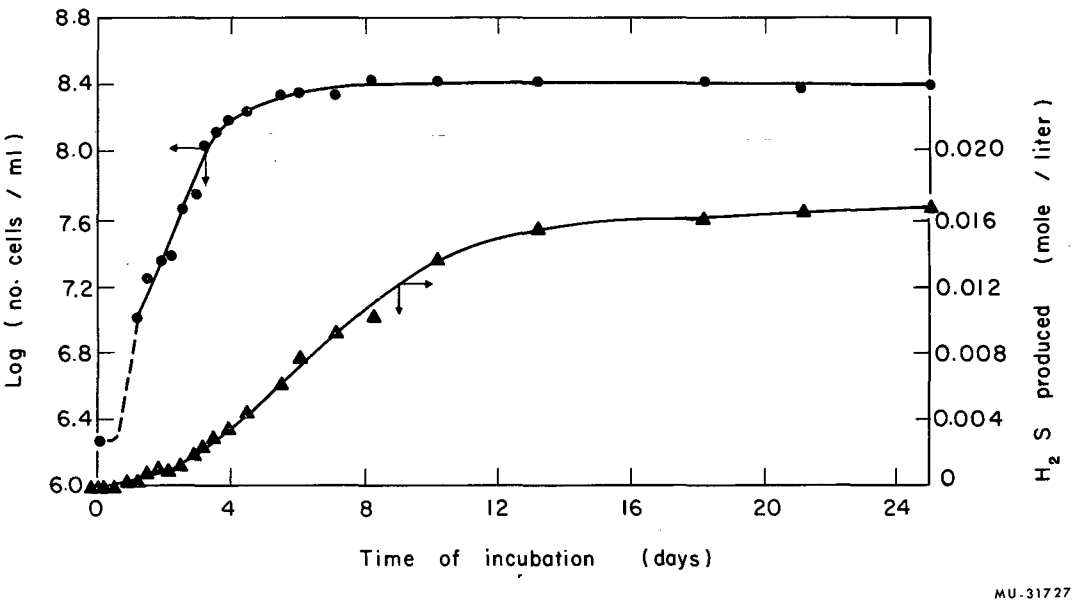

Fig. 1. Growth curve of Desulfovibrio in complex lactate medium at 30°C. Experiment No. 1.

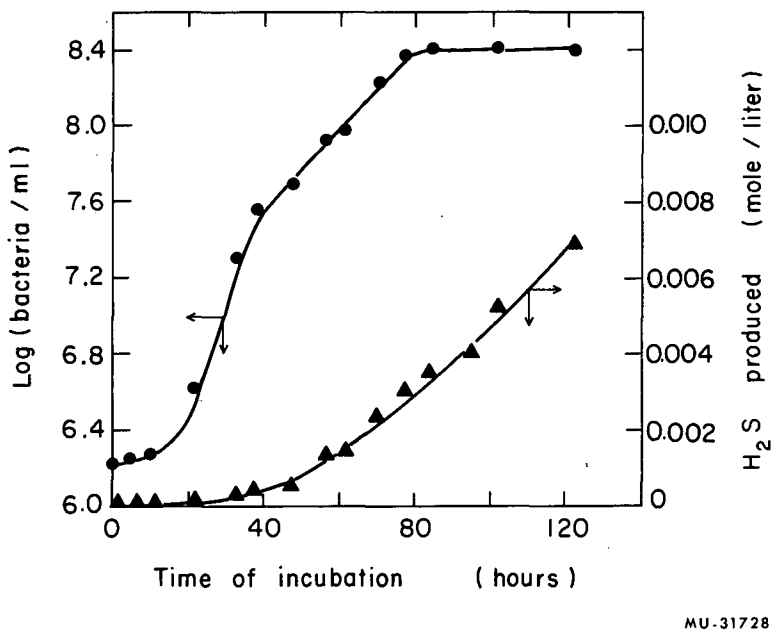

Fig. 2. Growth curve of Desulfovibrio in complex lactate medium at 30°C. Experiment No. 2.

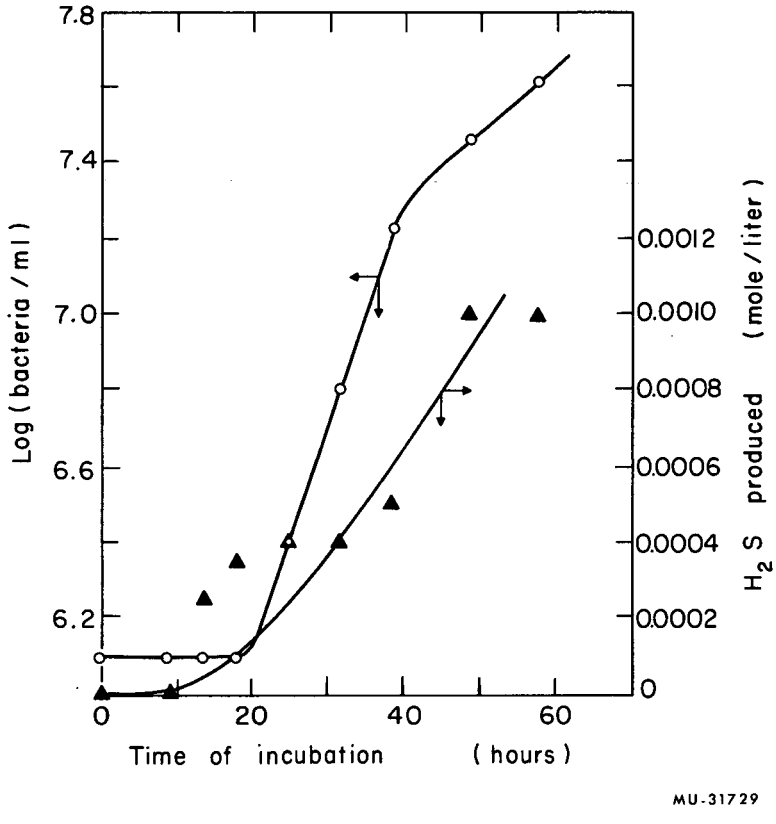

Fig. 3. Growth curve of Desulfovibrio in complex lactate medium at 30°C. Experiment No. 3.

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 believed to be 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 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 vitamin or 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 plotted in Figs. 4 and 5 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. The 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, $t_G = 14.65$ hours). Then there is another lag phase lasting one day, followed by much slower exponential growth (duration = 3 days, $t_G = 115$ hours). 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.

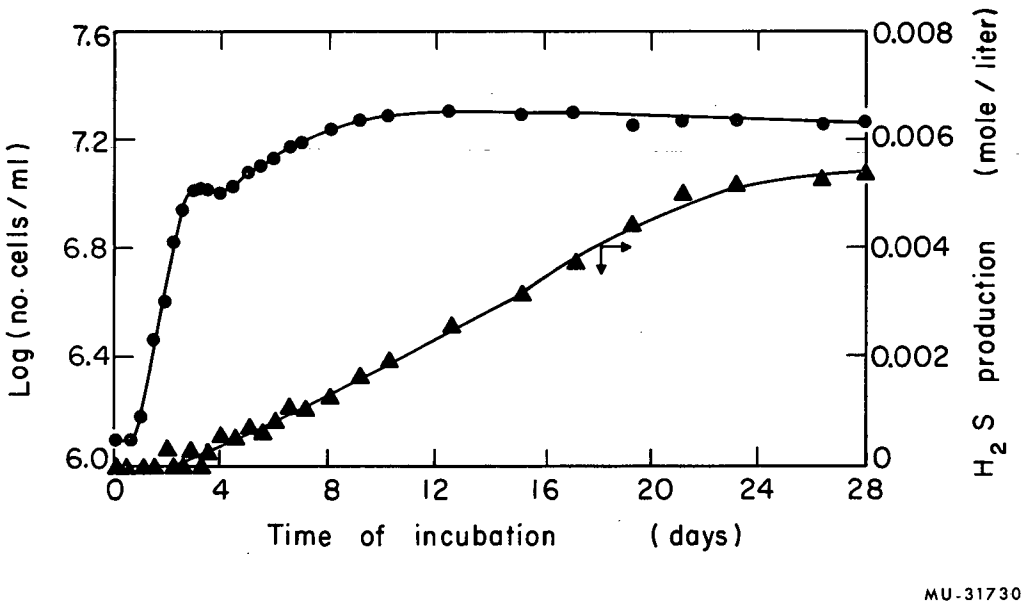

Fig. 4. Growth curve of Desulfovibrio in simple lactate medium at 30°C.

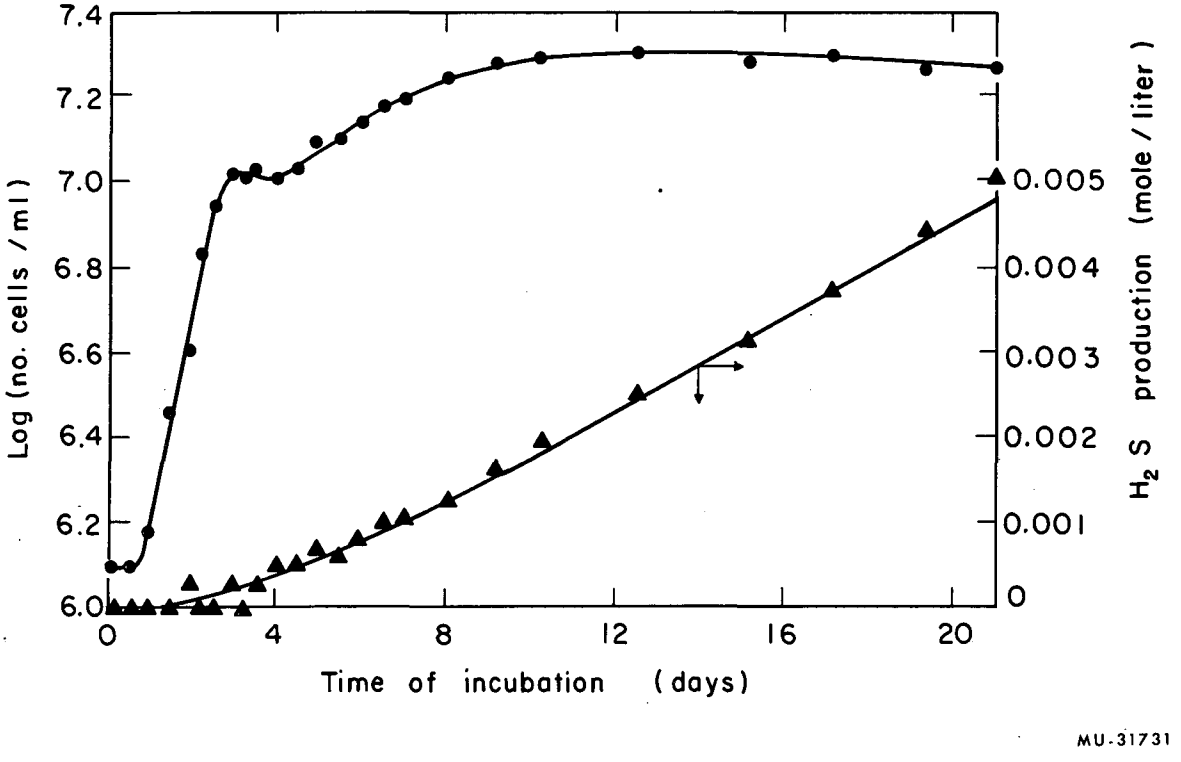

Fig. 5. Growth curve of Desulfovibrio in simple lactate medium at 30°C (same as Fig. 4, but larger scale).

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[7] 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 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 bacterial 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

The specific growth rate k, is given by

$$k = \frac{1}{n}\frac{dn}{dt} \qquad (1)$$

where: n is the number of bacteria

t is the time, days.

The above expression can be written as

$$k = \frac{d \ln n}{dt}, \qquad (2)$$

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 plotted in Fig. 6. In these curves, the characteristic growth behavior in the two media plainly stands out.

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 II.

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 per liter.

These sulfate concentrations were plotted versus time of incubation and from the slopes of these curves, the sulfate utilization or reduction rates $d[SO_4^{--}]/dt$ for different incubation times were obtained.

These calculations 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

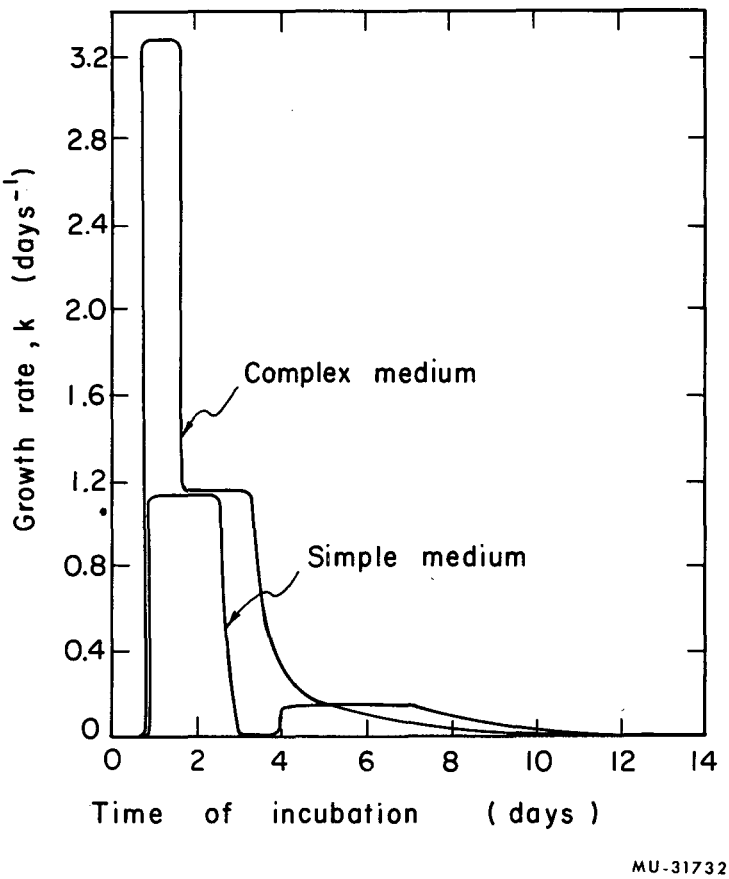

Fig. 6. Growth rate of Desulfovibrio in complex and simple lactate medium at 30°C.

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. Because the bacterial populations in the two experiments were very different, comparison of specific rates of sulfate reduction is more meaningful.

4. Rate of Sulfate Reduction Per Bacterial Cell

By dividing the sulfate reduction rates per volume of culture, computed in the preceding section, by the bacterial counts obtained from the growth curves, sulfate reduction rates per single bacterial cell are obtained. These rates are plotted in Figs. 7 and 8 versus time of incubation.

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 activities 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 untill 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. However, no correction was made for the small increase in cell diameter.

5. Effect of Yeast Extract in MacPherson's Medium

In a nutritional study of a fresh-water strain of sulfate-reducing bacteria, MacPherson and Miller[8] reported the development of a chemically defined culture medium that did not form a precipitate on autoclaving as did Medium E. The composition of the MacPherson's medium is given in Appendix II. Such a medium is desirable in a kinetic study and experiments were performed with this medium as well as with complex and simple media described above. To obtain large yields, it proved necessary to add yeast extract. The

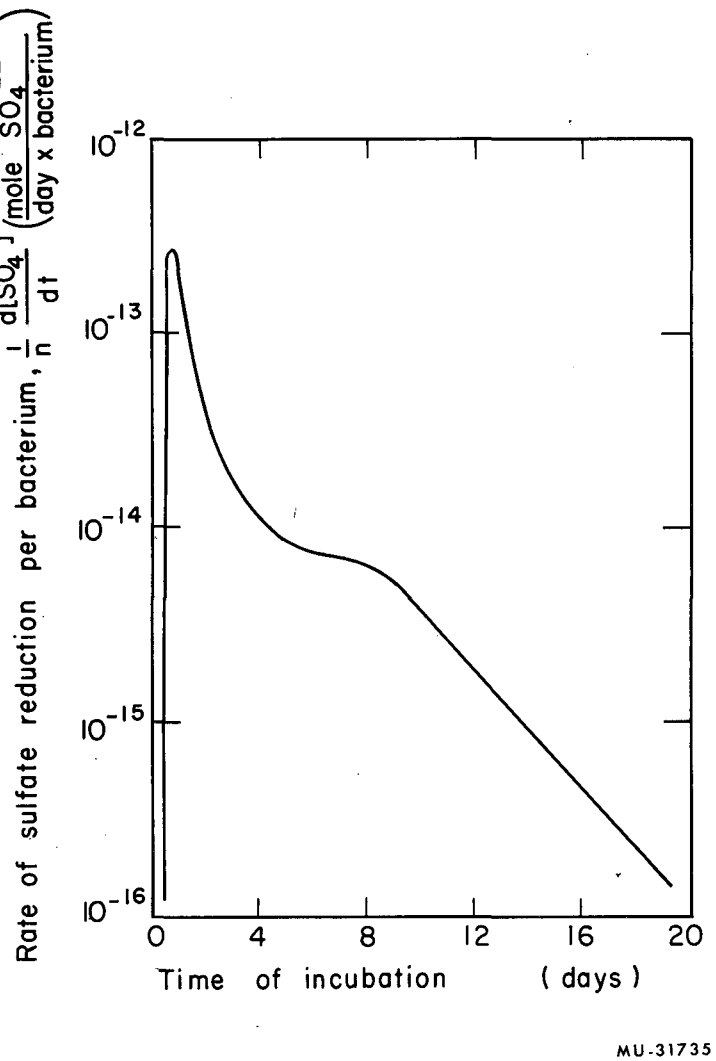

Fig. 7. Sulfate reduction rate per bacterium in complex lactate medium.

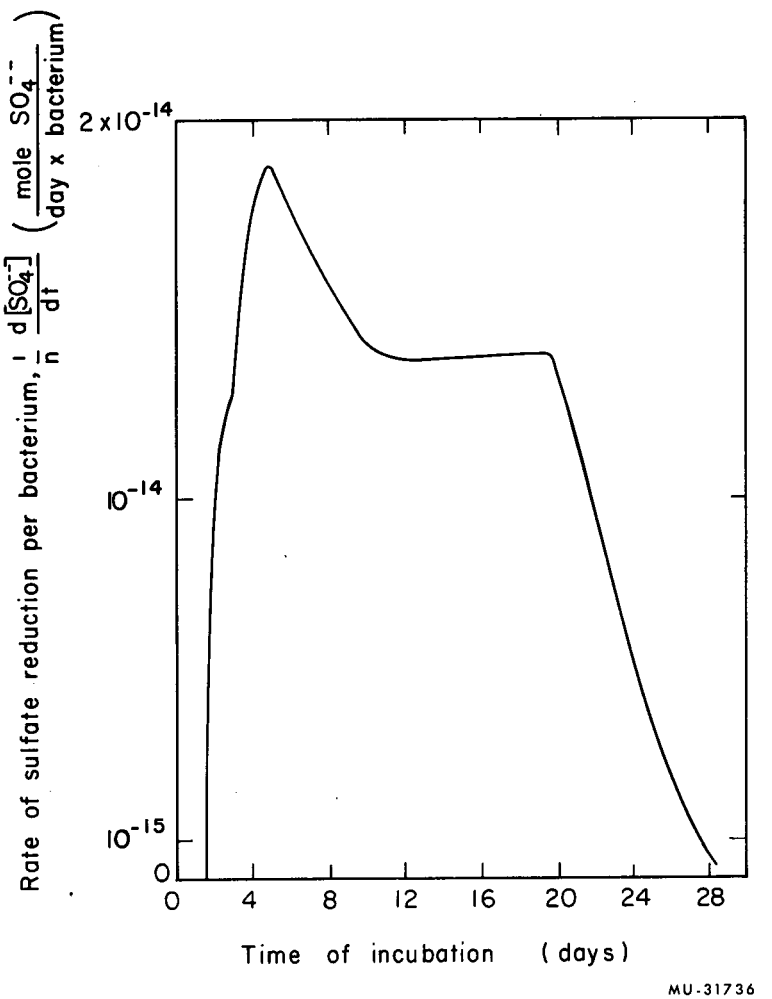

Fig. 8. Sulfate reduction rate per bacterium in simple lactate medium.

considerable effect of yeast extract on the kinetics of sulfate reduction was undesirable in a kinetic study because the components of the yeast extract important to the culture were unknown and could not be measured. For this reason, an experiment was performed to determine if a concentration of yeast extract could be found at which it would not play a predominant role in the kinetics of sulfate reduction. Seven sets of MacPherson's medium were prepared. In each set of media, the concentration of yeast extract was changed from the standard value. Media were prepared and inoculated that contained zero, 0.05, 0.1, 0.2, 0.3, 0.5, and 1.0% (w/v) yeast extract. The standard composition was also altered to contain 85 mM/l lactic acid instead of 100 mM/l because inhibition had been observed at the higher lactic acid concentration. The technique of batch culture in glass bottles was followed. After inoculation, periodic measurements of sulfide and cell concentration were made on the batch cultures. Dry weight measurements were also made initially and four days after inoculation. Table I lists yields for the various initial concentrations of yeast extract. Cell yield was calculated as the average of optical counts on a culture after the growth had reached an approximately constant value. Dry weight measurements which were all made after four days of batch growth cannot be regarded as final values for the cultures containing 0.5 and 1.0% yeast extract, because of inhibition by these higher concentrations. However, these dry weights still bear a rough proportionality to the initial concentration of yeast extract.

Sulfide concentrations after 4 days and after 16 days of incubation are also presented in Table I. It can be seen that yeast extract at all concentrations has an important effect on the kinetic behavior of sulfate-reducing bacteria. Increasing concentrations of yeast extract led to increasing values of cell yield and sulfide production. But growth rates were near their maximum at 0.1% yeast extract and appreciable reductions in growth rate and sulfide production rate were observed at concentrations of 0.3% and above. On the basis of these results, it was concluded that changes in the yeast extract concentration would have the smallest effect at a value of 0.25% (w/v).

Table 1. The Effect of Yeast Extract on Yields in Medium M.

Initial concentration of yeast extract [% (w/v)]	Cell yield (10^8 cells/ml)	Dry weight after 4 days (g/l)	Sulfide after 4 days (mM/l)	Sulfide after 16 days (mM/l)
0	0.011	—	0.6	—
0.05	0.45	—	9.1	—
0.1	1.23	0.066	11.0	23.7
0.2	2.52	0.140	16.3	27.1
0.3	2.54	0.180	14.4	27.7
0.5	2.34	0.174	5.6	27.2
1.0	3.69	0.286	1.8	28.7

B. Effect of Sulfate Concentration

To determine how sulfate reduction is affected by the initial sulfate concentration, an experiment was conducted in which a batch of complex lactate medium was inoculated and then distributed into bottles containing different amounts of sterile sulfate. Four concentration levels of sulfate were employed: zero, 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 amount although it was probably inhibitory at the highest concentration.

As in the previous experiments, the residual sulfate concentrations were based on the experimental hydrogen sulfide determinations. The results are shown in Table II. The ratios of sulfate to the initial sulfate concentration, which are indicative of the relative rate of sulfate utilization, are plotted in Fig. 9.

Average reduction rates were obtained by dividing total sulfate reduced by the time period during which the reduction was accomplished. The average rates were calculated because of the insufficient number of experimental points to permit accurate evaluation of instantaneous reduction rates versus time. The average sulfate reduction rates, and the final per cent conversions of initial sulfate, are plotted in Fig. 10.

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.[9, 10] 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

Table II. 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. = 0.0021 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	9.0851	0.842	16
13.0	0.0821	0.812	19
16.17	0.0789	0.78	22
22.5	0.0753	0.745	26

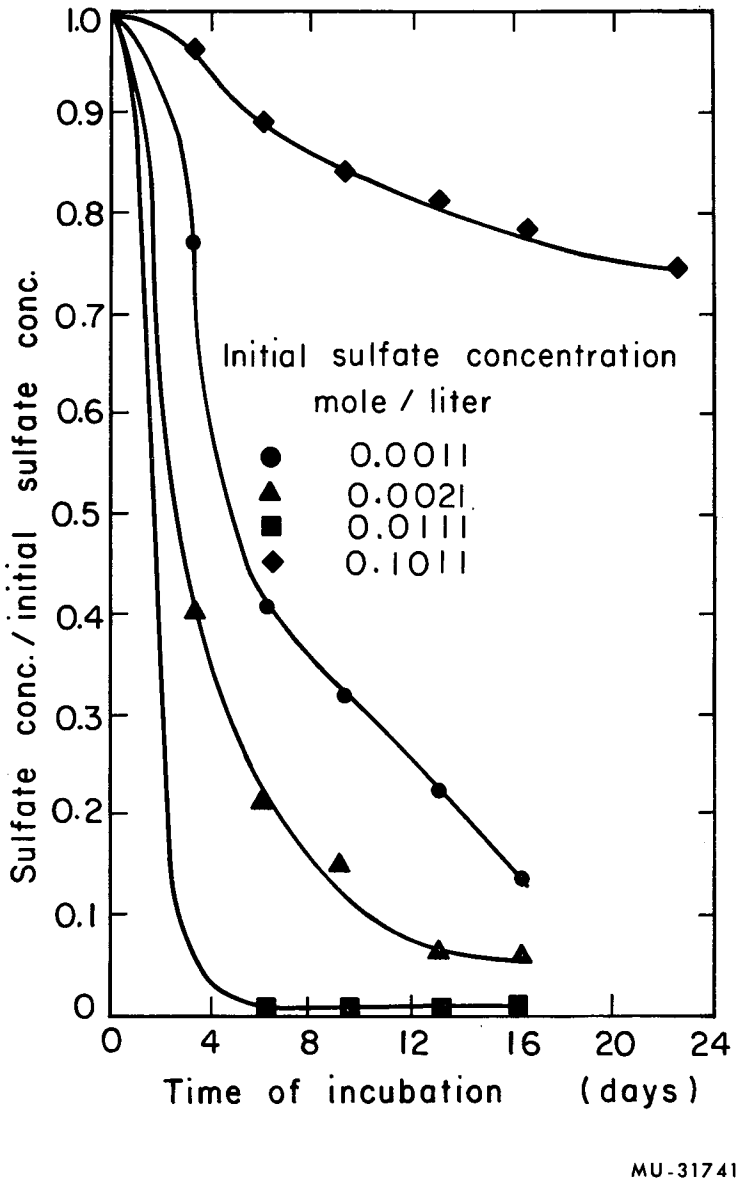

Fig. 9. Fraction of sulfate reduced at various initial sulfate concentrations.

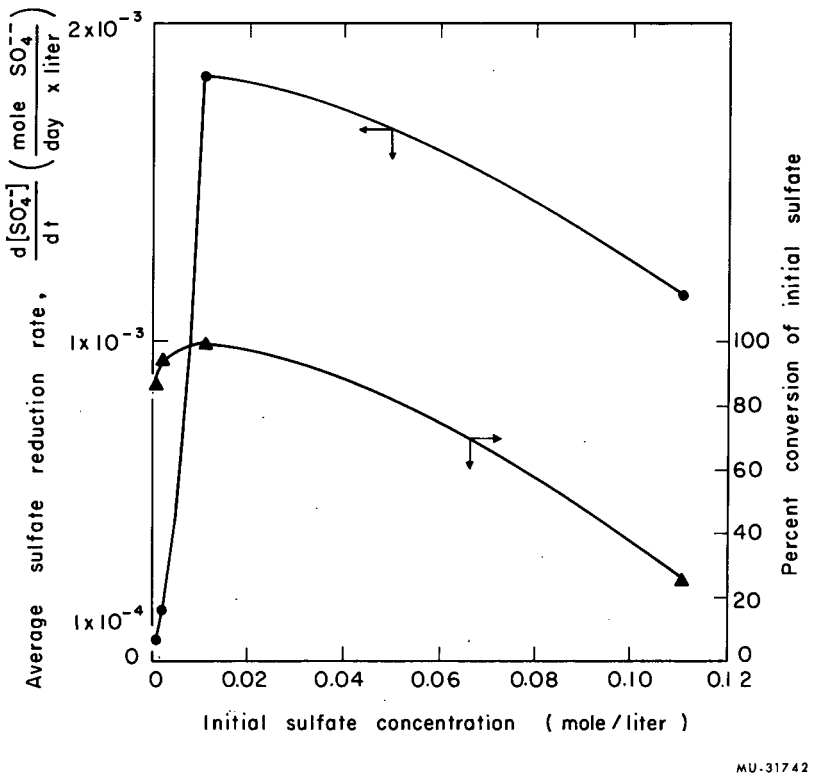

Fig. 10. Rates and efficiencies of sulfate reduction as a function of initial sulfate concentration.

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 of metabolic activities in Case D is believed due to inhibiton by H_2S, which reached a concentration of 0.0258 moles/liter, compared to only 0.011 moles/liter in Case C. From the above discussion it appears that the initial sulfate concentration has a strong effect on the reduction rates, but only in an indirect way. The results suggest that if high reduction rates 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.[11]

The overall per cent conversion of sulfate to sulfide (Fig. 10) is also highest at 0.0111 molar sulfate and lowest at 0.1111 molar in agreement with the assumption of hydrogen sulfide inhibition.

An experiment was performed to ascertain the effect of sulfate concentration in MacPherson's medium. Fig. 11 shows the effect of reducing the initial concentration of sodium sulfate by one half in a batch culture. Both cell yield and sulfate reduction were decreased, although the change did not seem to have an effect on the rates of growth and sulfate reduction until a sulfate concentration of about 4 mM/l was reached. An attempt was made to fit the Michaelis-Menton[12] model for substrate-limited enzyme kinetics to the specific rate of sulfate-reduction by measuring slopes of the sulfide production curve and estimating sulfate concentrations from sulfide concentrations and the initial sulfate concentrations. The model was fit to the data in the conventional manner of plotting the reciprocal of the specific rate of sulfate reduction against the reciprocal of the sulfate concentration. The line through the data is given by the equation:

$$\frac{1}{n}\frac{d[SO_4^{--}]}{dt} = \text{specific rate of sulfate reduction}$$

$$= -2.5 \times 10^{-10} \frac{[SO_4^{--}]}{17 + [SO_4^{--}]} \qquad (3)$$

where n = cell concentration in cells/ l

$[SO_4^{--}]$ = sulfate concentration in mM/l

t = time in days

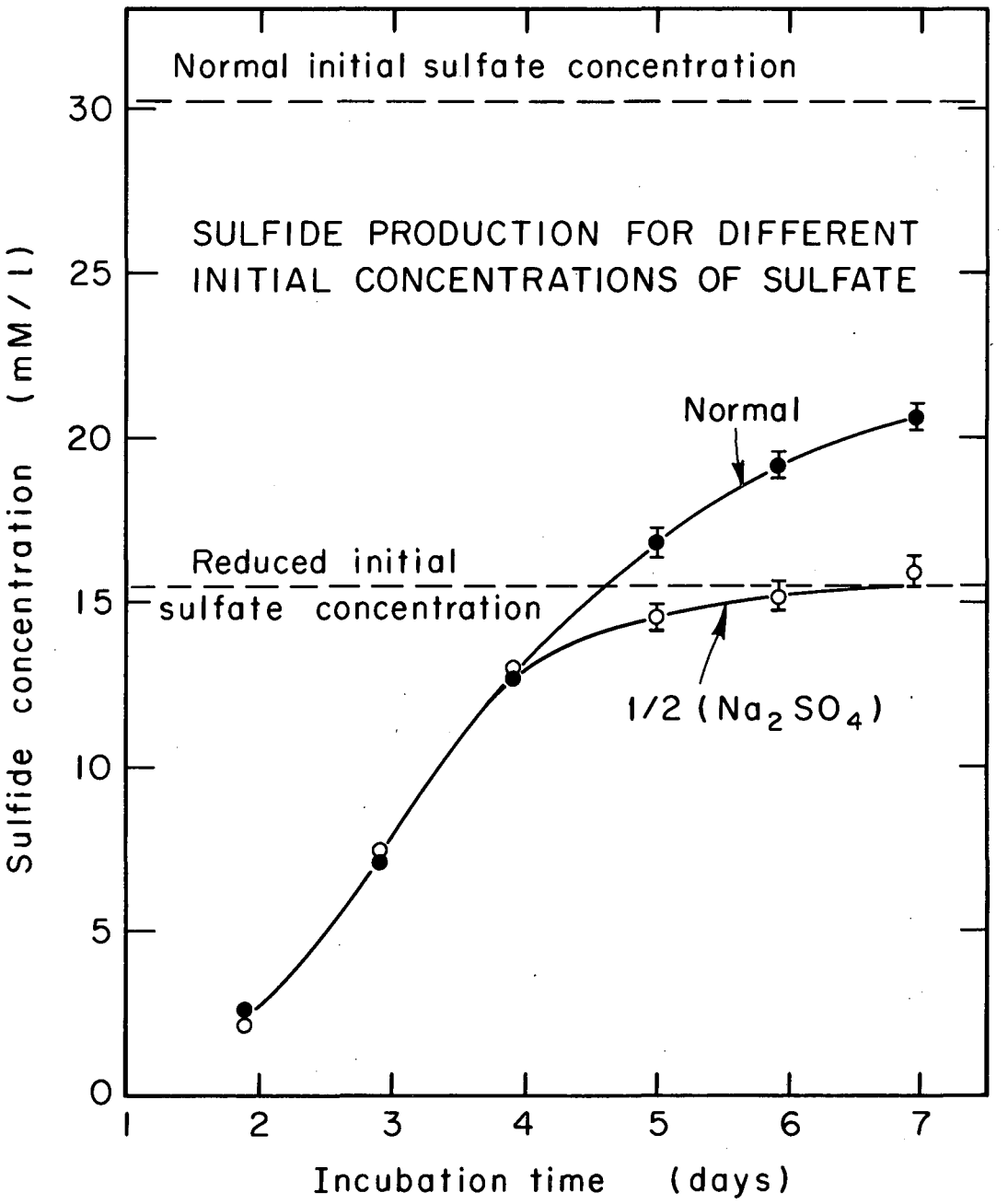
Normal initial sulfate concentration
SULFIDE PRODUCTION FOR DIFFERENT
INITIAL CONCENTRATIONS OF SULFATE
Normal
Reduced initial
sulfate concentration
1/2 (Na_2SO_4)
Sulfide concentration (mM/l)
Incubation time (days)
0
5
10
15
20
25
30
1
2
3
4
5
6
7
MUB-7952

Fig. 11.

Fig. 12 is a graph of the specific rate of sulfate reduction as a function of the sulfate concentration. In the figure, the locus of the preceding equation is compared to the observed data. Agreement is best for low sulfate concentrations, as would be expected. Specific rates calculated from the data for the unmodified medium are much lower than those for the case where sulfate is initially present at a lower concentration. Possible causes for failure of the model to apply at higher sulfate concentrations include the effects of yeast extract, of sulfide concentration, and of the phase of growth of the organisms, because the data were calculated with data from both lag-phase and post-lag-phase cultures. Further investigation of this point is underway.

C. Effect of pH

The hydrogen ion activity is known to have an important influence on many biological rate processes. The next experiment illustrates the effect of pH on growth and sulfate-reduction in Medium M. Five liters of medium was prepared and divided equally among five bottles. After sterilization, the pH of each bottle was adjusted to a different value using aseptic technique. The initial pH values chosen for the experiment were 6.4, 6.95, 7.60, 7.99, and 8.60. As before, optical count and sulfide concentration were monitored after inoculation and distribution among smaller bottles. Growth was obtained only at the three highest initial values of pH. Figure 13 compares optical counts for these three cultures. Figure 14 is a comparison of sulfide production in the same three cultures. In each case, the pH declined during the experiment. No great differences in the specific rates of growth and sulfide production were noted. However, it can be seen that a shorter lag phase was obtained with the initial pH at 7.99. In future work, Medium M was adjusted to an initial pH of 8.0 before use.

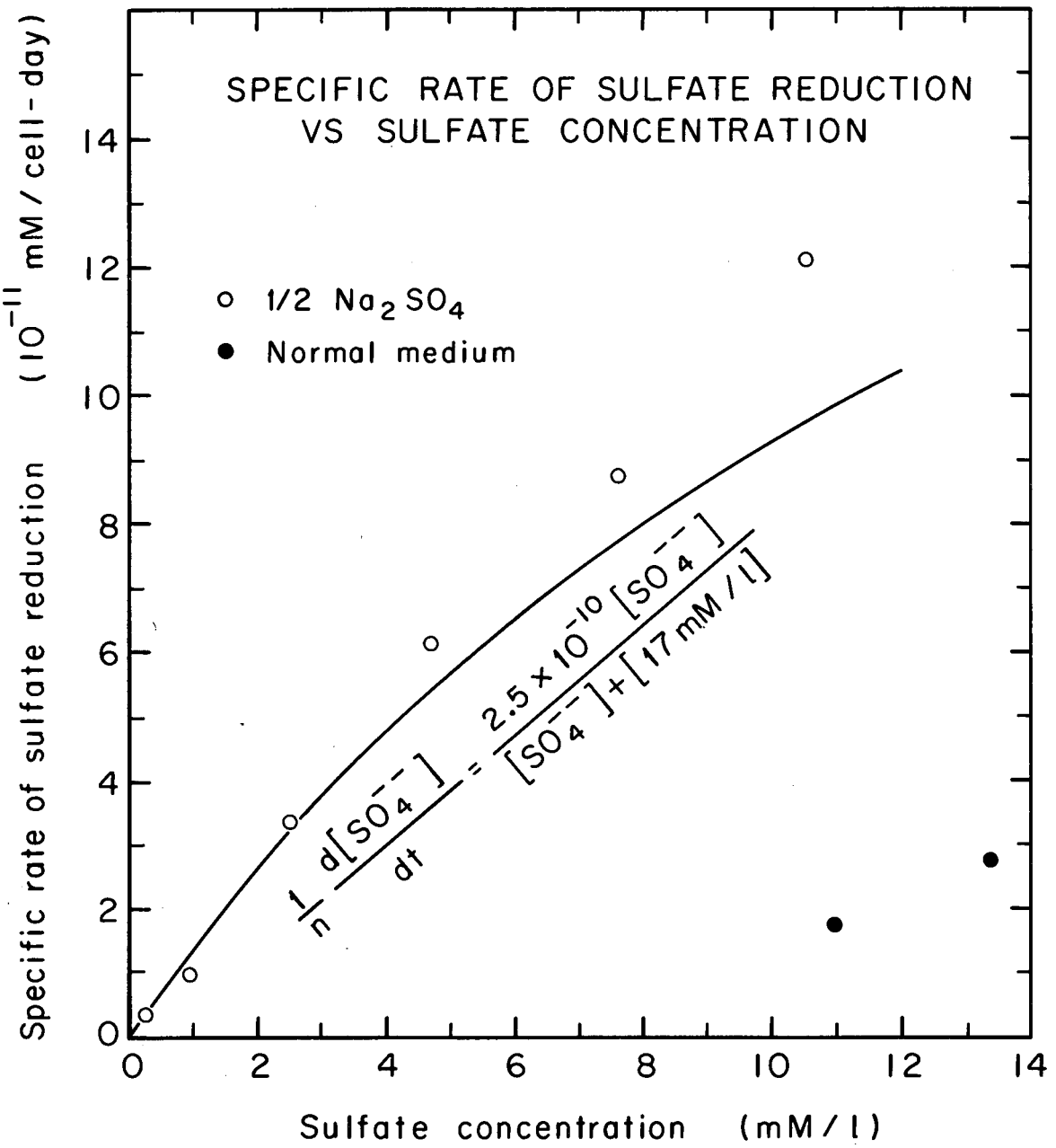

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Fig. 12.

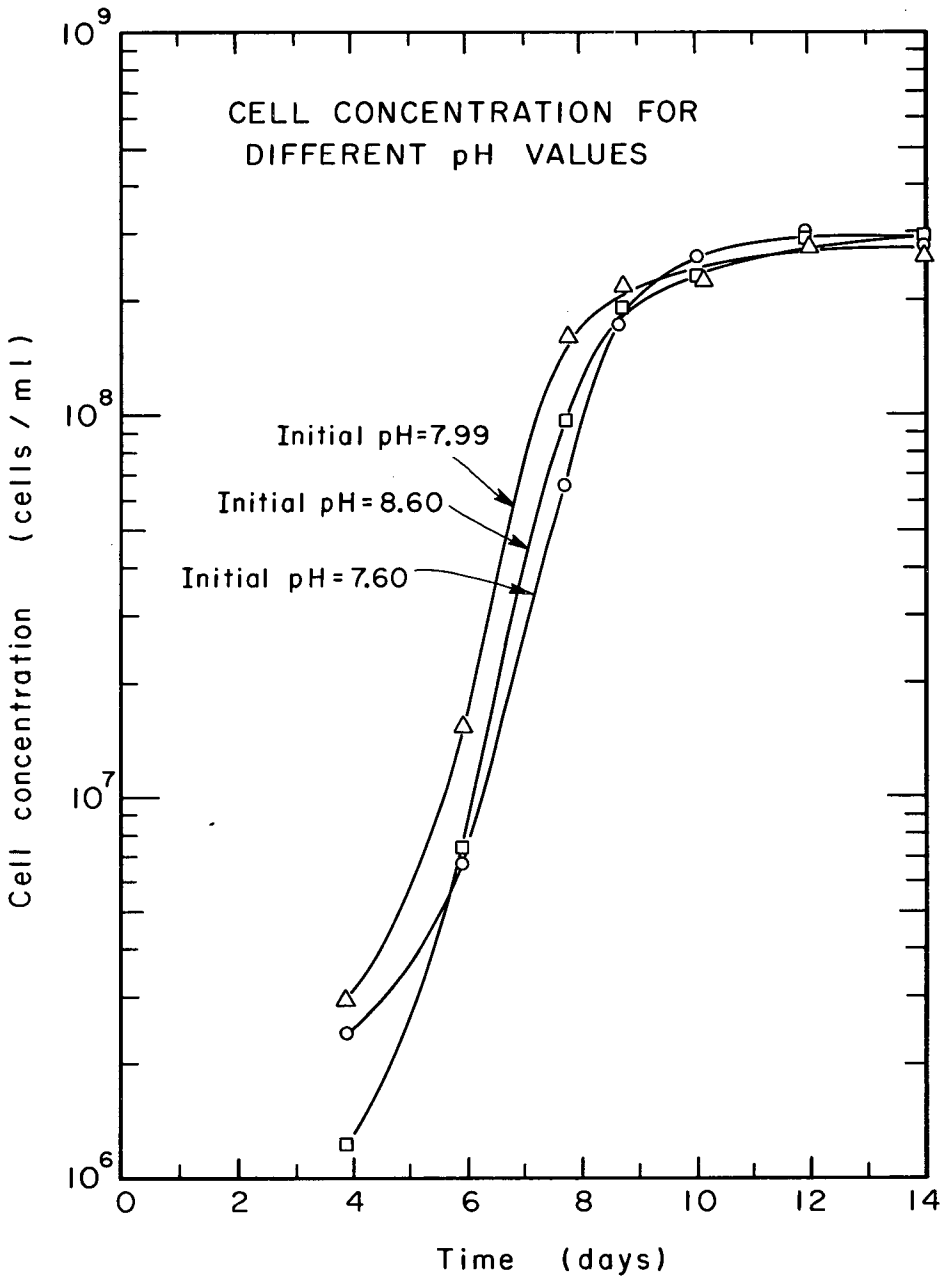

CELL CONCENTRATION FOR
DIFFERENT pH VALUES
Initial pH=7.99
Initial pH=8.60
Initial pH=7.60
Cell concentration (cells / ml)
Time (days)
10^9
10^8
10^7
10^6
0
2
4
6
8
10
12
14
MUB-7955

Fig. 13.

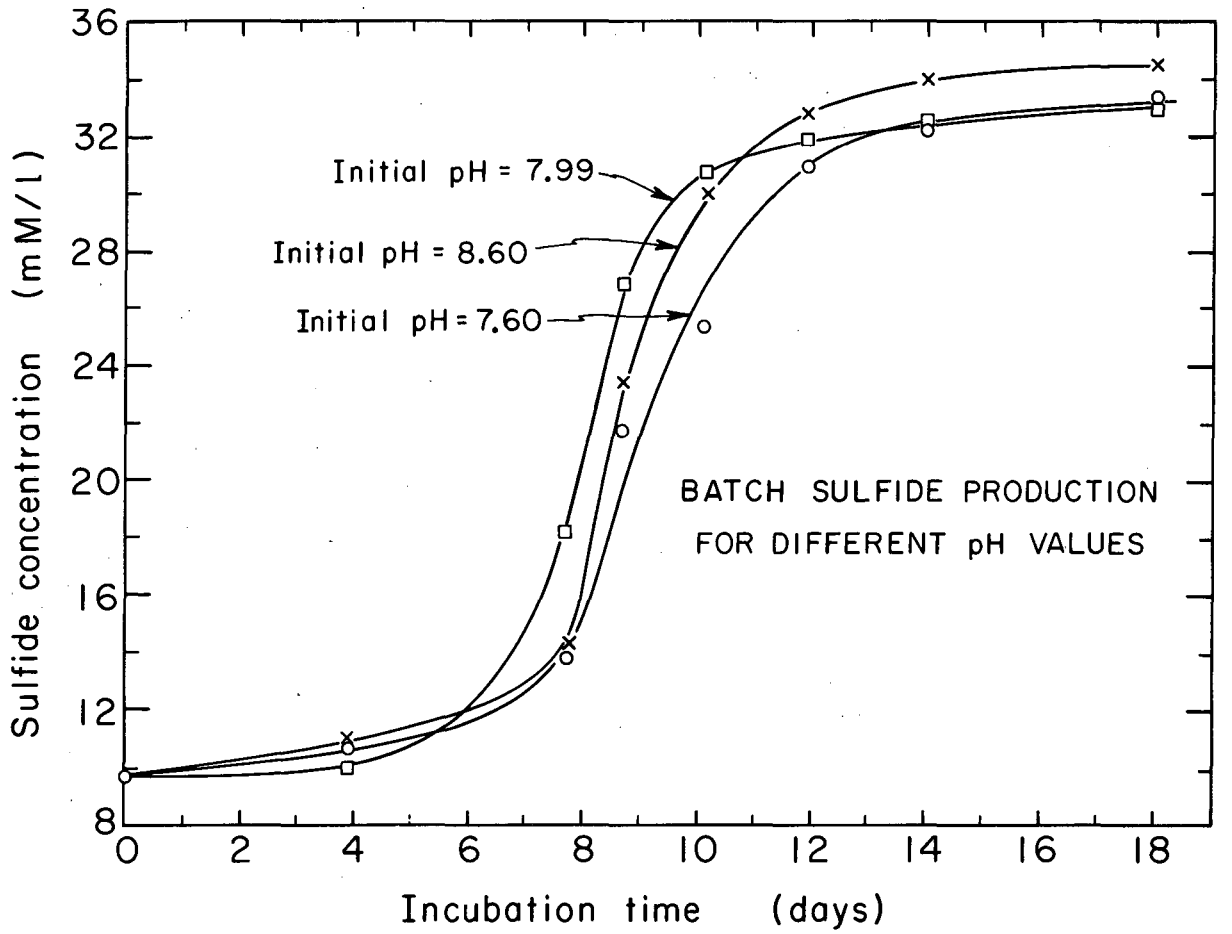

Sulfide concentration (mM/l)
36
32
28
24
20
16
12
8
0
2
4
6
8
10
12
14
16
18
Incubation time (days)
Initial pH = 7.99
Initial pH = 8.60
Initial pH = 7.60
BATCH SULFIDE PRODUCTION
FOR DIFFERENT pH VALUES
MUB-7951

Fig. 14.

D. Effect of Salinity

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 unknown rod-shaped bacteria (see Appendix I). The second experiment was done with a pure culture of Desulfovibrio, and the third one with an "adapted culture" discussed in the next section. Results obtained with the impure culture are shown in Fig. 15(a). The curves are not for the entire growth range, and sulfide production is shown directly instead of the usual sulfate concentration.

In the range from 1% to 10%, the effect of NaCl on the growth is very small, as seen in Fig. 15(a). 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. Sulfide production and the total yield of sulfide drop off rapidly as the NaCl concentration is increased above 10%. Results for the pure culture are shown in Fig. 15(b). 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.

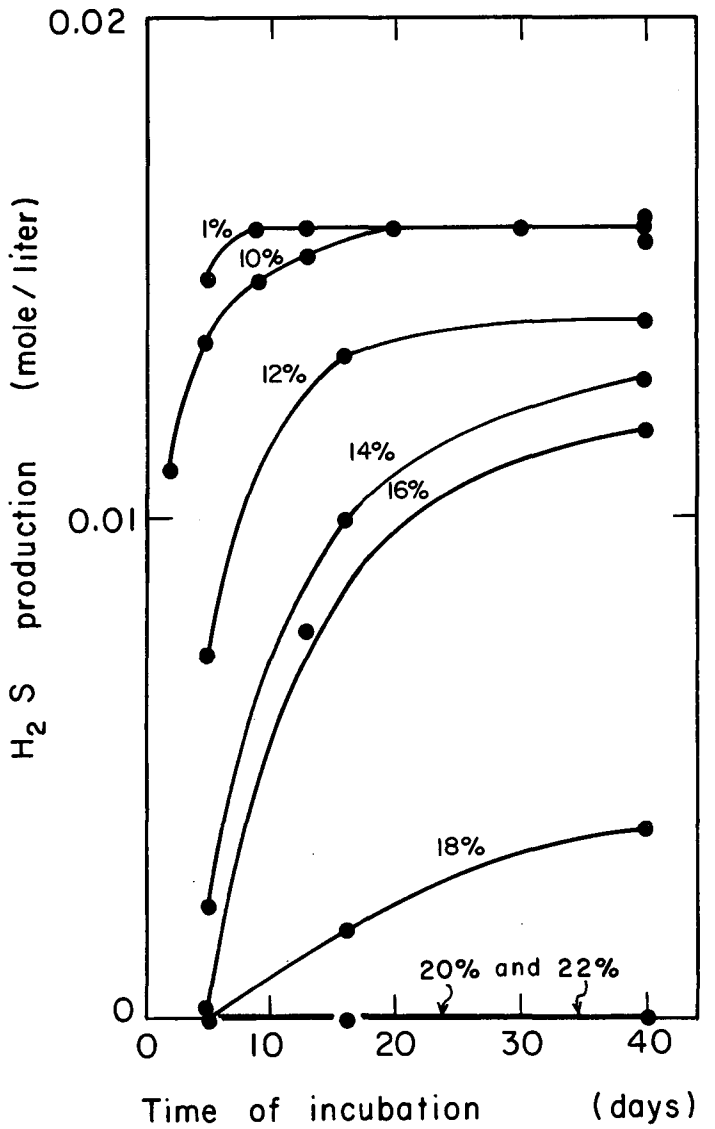


Fig. 15(a). Sulfide production by an impure culture of Desulfovibrio at different salinities.

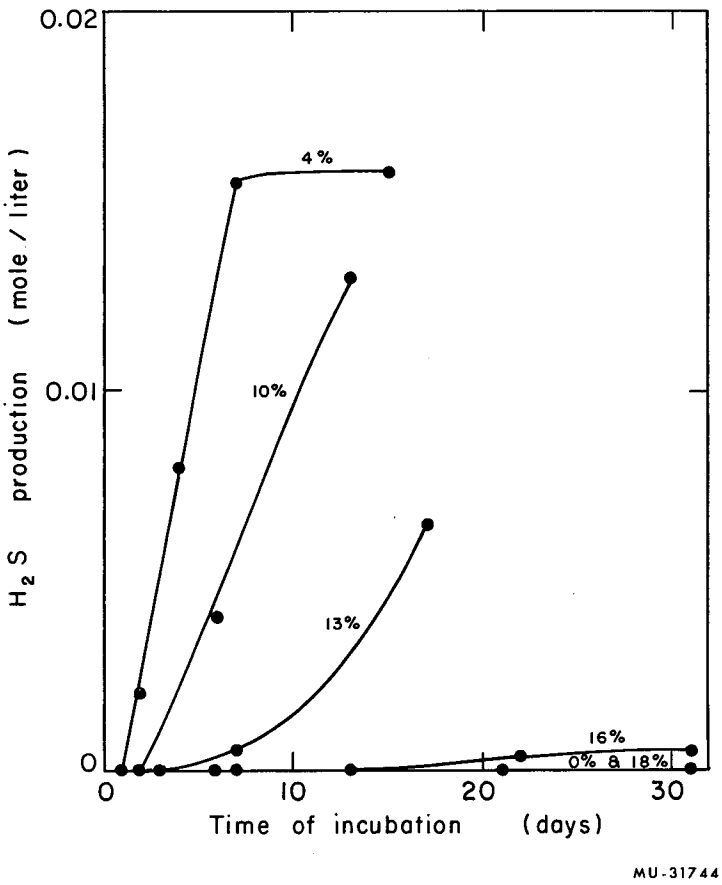


Fig. 15(b). Sulfide production by a pure culture of Desulfovibrio at different salinities.

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%. This suggests that the isolated strain is not a halophile, but that it is merely able to tolerate high salt concentrations. No growth was obtained on NaCl-free media with either the pure or impure cultures, but it is possible that the requirement for NaCl would be removed by adaptation to lower salt concentrations.

2. Adaptation to Higher Salinity

Adaptability of Desulfovibrio to salt concentrations higher than those of the natural habitats has been reported in the literature.[13,14,15] 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.

Comparing the curves obtained from growth at 16% NaCl of the normal pure culture and the adapted culture (Fig. 16), it is apparent that the NaCl sensitivity of Desulfovibrio was considerably lowered by the adaptation process.

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.

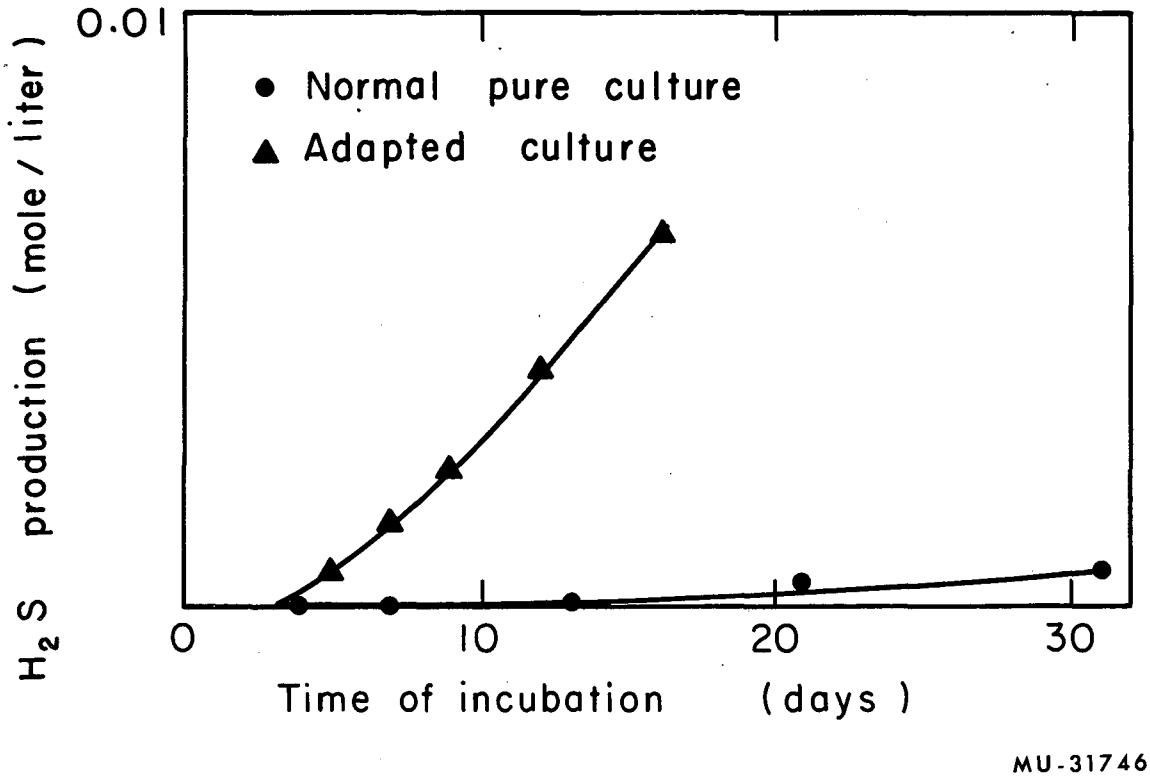


Fig. 16. Sulfide production in 16‰ salinity by the normal pure culture and by the adapted culture of Desulfovibrio.

E. Continuous Culture Experiments

Continuous culture of sulfate-reducing bacteria is of value in either the study of their kinetics or the development of an industrial process using them. In the kinetic study, the continuous culture technique is valuable because greater accuracy can be obtained by the use of repetitive measurements on a steady-state culture and by computation of specific rates of growth and product formation without depending on the use of estimated slopes of experimental curves. In industrial applications of bacterial processes, the technique is important because no means exists of predicting with certainty the behavior of a continuous culture from batch culture data.

A few preliminary continuous culture studies were made with Medium M for comparison with the batch culture results. These will be described briefly. The reactor is constructed of glass and contains 2.3 liters of liquid when operating. Baffling is achieved by vertical crimps in the walls. The reactor was jacketed to allow temperature control. Stirring is achieved with a teflon-coated bar magnet. Mixing was sufficient to completely disperse an ink drop in about four seconds.

Figure 17 is a flow diagram of the system. Separate supplies of hydrogen sulfide and nitrogen gas were used to control the sulfide concentration in the reactor. Regulation of the flows was achieved with rotameters, pressure regulators, and needle valves in combination. Gases entering and leaving the reactor were passed through a sterilized glass wool filter. Fresh medium was fed to the reactor with a peristaltic pump. Rates were monitored with rotameters and by collection. Feed was introduced close to the liquid level to reduce splashing. Level control was maintained in the reactor by an overlow line. Temperature control was maintained with $\pm.1^{\circ}$ C in the reactor by flowing constant temperature water through the water jacket of the reactor. All lines were constructed of glass or polyvinyl chloride tubing with teflon stopcocks. Sterilization of the system was accomplished with ethylene oxide gas.

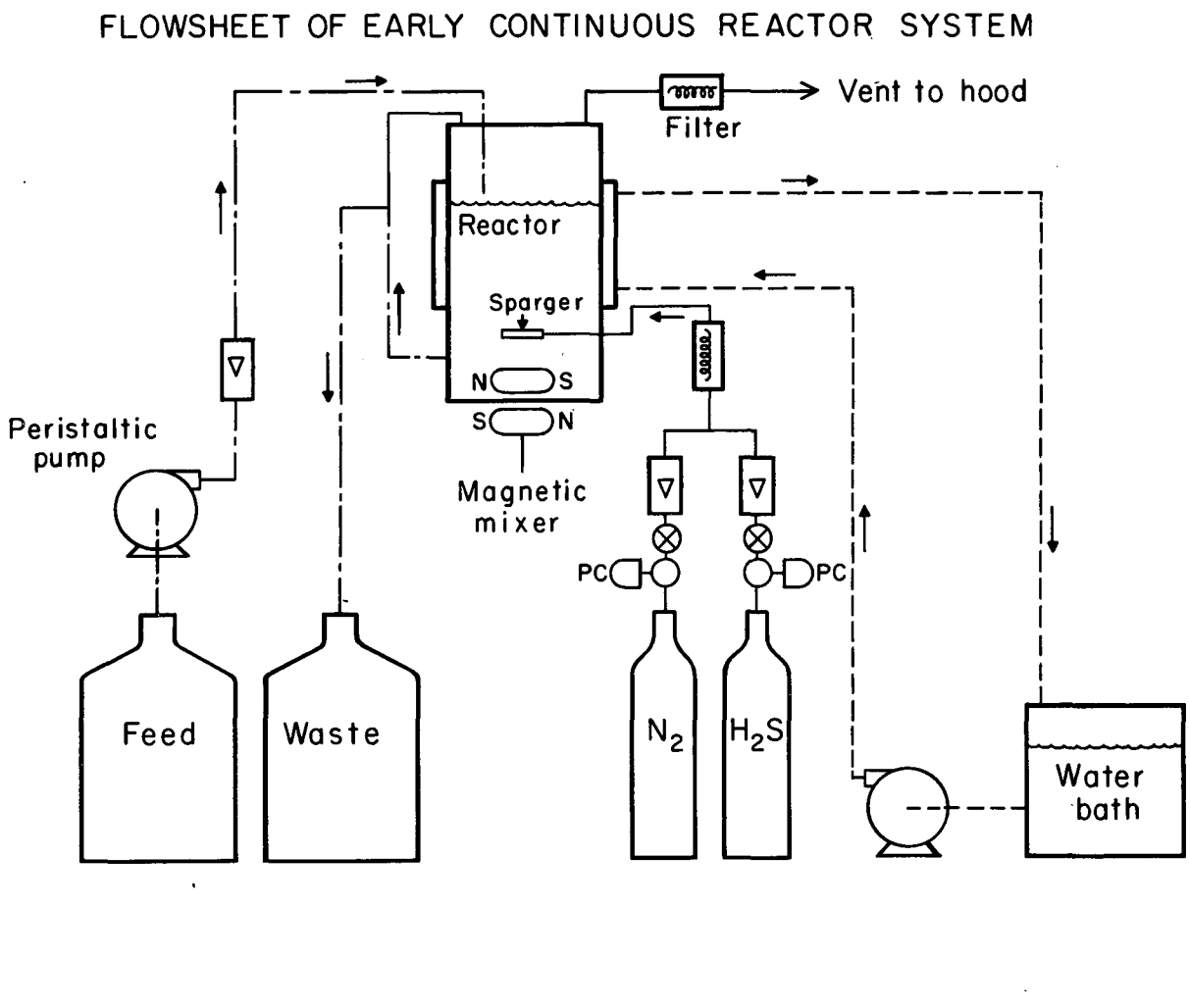

FLOWSHEET OF EARLY CONTINUOUS REACTOR SYSTEM
Vent to hood
Filter
Reactor
Sparger
N
S
S
N
Magnetic mixer
Peristaltic pump
PC
PC
Feed
Waste
N_2
H_2S
Water bath
MUB-7958

Fig. 17.

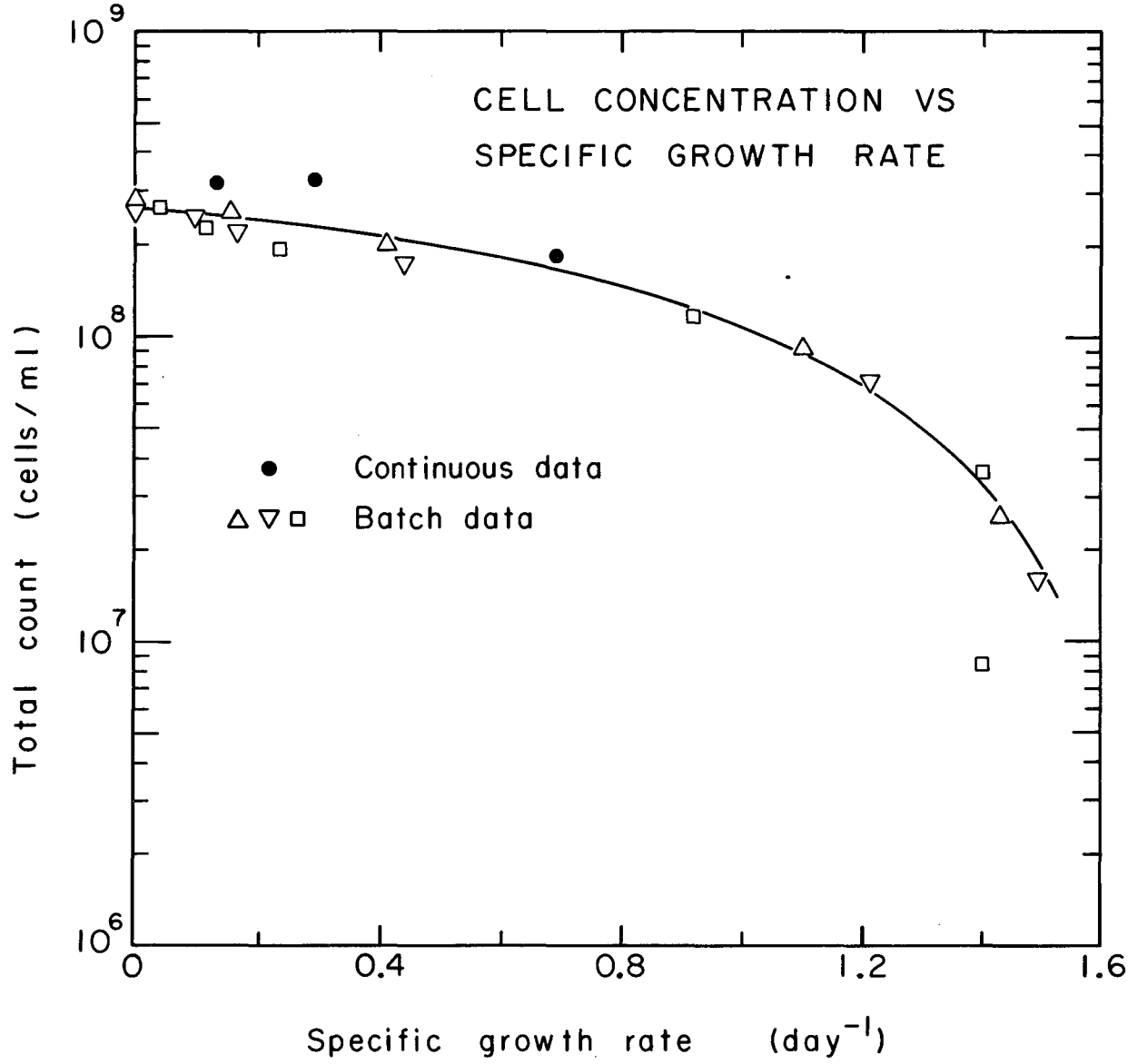
After sterilization and rinsing with the medium, more sterilized nutrient solution was added to the 20-liter feed supply and a continuous culture experiment begun by filling the reactor to the 2.3-liter mark and inoculating with a culture of sulfate-reducing bacteria. The cell concentration was allowed to reach a sufficiently high value to indicate exponential growth had begun and continuous addition of nutrient solution, nitrogen gas, and hydrogen sulfide gas to the reactor was begun. Cell concentration and sulfide concentration, as well as flow rates and temperature were monitored several times daily until steady values were reached. At the steady-state, pH, cell concentration by optical count, cell dry weight concentration, sulfate concentration, sulfide concentration, and flow rates were measured. At the completion of the measurements, the liquid feed rate was changed to a new value and the procedure begun again. All experiments were performed at a temperature of 30° C. Difficulty was met in obtaining and maintaining steady-states because of the lack of pH control and because of difficulties with flow and temperature controls, so that measurements at only three steady-states are presented. In Fig. 18 the cell concentration is plotted as a function of specific growth rate (dilution rate in continuous culture) for the data obtained in the continuous culture experiment using Medium M and for the three batch cultures of Section C of this work. Fairly good agreement is shown between batch and continuous culture data. It should be recognized, however, that these results are of a limited preliminary character.

F. Fitting Sulfate Reduction Rate Data into a Generalized Correlation

A commonly used expression for fermentation relates the product formation linearly to the growth rate and to bacterial density:[16]

$$\frac{dP}{dt} = \alpha \frac{dn}{dt} + \beta n \qquad (4)$$

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



MUB-7954

Fig. 18.

The form of Eq. (4) 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 culture is given by dn/dt, and the basic metabolic activities are proportional to the quantity of bacteria present, n.

In the case of lactic acid fermentation at constant pH, Eq. (4) was found to apply over the entire batch fermentation, except possibly in the lag phase and at the very end of the fermentation.[17] 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. (4) can be written

$$\frac{d[H_2S]}{dt} = \alpha \frac{dn}{dt} + \beta n. \tag{5}$$

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

$$\frac{d[H_2S]}{dt} = - E \frac{d[SO_4^{--}]}{dt}, \tag{6}$$

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.

By substituting Eqs. (1) and (6) into Eq. (5) and rearranging one obtains

$$- \frac{1}{n} \frac{d[SO_4^{--}]}{dt} = \alpha k + \beta , \tag{7}$$

where $\frac{1}{n} \frac{d[SO_4^{--}]}{dt}$ is the rate of sulfate reduction per bacterium.

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. 19 and 20.

For the complex medium (Fig. 19), the line drawn has a slope $\alpha = 2.2 \times 10^{-14}$ and an ordinate intercept $\beta = 7 \times 10^{-15}$. The equation of the line is therefore

$$-\frac{1}{n}\frac{d[SO_4^{--}]}{dt} = 2.2 \times 10^{-14}k + 7 \times 10^{-15}. \qquad (8)$$

From the plot in Fig. 19 it can be seen that Eq. (8) 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, a continuous cultivation could operate entirely in the region where Eq. (8) applies.

In the case of a simple medium, Eq. (7) could not be applied over the entire batch process. However, for the period starting with the second exponential growth, Eq. (7) fits the data very well, as seen in the Fig. 20. The equation of the line is

$$-\frac{1}{n}\frac{d[SO_4^{--}]}{dt} = 3 \times 10^{-14}k + 1.34 \times 10^{-14}. \qquad (9)$$

Actually, Eq. (9) 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.

Specific rate data were also obtained with Medium M in both batch and continuous culture as described in Sections C and E. A tentative correlation between specific rate of sulfate reduction and specific growth rate shown in Fig. 21, may be expressed by the equation:

$$-\frac{1}{n}\frac{d[SO_4^{--}]}{dt} = 10^{-13}[0.069 + 0.505k]. \qquad (10)$$

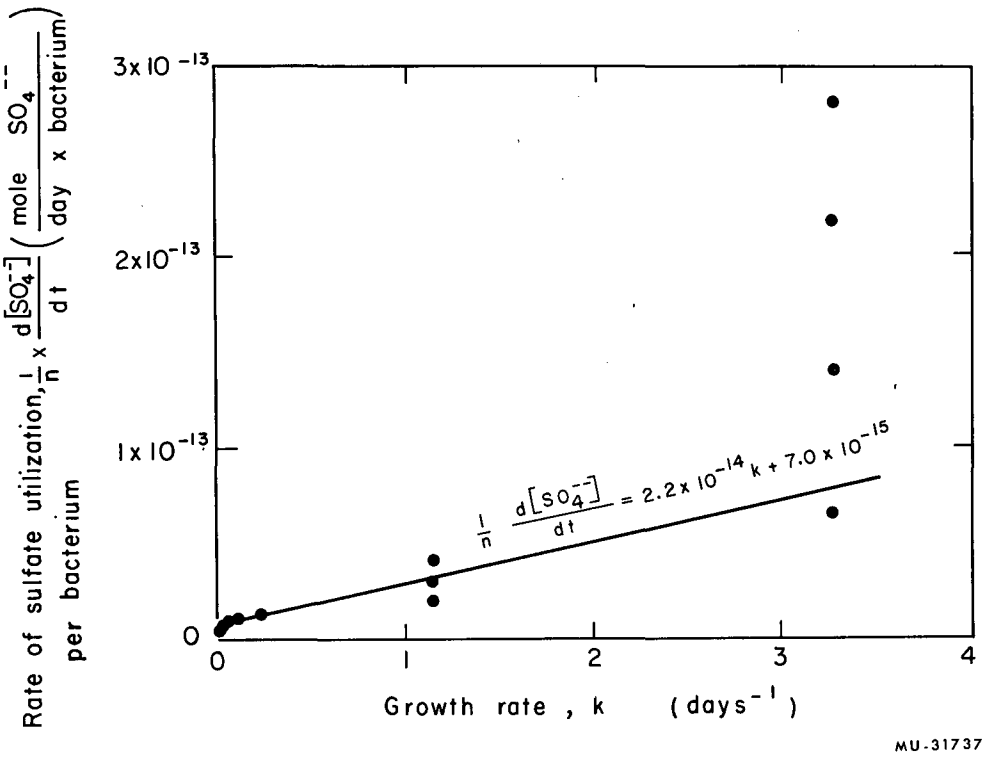


Fig. 19. Correlation for sulfate reduction in complex medium.

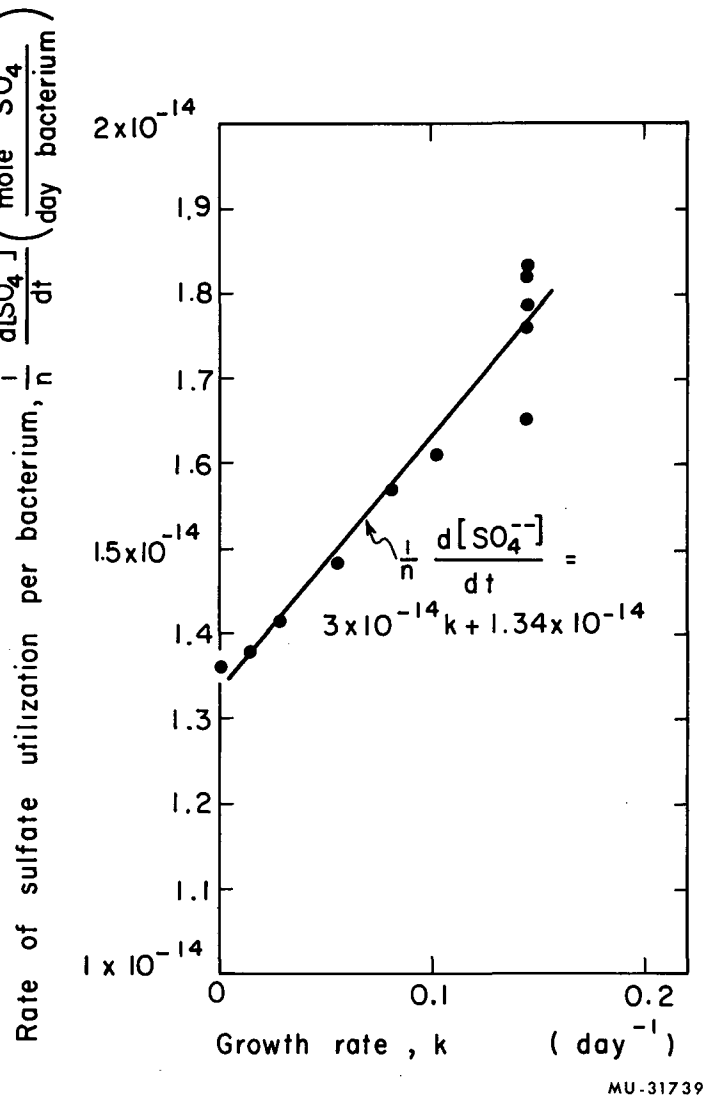


Fig. 20. Correlation for sulfate reduction in real simple lactate medium.

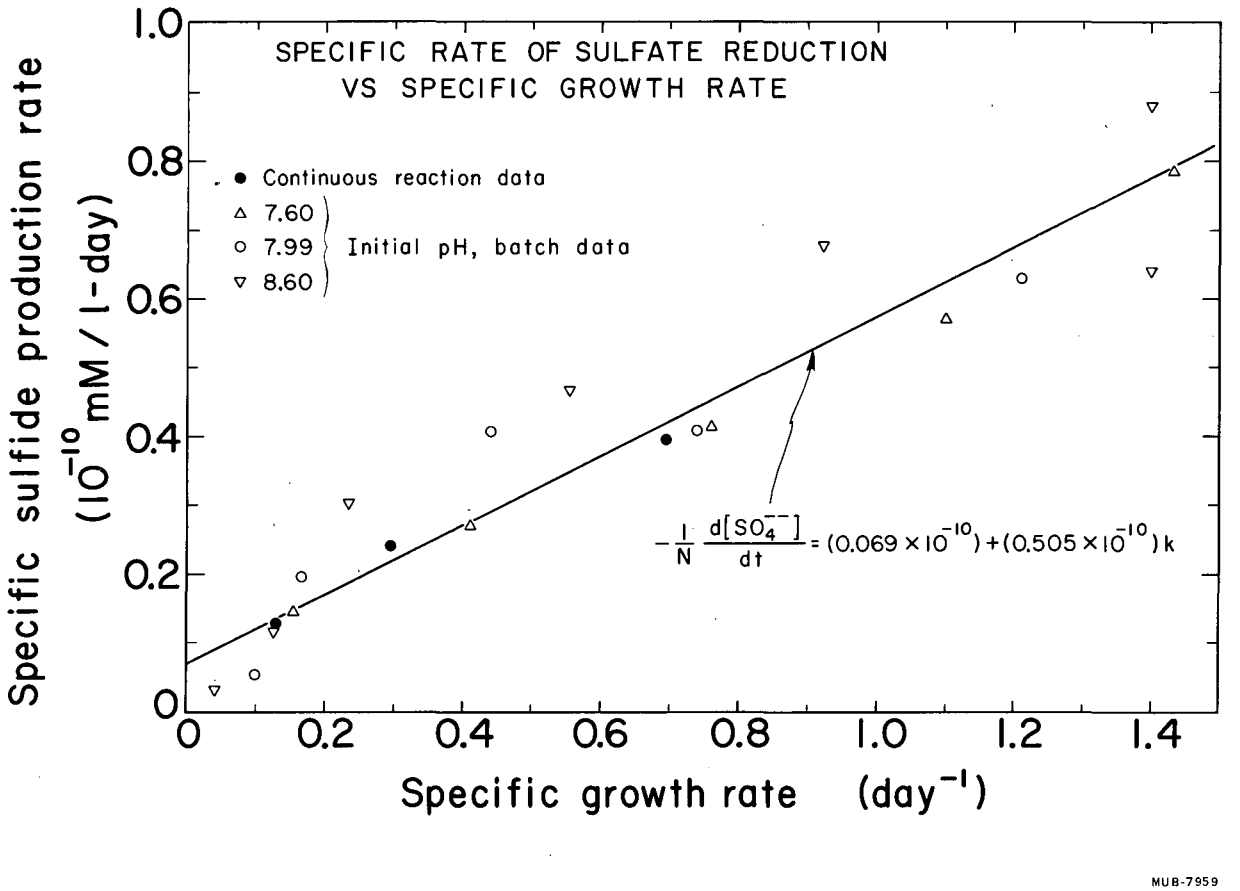

SPECIFIC RATE OF SULFATE REDUCTION
VS SPECIFIC GROWTH RATE
Continuous reaction data
7.60
7.99
8.60
Initial pH, batch data
$-\frac{1}{N}\frac{d[SO_4^{--}]}{dt} = (0.069 \times 10^{-10}) + (0.505 \times 10^{-10})k$
Specific sulfide production rate
$(10^{-10}$ mM / l-day)
Specific growth rate (day^{-1})
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
MUB-7959

Fig. 21.

Fairly good agreement is obtained between batch and continuous results by this method, although confirmation is still needed at higher dilution rates and under more precisely controlled continuous culture conditions. Further experiments are in progress in a new apparatus (see Appendix III).

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 per liter

t = time, hours or days

$[SO_4^{--}]$ = concentration of sulfate, moles per liter in equations (8) and (9), millimoles per liter in equations (3) and (10).

Acknowledgments

The authors gratefully acknowledge the assistance of Mr. Ronald Charron, for his many contributions to the design and construction of the improved continuous reactor system and of Professor Alvin J. Clark and other members of the Bacteriology Department who provided many helpful suggestions about bacteriological techniques.

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Appendix I

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. 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 mud 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 II 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.

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 II) to which 2.5% agar has been added. A reducing agent must be added to the solid media to make growth of Desulfovibrio possible. Cysteine, 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 occurred.

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 that 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, bisulfide ions, 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, 0.003 mole per liter H_2S). After eight days of incubation at 30° C 0.011 mole per liter H_2S was produced (55% conversion). Microscopic examination

revealed an apparently pure culture of vibrios of the type shown in Fig. 23(a). However, the second subculture in the same medium contained besides vibrios also some Gram-negative straight rods shown in Figs. 22(c) and 22(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 mobility 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 mobility 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 2 μ in length, and about 0.5 μ in diameter [Figs. 22(a) and 22(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. 22(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 15 μ, 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. 23(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 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 2-μ long, and have one curve. On the other

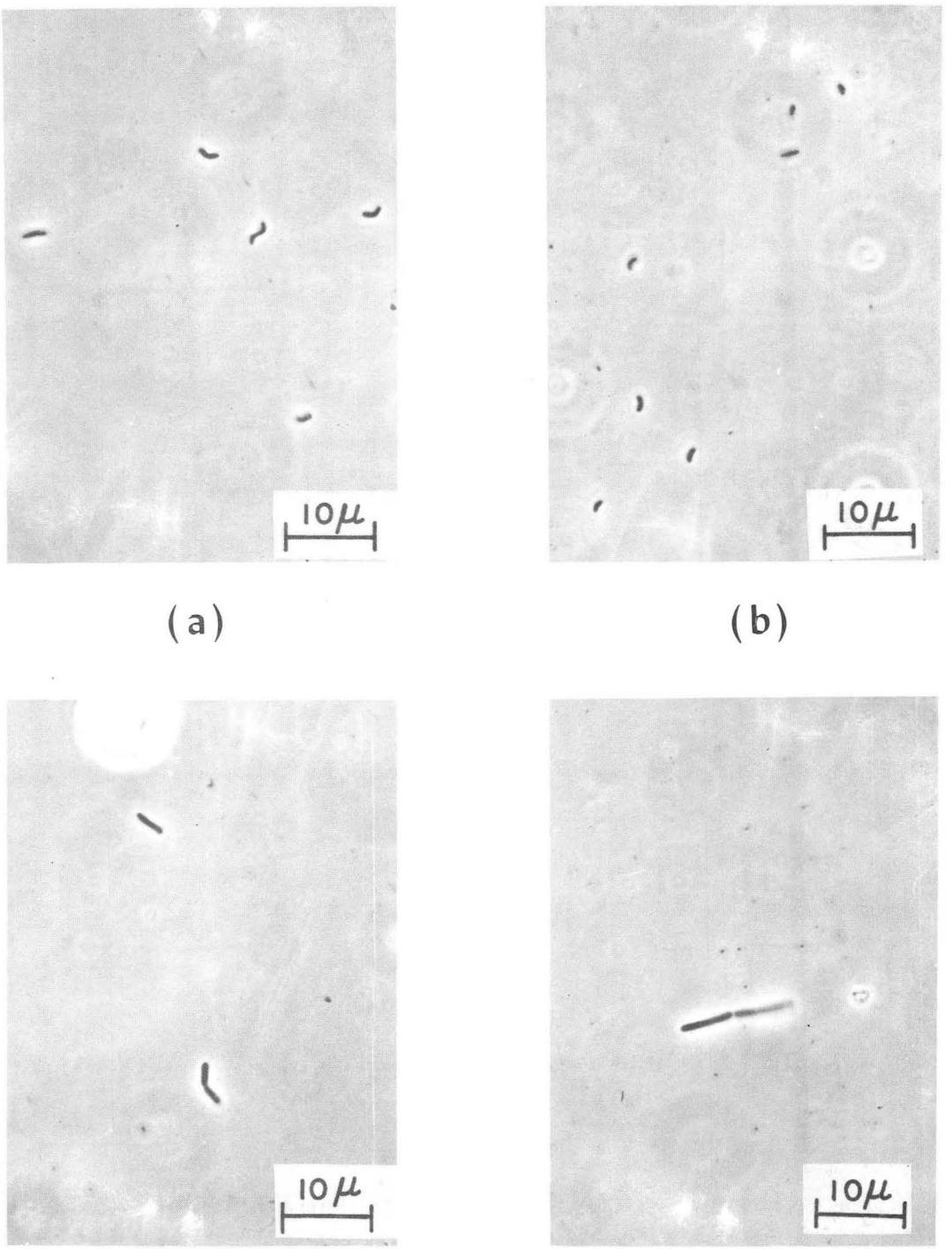

Fig. 22. Desulfovibrio and the unknown contaminating organism. (A) and (B), Desulfovibrio grown on complex lactate medium containing 3% sodium chloride. (C) and (D), the unknown bacteria. Phase contrast (x970).

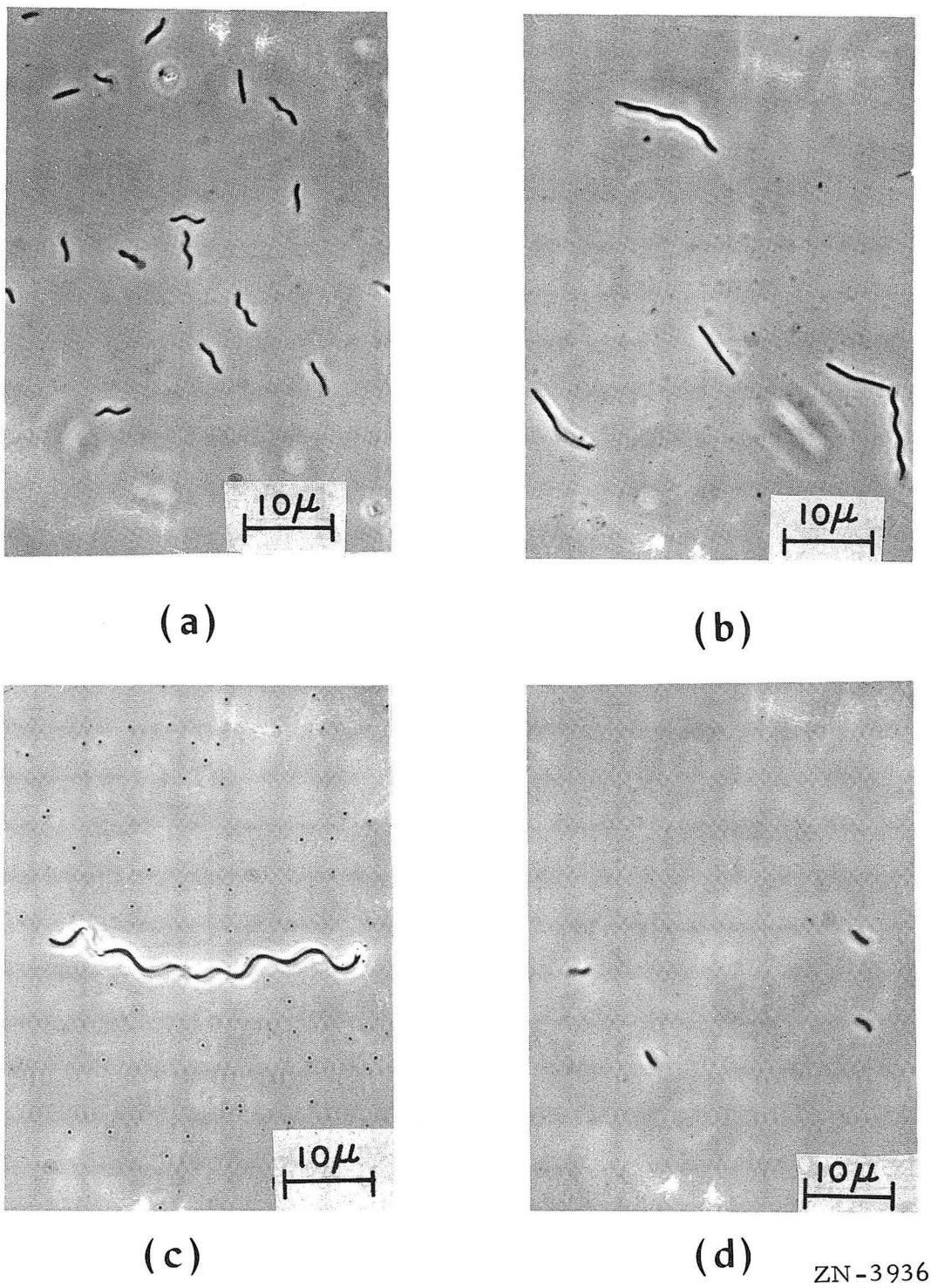

Fig. 23. Morphological variations of Desulfovibrio. (A) young cells grown on complex lactate medium containing 10% NaCl. (B) cells grown on complex lactate medium containing 16% NaCl. (C) old cells grown on simple lactate medium (10% NaCl). (D) cells grown on complex ethyl alcohol medium (10% NaCl). Phase contrast (x970).

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. 22(c) and 22(d)].

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

Appendix II

METHODS

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
$FeCl_2 \cdot 4H_2O$	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.

8. MacPherson's Medium	Weight (grams)
Lactic acid	9.01
KH_2PO_4	0.340
$CaCl_2$	0.056
$MgSO_4 \cdot 7H_2O$	0.0616
Na_2SO_4	4.26
NH_4Cl	0.535
Yeast extract	1.0
Sodium chloride	100.0
$FeSO_4 \cdot 7H_2O$	0.007
$Na_2S \cdot 9H_2O$	0.240

9. Medium M	Weight (grams)
Lactic acid	7.66
$CaCl_2$	0.111
$MgSO_4 \cdot 7H_2O$	0.123
Yeast extract	2.5

The other components of MacPhenson's Medium are present in the same concentrations in Medium M.

10. Yeast Dextrose Agar Medium	Weight (grams)
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.5
Dextrose	20.0
Yeast extract	5.0
Agar	2.0

11. N.I.H. Thioglycollate Broth	Weight (grams)
Yeast extract	5.0
Casitone	15.0
Dextrose	1.0
Sodium chloride	2.5
l-cystine	0.05
Thioglycollic acid	0.3 ml
Agar	0.75 g

C. Chemical Methods

In the determination of sulfide, 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.

Sulfate was determined by precipitation as barium sulfate, followed by digestion, ignition, and weighing of the precipitate.

Hydrogen ion activity was determined with a Beckman Zeromatic pH meter equipped with glass and calomel reference electrodes (manufactured by Beckman Instruments, Inc., of Fullerton, California).

D. Total Counts

Cell concentrations were commonly determined by conventional optical counting techniques using a Petroff-Hausser counting chamber with an improved Neubauer ruling (manufactured by C. A. Hausser and Son of Philadelphia, Pa., U.S.A.). The same counting chamber was used throughout the work and the precautions suggested by Norris and Powell[18] and by Cook and Lund[19] were followed, short of measuring the chamber depth. In counts on successive slides of the same sample gave a standard deviation of 11.9%. In measurements during kinetic experiments, three successive slides were prepared and counted to minimize the random error.

E. Dry Weights

Dry weight measurements were made by passing a measured volume of culture, usually 30 to 50 ml, through weighed cellulose acetate filters with an average pore size of .45microns (manufactured by Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.). The filter and collected organisms were then dried at 95 degrees Centigrade for 48 hours and weighed again. As a control, a similar procedure was followed with a second filter pad and the filtered culture fluid. The dry weight of organisms present in the sample was calculated as the weight increase of the experimental filter pad less the weight increase of the control filter pad. A precipitate of ferrous sulfide was present during all measurements but calculations show that it could not contribute more than 1% to the dry weight of the organisms at the cell concentrations studied. Unfortunately, the precipitate did prevent meaningful optical density measurements or dry weight measurements at low cell concentrations.

F. Culture Purity

Cultures were periodically checked for purity following the procedures reccommended by Postgate[20]. Additional precautions included aerobic plating on yeast dextrose agar and medium E to test for facultative anaerobes and anaerobic stabbing into N.I.H. Thioglycollate broth to test for clostridia.

Appendix III

FUTURE EXPERIMENTAL WORK

Emphasis in our current work with sulfate-reducing bacteria is on growth kinetics. As the salt-tolerant strain discussed in the body of this report required unknown factors in yeast extract for rapid and abundant growth, we have decided to perform future experiments with a strain that could be grown in a chemically-defined medium. The Hildenborough strain of Desulfovibrio desulfuricans (NCIB No. 8303) was selected because its fundamental properties are already known from previous studies and it was for this organism that the chemically-defined medium known in this report as MacPherson's medium had been developed. Our work with this strain will be done for the most part in continuous culture. Both steady-state and unsteady-state measurements will be made with lactic acid, the carbon source, present in limiting amounts. Steady-state measurements will be made at a series of dilution rates for several feed concentrations of lactic acid and various mathematical models for growth kinetics fit to the data. Unsteady-state measurements will then be made between known steady-state operating points. Disturbances will be in flow rate and in concentration of the limiting substrate in the feed to the reactor. Impulse and step disturbances and combinations of these will be used. Kinetic models can then be tested by comparing the predicted and observed response of the culture to the imposed disturbances.

An improved continuous culture system will be used (described later in this section) that allows control of pH, sulfide concentration, carbonate concentration, and temperature. These variables will be monitored in addition to the optical density of the culture, the redox potential, and the concentrations of lactic and acetic acid, sulfate, and ammonium ion. Optical density, pH, and redox potential of the culture will be measured and recorded continuously. Measurements on the cells in the culture will include viable cell count, dry weight concentration of cellular material, packed cell volume, total nitrogen, and RNA concentration. The cell size distribution and concentration will be measured electronically and an attempt made to correlate these results with other measurements on the cells. It is hoped that from the information obtained, a comparison of present kinetic models can be made and that an improved model can be proposed if the need exists. A flow diagram of the improved continuous culture system is shown in Fig. 24.

FLOW DIAGRAM OF CONTINUOUS CULTURE APPARATUS

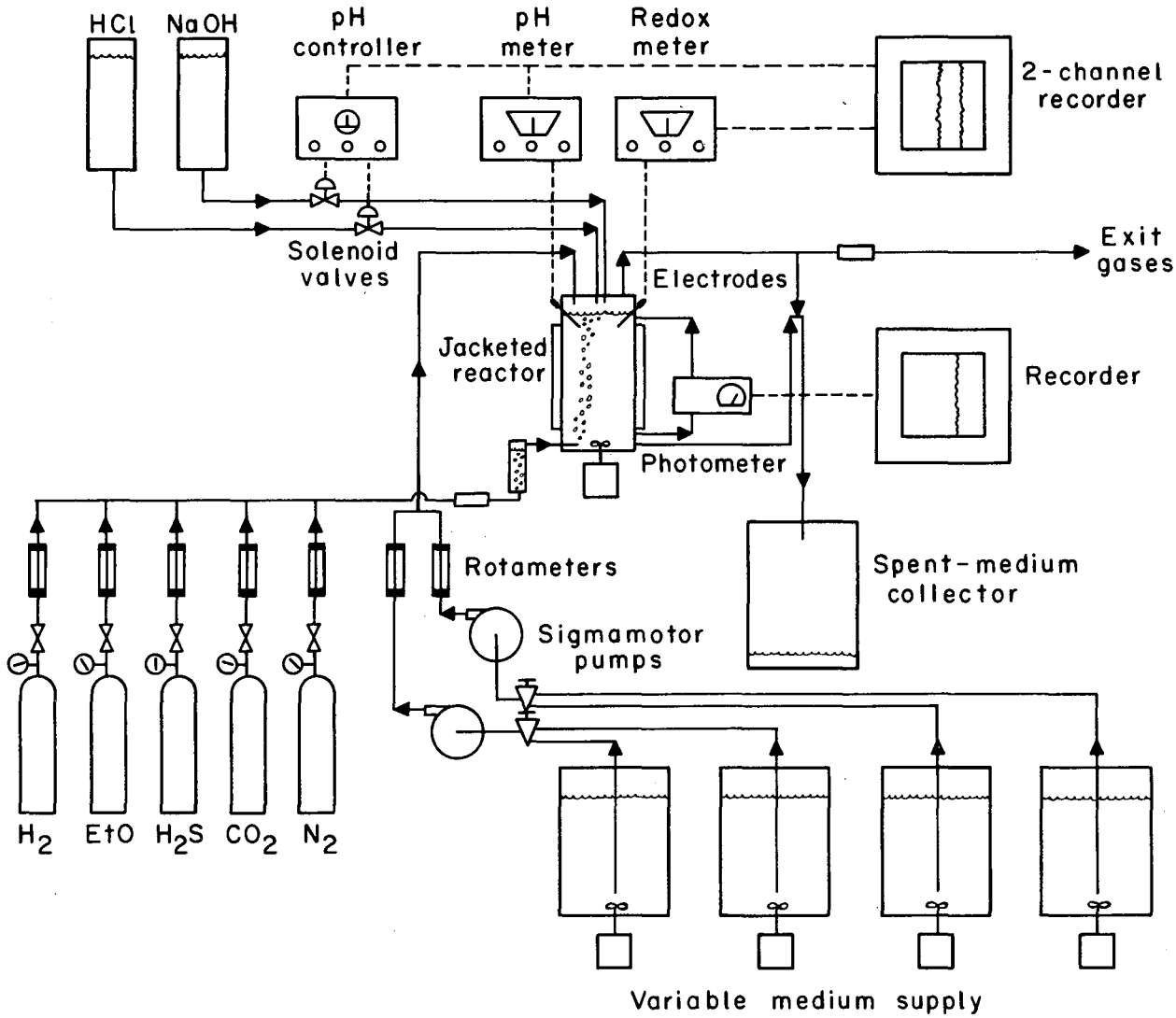

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Fig. 24.

The reactor is a cylindrical glass vessel of 2.3-liter capacity fitted with a removable ground-glass top. Water at a constant temperature is circulated through a jacket on the reactor. An inoculating culture may be added through either of 2 serum caps. Samples may be withdrawn through the serum caps or by opening a Teflon stopcock in a glass sampling line. Similar stopcocks are on the gas inlet port and the medium outlet port. (Stopcocks, serum caps and similar details are not shown on the flow diagram.) Using ground-glass joints, electrodes for pH and redox measurement are mounted through the wall of the reactor at a 45° angle with the wall. Acid and base for pH control are introduced through capillary tubing sealed into a serum cap in the top of the reactor. Gases are brought in through a porous glass sparger near the bottom of the reactor. Agitation is effected by a 3-inch magnetic stirring bar. The stirring action also pumps a stream of the fluid in the reactor through a spectrophotometer cell in an external leg. The output of the spectrophotometer is displayed on a potentiometric recorder, providing a continuous record of cell concentration.

The level in the reactor is controlled by an overflow leg connected to the lower part of the reactor. Spent medium can be collected in either of two 20-liter bottles.

Four 20-liter glass bottles serve to supply the reactor with medium. Each bottle can contain a medium of different composition so that the composition of the reactor feed may be varied. The bottles can be filled quite rapidly through a permanently installed filling system without disturbing the operation of the reactor. Medium is sterilized by passage through a sterile stainless steel Millipore filter assembly directly into the appropriate medium bottle. Details of the filling system are not included on the flow sheet. In order to retard growth of any contaminating organisms in the medium prior to use, it is kept at about 8° C by passing refrigerated water through cooling coils in each medium bottle.

The medium is pumped from the bottles to the reactor through one or both of two Sigmamotor peristaltic pumps. One pump is used when low flow rates are required, while the other is used for delivering higher rates. By adjusting the rates of the two pumps, the two streams may be blended to give

a medium of the desired composition and flow rate. Flow rates are monitored with rotameters. The entire medium supply system, including the filling lines, is sterilized in place with ethylene oxide. In preliminary tests, no contamination was encountered.

Gaseous nitrogen, carbon dioxide, hydrogen sulfide, hydrogen and ethylene oxide can be introduced singly or in combination through a porous glass sparger near the bottom of the reactor. The sparger breaks the gas stream into fine bubbles, providing good mass transfer with the liquid in the reactor. The flow rate of each gas is controlled with a needle valve and a rotameter. All rotameters are mounted on a single panel for quick reference. Before entering the reactor the gases pass through a bacteriological filter and through a water saturator, which helps reduce evaporation losses in the reactor. Exit gases are vented to a hood through a bacteriological filter. Samples of the exit gas may be readily taken for analysis.

The pH and redox potential of the reactor solution are measured continuously. The redox potential is measured with a Beckman Zeromatic pH Meter and recorded on one channel of a 2-channel recorder

A Leeds and Northrup Model 7501 expanded Scale pH Meter with Beckman pH electrodes is used for pH measurement and control. The output of the meter is sent to the second channel of the 2-channel recorder for recording of a 2-pH unit span full-scale.

The output from the pH meter is also used as the control signal for a bidirectional controller that was built following the design appearing in an article by Cotman and Smith,[21] who reported control to within ±.02 pH units. The controller governs the on-off actions of solenoid valves which allow acid or base to flow into the reactor for pH correction. The set point and control span are selected by the turn of appropriate dials.

Figure 25 shows the equipment as mounted in the laboratory.

The classical methods of measuring the concentration and character of microbial cells do not give quantitative information about the distribution of properties among the members of the population. In 1957, Coulter patented[22] a technique for counting and sizing small particles in a conducting solution.

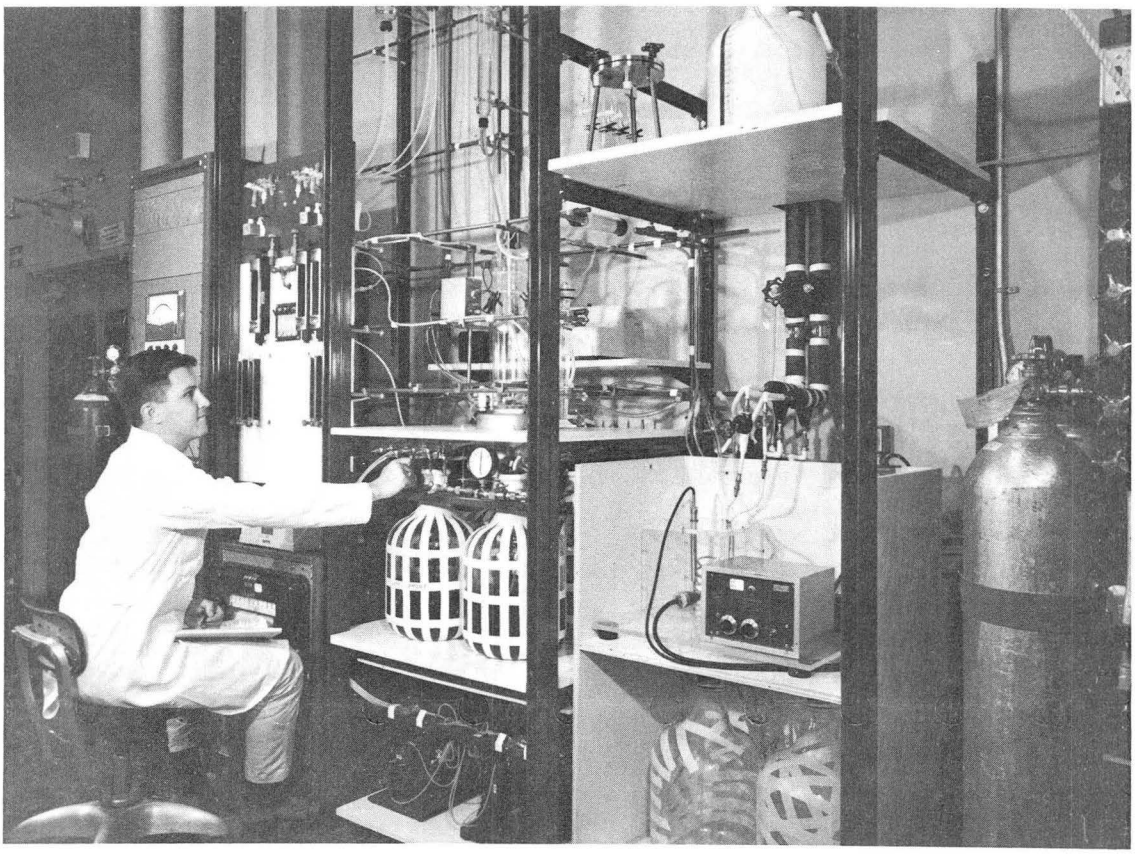

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Fig. 25

The Coulter counters, based on this principle, determine particle size and concentration electronically by measuring changes in the electrical conductivity of a very small aperture as a suspension of the particles is sucked through the aperture. The response of the counter is roughly proportional to the volume of each particle.

The Coulter counting system may be divided into two parts. The first is the aperture, measuring electrodes, sample-holder, and the apparatus for drawing a precisely measured volume of sample through the aperture. The second part is the electronic equipment designed to measure changes in the electrical conductivity of the solution passing through the aperture and to interpret them as the concentration and size distribution of particles in the liquid suspension. The electronic equipment consists of a preamplifier and amplifier to magnify the pulses in conductivity, an oscilloscope to display the pulses, and a device to count those pulses from the sample that fall between variable upper and lower thresholds chosen by the experimenter. If desired, a plotter may be added to the basic combination that presents the population's size distribution graphically.

We are planning to employ the Coulter aperture with a modified electronic system to obtain the size distribution and cell count. A diagram outlining the main features of the system is shown in Fig. 26. Fig. 27 is a photograph of the system. The aperture tube and associated system used to maintain a constant pressure drop across the aperture is of the standard Coulter design. A biasing voltage is supplied across the two electrodes on the two sides of the aperture and pulses in the electrical current through the aperture resulting from the passage of particles are amplified by the preamplifier and amplifier. The amplified signal is then fed from the amplifier to a cathode-ray oscilloscope that displays the electrical pulses and to a 128-channel pulse-height analyzer that sorts the pulses according to electrical size and counts the pulses of different sizes. The pulse-height analyzer stores the number of counts of each size in its memory and the resulting size distribution can then be displayed in a number of ways. At present, it is possible to display the information as a picture on a cathode-ray oscilloscope or as a graph on an x-y plotter. Under development is the use of a typewriter digital display and a paper-tape punch.

DIAGRAM OF PARTICLE COUNTING SYSTEM

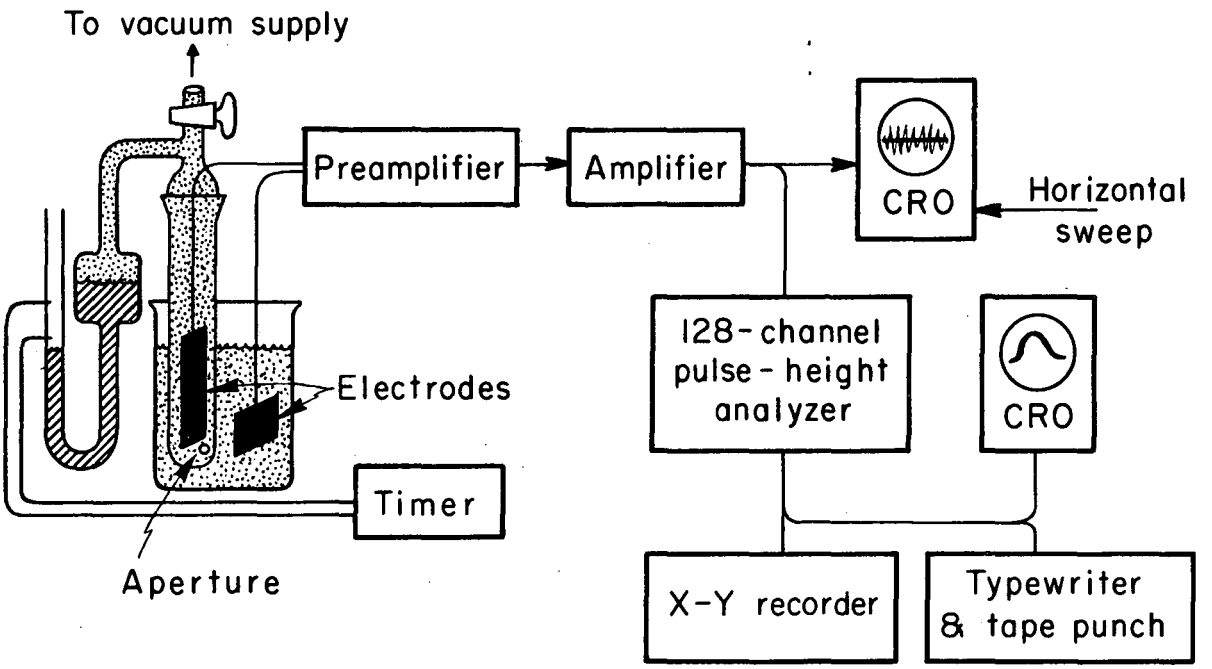

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Fig. 26

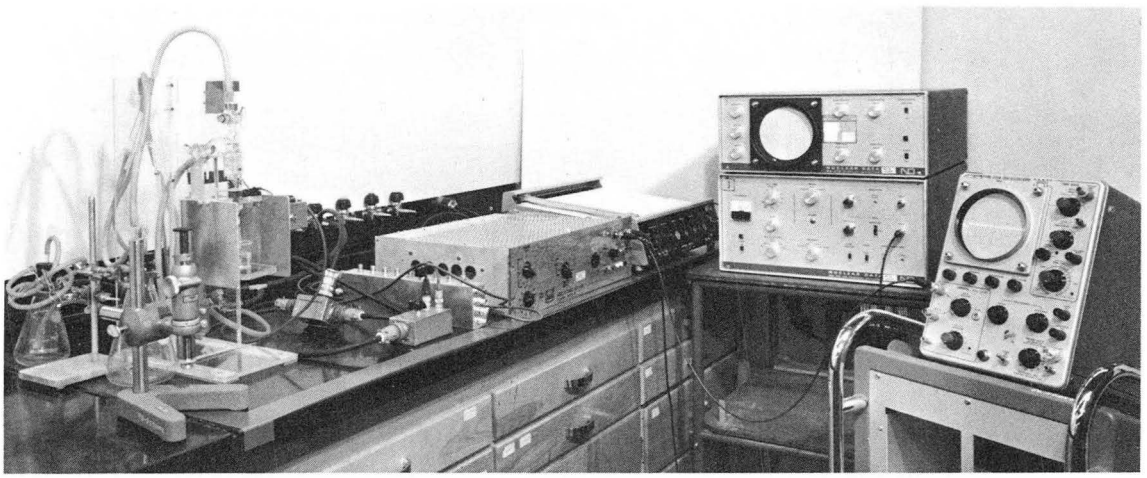

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Fig. 27

for feeding the information into a computer. This last feature is important because we have developed a technique for correcting the measured size distribution of particles in a population for the error caused by coincident passage of two or more particles through the aperture and the technique requires rather tedious calculations. The computer will also be used to calculate the number concentration of cells in the culture by numerical integration of the corrected size distribution and the volume concentration of the cells by numerical integration of the product of cell concentrations and corresponding volumes.

An effort will be made to correlate the measurements made with the counting and sizing system with the other measurements normally made on the cultures studied in the course of our kinetic experiments. Of particular interest will be the degree of correlation between percent by volume bacteria as computed from the measured size distribution and conventional indices such as dry weight, optical density, and packed cell volume.

Sulfate-reducing bacteria have been implicated in a wide variety of phenomena of economic importance, such as, corrosion, the formation of mineral deposits, and the plugging of oil deposits. The kinetic information obtained will be used to evaluate quantitatively some of these current economic activities, as well as, potential applications of sulfate-reducing bacteria, including the removal of sulfate from brine solutions with the salt-tolerant strain.