ENDOGENOUS FERMENTATION IN THIORHODACEAE[1]

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The photosynthetic bacteria grow well anaerobically in the light, and many members of the group are strict anaerobes, but their abilities to ferment added substrates appear limited and no one has succeeded in growing these organisms anaerobically in the dark on any substrate (Gest, 1951). On the other hand, it has been known since the work of Gaffron (1934) and Roelofsen (1935) that a strain of purple sulfur bacteria (Chromatium strain D), when grown on a medium containing thiosulfate or H_2S, may exhibit an active endogenous fermentation over a period of many hours.

Gaffron (1934, 1935) believed he had shown that the photochemical oxidation of H_2S to sulfate by this organism is a reversible reaction, and that the endogenous fermentation represents an oxidation of reserve food materials coupled with the reduction of sulfate to H_2S.

The evidence for this was that the addition of sulfate stimulated the endogenous fermentation and apparently was necessary for the photoassimilation of malate and butyrate. When incubated in the dark, the bacteria were also found to produce large quantities of an iodine-reducing material which gave a qualitative test for H_2S.

Roelofsen (1935), however, was unable to demonstrate any influence of sulfate on gas and acid production in the dark and Van Niel (1936) showed that the effect of sulfate on the light metabolism of organic substrates was a non-specific salt effect.

Van Niel did, however, confirm the production of an iodine-reducing material at a rate comparable to that reported by Gaffron, but the reductant appeared to be largely nonvolatile in acid solution and therefore not H_2S. The actual rate of production of H_2S, specifically determined by trapping it in acidified $CdSO_4$ solution, proved to be barely 10 per cent of rates reported by Gaffron for the production of iodine-reducing material. If the cells were devoid of stored elemental sulfur, the rate of H_2S production was even lower and could not be increased by the addition of sulfate.

Van Niel concluded that H_2S production was not an important metabolic activity of the purple sulfur bacteria, and that the traces which were formed arose from reduction of sulfur rather than sulfate.

In the following study the endogenous fermentation of Chromatium strain D has been reinvestigated. The results support Gaffron's belief that the iodine-reducing material produced in the dark is indeed H_2S. However, it appears that only the first step of the photochemical oxidation of H_2S is readily reversible, since colloidal sulfur and not sulfate proved to be the source of the H_2S, in accord with the results of Van Niel (1936).

Other products of the endogenous fermentation by Chromatium and the reduction of colloidal sulfur by baker's yeast were also investigated.

MATERIALS AND METHODS

The purple sulfur bacterium used for most of this work was "strain D," a strictly anaerobic organism which has been variously called a Thiocystis or Chromatium species. A culture was kindly supplied by Dr. Wilson of the University of Wisconsin, who had been maintaining it on inorganic medium. According to the account of Roelofsen, strain D was isolated by him from an enrichment culture used by Gaffron and was subsequently used by both Roelofsen (1935) and Van Niel (1936). Its favorable characteristics for metabolic studies are rapid growth on inorganic medium with negligible formation of mucoid clumps and films.

Culture medium. The composition of the culture medium is shown in table 1. An essentially inorganic medium was used so as to approximate the cultural conditions in the experiments of Gaffron (1934, 1935) and Roelofsen (1935).

[1] Part of this paper has been abstracted from a thesis submitted by the author in partial fulfillment of the requirements for the Ph.D. degree.

TABLE 1

Composition of medium

Solution A
1.0% NaCl
0.2% NH_4Cl
0.1% KH_2PO_4
0.1% $MgCl_2 \cdot 6H_2O$
0.02% $CaCl_2$ anhyd.
trace elements (Larsen)
1.0 mg % indigo carmine
0.90% v/v conc. HCl

Solution B
1.26% Na_2CO_3 anhyd.
0.6% $Na_2S_2O_3 \cdot 5H_2O$
0.02% disodium ethylene diamine tetraacetate
(0.01% $Na_2S \cdot 9H_2O$ added just before autoclaving)

In pyrex-distilled water

Parts A and B are autoclaved separately and mixed in equal volume after cooling. The resulting pH should be near 8.0. The final concentrations of NH_4Cl, KH_2PO_4, $MgCl_2 \cdot 6H_2O$ and trace elements are those of Larsen's medium (Larsen, 1952) with the exception that the iron concentration is increased 50 per cent. Indigo carmine is used as an indicator for absence of oxygen (Hutner, 1950). With the addition of 2 to 2.5 per cent agar this medium was used for maintaining pure cultures for inoculation.

The bacteria were grown in a continuous culture apparatus shown in figure 1. In order to maintain a relatively constant pH and pCO_2, a stream of 4 per cent CO_2 in nitrogen was bubbled through the culture flask. Traces of oxygen in the nitrogen (prepurified grade, Matheson Co.) were removed by washing the gas with a solution of chromous sulfate. Hydrogen sulfide can be admitted to the gas stream to cause reduction of dissolved oxygen initially present in the culture medium or to supply an additional feeding after the thiosulfate has been consumed. After harvesting about 90 per cent of the bacteria, new culture solution is injected by gravity-feed from sterile reservoirs of solutions A and B. Injections are made simultaneously from the two reservoirs through 20-gage stainless steel syringe needles thrust, with sterile precautions, through the thick rubber of the sampling outlet.

Centrifugations of the bacterial suspensions

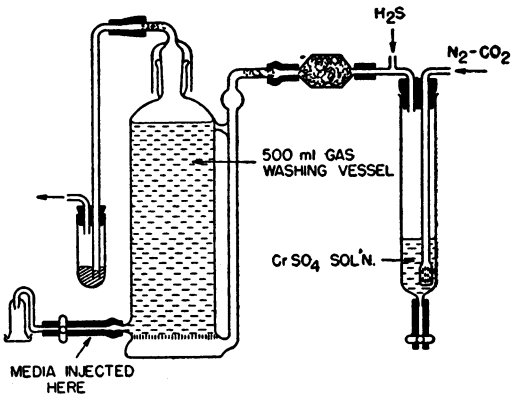

Figure 1. Anaerobic culture apparatus

were made in completely filled and stoppered tubes. Dissolved oxygen was removed by the addition of 0.01 per cent $Na_2S \cdot 9H_2O$.

Advantages of this continuous culture method over the filled bottle technique of van Niel (1931) may be summarized as follows: (1) a large inoculum of cells from the previous culture is always available; (2) a pure culture can be maintained with little effort over a period of months; (3) pH fluctuations are minimized since the pCO_2 is held constant; (4) temperature fluctuations do not cause entrance of air; and (5) additions and samplings can be readily made with little fear of contamination. With a 10 per cent inoculum full growth is achieved within two days at 30 C.

Samples from continuous cultures were checked from time to time for purity by both microscopic examination and incubation in the dark both aerobically and anaerobically on "A C medium" (Difco), with the addition of 0.3 per cent sodium sulfate to detect sulfate reducers. In only one instance was a continuous culture vessel found to be contaminated.

For some of the work described here bacteria were cultured in a stream of 4 per cent CO_2 in hydrogen freed of oxygen by passage through a palladium catalyst (Baker and Co., Inc.). The medium used was that shown in table 1 with the exception that thiosulfate was eliminated, $(NH_4)_2SO_4$ was substituted for NH_4Cl, and the final concentration of $NaHCO_3$ was reduced from 0.5 per cent to 0.25 per cent. Growth of the bacteria in this medium to a density of 13-ml cells per liter of medium has proved possible.

Cell volume determinations were made by sedimenting cells to a constant volume at 2,000

× G. One ml of packed cells of sulfur-free bacteria has a dry weight of approximately 0.34 g. In some instances in this paper the amount of H_2S evolved by the bacteria is expressed in terms of "cell volumes." This means the volume of H_2S evolved, corrected to standard conditions, divided by the volume of cells.

Determinations of CO_2 and total acid production were made by the Warburg manometric method. Hydrogen production was usually measured by the difference between two vessels, one containing palladium black to absorb hydrogen (Roelofsen, 1935). Hydrogen sulfide was continuously removed by precipitation of ZnS on a large roll of filter paper in the center well saturated with a solution 0.25 M in $ZnSO_4$ and 0.5 M in malic acid adjusted to pH 5.1 with NaOH. This mixture quantitatively trapped the H_2S without interfering with the CO_2 measurements. At the end of the incubation period H_2SO_4 was tipped into the suspension to bring the pH to below 2. After 20 min the vessel was opened and the filter paper was removed and submerged in excess acidified iodine solution. The remaining iodine was back-titrated with thiosulfate. Control experiments indicated that 95 per cent or more of 4 μM H_2S added could be recovered by this method.

When only H_2S was to be measured, the bacterial suspensions were incubated in 5- to 30-ml syringes. Advantages of syringe methods for anaerobic studies have been noted by Kirk and Hansen (1951). Samples were withdrawn, free from contact with air, with the aid of a 0.25- or 1.0-ml syringe. Each sample was then injected through a rubber seal into the center compartment of a Conway diffusion dish where the H_2S was liberated by contact with acid. Further steps in the analysis followed the method of Archibald and Gordon (1953).

Total fatty acids were determined by steam distillation of the supernatant from the acidified bacterial suspension after centrifugation. The distillates were titrated with 0.1 N $Ba(OH)_2$ in a stream of CO_2-free air. Excess standard acidified iodine was added to an aliquot of the resulting solution and back-titrated with thiosulfate to give a measure of the traces of SO_2 usually present. Excess $Ba(OH)_2$ was added to the remainder of the distillate and the solution reduced to a volume of 0.2 to 0.4 ml. The barium was precipitated as the sulfate by the addition of excess $(NH_4)_2SO_4$. Aliquots of the supernatant were used for paper chromatography on Whatman No. 43 paper with a solvent consisting of 100 ml 2-butanol + 25 ml 1 M NH_4OH aq. (Kennedy, 1953, *personal communication*). After solvent development the chromatograms were air-dried and sprayed with 0.08 per cent phenol blue in 25 per cent aq. ethanol 0.005 N in H_2SO_4.

Colloidal sulfur was prepared by the methods of Odén (1913). The final fraction used flocculated in 0.2 M NaCl. This fraction contained relatively little adsorbed pentathionate (Freundlich and Scholz, 1922). Kurtenacker's (1938) $HgCl_2$ method was used for the determination of adsorbed pentathionate and tetrathionate. In the experiments to be reported, the amount of pentathionate present never amounted to more than 15 per cent of the H_2S produced.

In the analysis for the radioactive isotope S^{35}, carrier sulfate was added to samples containing organic sulfur which were then dried and fused in a platinum crucible with 4 g NaOH and 0.5 g KNO_3 to effect oxidation of the sulfur to sulfate (Treadwell and Hall, 1942). Precipitations of $BaSO_4$ were made in dilute solution 0.01 N in HCl at 90 C. After being dried and weighed the $BaSO_4$ was pressed into a circular depression in a counting planchet to make an "infinite thickness" layer and the radioactivity measured with a flow counter.

RESULTS

Identity of the reducing substance. When washed suspensions of bacteria grown in thiosulfate medium were directly titrated with iodine solution before and after incubation in the dark, an increase in iodine titer always occurred. During illumination the titer decreased. These results confirmed earlier observations by Gaffron (1935) and Van Niel (1936). The suspensions, taken from Warburg vessels or syringes, were treated with an excess of iodine at a pH of approximately 2 and immediately back-titrated with standard thiosulfate solution.

Contrary to the results of Van Niel, however, the reducing substance was found to be largely volatile in acid solution. When the cells were separated from the supernatant, most of the reducing substance remained in the supernatant. The reducing agent in the supernatant was found to be acid labile, and precipitation with zinc and lead salts showed it to be H_2S. A reduc-

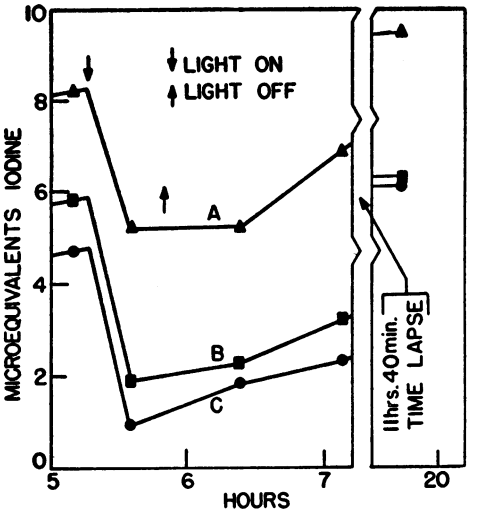

Figure 2. Change of iodine-reducing titer during illumination. *A*, Titer of entire suspension. *B*, Titer of supernatant. *C*, Sulfide titer in supernatant. Each Warburg vessel contained 0.052 ml bacteria grown in thiosulfate medium, 9 μM bicarbonate, 500 μM NaCl, in a total volume of 2.8 ml. The gas phase was 2 per cent CO_2 in purified nitrogen.

ing capacity of 13.05 to 13.23 μEq I_2 could be recovered as ZnS or PbS from a supernatant with a total iodine titer of 13.60 μEq. ZnS was precipitated in the presence of ammonia and ammonium ion, and PbS in excess NaOH. Control experiments showed that under these conditions thiosulfate, sulfite, cysteine, and methyl mercaptan stay in solution.

In another experiment the H_2S production in the dark from two mucus-forming strains of Thiorhodaceae was measured. In each case at least 80 per cent of the iodine-reducing titer of the supernatant could be precipitated as ZnS.

Although H_2S does not account for the entire iodine titer of a whole cell suspension, it is apparently responsible for the changes in titer during light and dark periods. Figure 2 shows changes in light and dark periods of the iodine titer of a suspension, the supernatant titer, and the H_2S titer. Each set of points was determined from three samples withdrawn by syringe from an alkalinized suspension in a Warburg vessel, avoiding contact with air. The titer of the suspension was obtained by adding one sample to an excess of acid iodine solution and immediately filtering out the cells under pressure through asbestos. The titer of the supernatant

TABLE 2

Rate of H_2S production

In each experiment, washed cells of Chromatium strain D, grown in a medium containing the specified H-donor, were suspended in a bicarbonate solution under a gas phase containing 2–5 per cent CO_2 in purified nitrogen and incubated in the dark at 34–35 C, pH 7–8. In the experiments of Gaffron the change in iodine titer of the suspension is assumed to represent H_2S production.

Experiment	Cultural Conditions	Time of Incubation	Avg Rate in Cell Volumes H_2S per Hour
		hr.	
1	$S_2O_3^{-2}$ medium	2.5	0.46
2	$S_2O_3^{-2}$ medium	4.4	0.33
3	H_2 medium	5.6	0.08
4	H_2 medium*	4.9	0.002
Gaffron 1†	$S_2O_3^{-2}$ medium	1.5	0.42
Gaffron 2‡	$S_2O_3^{-2}$ medium	24	0.33
van Niel§	malate medium + H_2S feeding	24	0.045

* Cysteine, 0.01 per cent used instead of $Na_2S \cdot 9H_2O$, 0.01 per cent in harvesting and washing procedures.

† Gaffron, 1935, table VI, 4.

‡ *Ibid.*, table VIII, b), 1.

§ van Niel, 1936, p. 329, expt. 3, No. 2.

was determined by rapid filtration of another aliquot directly into acidified iodine solution. H_2S was determined by filtration of the third aliquot into ammoniacal $ZnSO_4$ solution, the ZnS being then washed and allowed to react with acid iodine solution. In each case, back titration with standard thiosulfate determined the amount of iodine reduced.

The results indicate no significant decrease in the cell titer, as measured by the difference between the whole suspension and supernatant titers, during the light period, or increase during the dark period. The differences between the total supernatant and H_2S titers are probably largely due to losses of ZnS during the washing operation. In other experiments, better recoveries of ZnS were obtained.

Rate of production of H_2S. Table 2 presents a few representative rates of H_2S formation collected from various experiments. Two rates from Gaffron's experiments (1935) and one from Van Niel's (1936) are included for comparison. It may be observed that the rates obtained with thiosulfate-grown cells in the present experiments are

in the same range as those reported by Gaffron. Since the rate of H_2S production falls with time, van Niel would doubtless have observed somewhat higher rates had he used shorter incubation times.

Influence of pH. H_2S production increases about 25 per cent as the pH is raised from 7.5 to 9.5, but drops rapidly to zero in weakly acid (pH 4.5) solution.

Effect of oxygen. It is important to maintain strictly anaerobic conditions when measuring H_2S production, since the bacteria catalyze a very rapid oxidation of H_2S by even traces of oxygen. For this reason 0.005 to 0.01 per cent $Na_2S \cdot 9H_2O$ was initially added to suspensions contained in syringes to reduce the dissolved oxygen present. In manometric experiments the gas mixtures used for flushing were freed from traces of oxygen by washing with a chromous sulfate solution or, in the case of hydrogen mixtures, by passage through a palladium catalyst.

Experiment with radioactive sulfate. The confirmation of H_2S production again raised the possibility of sulfate reduction. This possibility had been strengthened by a report of Nakamura (1939) that a species of Thiorhodaceae absorbed hydrogen in the dark when sulfate was added. The availability of the radioactive isotope S^{35} suggested an experiment which could reveal even traces of net sulfate reduction or exchange with reduced sulfur compounds in the cell.

The general plan of the experiment was to compare $S^{35}O_4^{-2}$ reduction in a bacterial suspension incubated in the dark with one incubated in the light. The soluble and insoluble fractions (the solvents being 0.01 N H_2SO_4 and boiling 80 per cent ethanol) were freed of unreacted sulfate and analyzed to find the percentage of the added counts that had been fixed. The incorporation of S^{35} into the H_2S formed in the suspension incubated in the dark was determined separately after the addition of $Na_2S \cdot 9H_2O$ carrier. The H_2S was trapped in a solution of 3 per cent H_2O_2 made 0.5 M in NH_4OH, where the sulfide was oxidized to sulfate (Treadwell and Hall, 1942).

A summary of the results is shown in table 3. Only a very small percentage of the S^{35} added could be recovered in the bacterial fractions. The total sulfate actually fixed is not known since the amount remaining in the washed cells at the start of the incubation was not determined. However, even if we assume a generous cellular concentration of 0.1 M for sulfate, the proportion of H_2S arising from sulfate could not have been greater than about 0.01 per cent assuming free exchange of intracellular and extracellular sulfate.

TABLE 3

Failure to reduce $S^{35}O_4^{-2}$

Each vessel contained 0.13 ml washed cells of Chromatium strain D grown on thiosulfate medium, 0.011 M $NaHCO_3$, 0.5 per cent NaCl, and 0.2 μM of sodium sulfate and sulfuric acid containing 6.1×10^6 counts/min of S^{35}. Liquid volume = 25 ml. Gas phase was 2 per cent CO_2 in nitrogen. Vessels were incubated in either light or darkness for 4 hours at 33–35 C. During incubation 12.5 μM H_2S were produced in the dark vessel. Approximately 10 per cent of the cells contained sulfur inclusions.

	Counts/Min	Per Cent of Added Counts/Min
Added as $S^{35}O_4^{-2}$	6.1×10^6	
Recovered as S^{-2} (dark vessel)	670	0.01
Recovered in insol. fraction, dark vessel	390	0.007
Recovered in insol. fraction, light vessel	640	0.01
Recovered in soluble fraction,* dark vessel	2,250	0.04
Recovered in soluble fraction,* light vessel	2,120	0.04

* Freed of $S^{35}O_4^{-2}$ by two precipitations with $BaCl_2$ of added carrier sulfate.

There was no measurement made of the permeability of these cells to sulfate but it would appear to be high, at least during illumination, since sulfate is an end product of the photometabolism of reduced sulfur compounds and must escape rapidly from the cell.

One type of sulfur intermediate which would not be detected by the procedures used is a nonvolatile compound which is oxidized quantatively back to sulfate by air in strongly acid solution in the presence of bacteria. With this possible exception we may conclude from this experiment that strain D, when grown on thiosulfate medium and still containing some elemental sulfur, reduces sulfate to a negligible extent in both light and dark compared to the production of H_2S in the dark.

The reduction of $S^{35}O_4^{-2}$ was not studied in cells entirely devoid of stored sulfur by growth

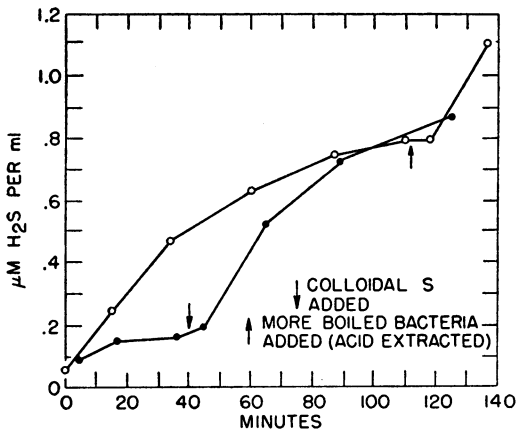

Figure 3. Stimulation of H_2S production by colloidal sulfur. ● = H_2-grown bacteria alone, colloidal sulfur added at 40 mins. ○ = H_2-grown bacteria + boiled thiosulfate-grown cells, additional boiled cells, acid extracted, added at 112 min. Each syringe contained 0.013 ml H_2-grown bacteria per ml of 0.02 M phosphate buffer pH 7.4 containing 0.01 per cent $Na_2S \cdot 9H_2O$. Temp = 34 C.

in a medium containing hydrogen as H-donor. It was found, however, that the addition of sulfate to such cells did not stimulate H_2S production. Van Niel (1936) found the same result using cells grown on a malate medium.

Desulfhydration. Many species of bacteria have been found to catalyze a desulfhydration of added cysteine and homocysteine (Fromageot, 1951). The over-all equation for the process may be written:

$$\text{cysteine (or homocysteine)} \rightarrow \alpha\text{-keto acid} + NH_3 + H_2S$$

If the H_2S produced by strain D were coming from intracellular cysteine, one might then expect to find a concomitant production of NH_3. However, in several experiments where NH_3 production in the supernatant was measured by direct nesslerization, it was found to be of the order of only $\frac{1}{10}$ the H_2S production. It is concluded that the production of H_2S cannot be accounted for by this mechanism.

Effect of boiled bacteria and colloidal sulfur. Purple sulfur bacteria grown in an atmosphere of hydrogen and CO_2, with sulfate as the sole sulfur source, exhibit very low rates of H_2S production. One example of this is given in experiment 3 of table 2. When, however, boiled cells of thiosulfate-grown bacteria were added to the hydrogen-grown bacteria a rapid production of H_2S ensued. Boiled cells of hydrogen-grown bacteria had no stimulatory effect. The factor in the boiled cells of thiosulfate-grown bacteria which caused the stimulation was not extracted from the insoluble matter by boiling in 0.1 N HCl followed by boiling in 0.5 M NH_4OH, and resisted oxidation by air. Colloidal sulfur was found to duplicate the effect of boiled cells of thiosulfate-grown bacteria. The effects of added sulfur and boiled cells on H_2S production in hydrogen-grown bacteria are compared in figure 3. Note the similarity in rates after the addition of sulfur and the second addition of boiled bacteria (this time acid-washed). The lag after the addition of the sulfur or boiled cells until the new rate is established can be shown to be due to oxidation of some H_2S by oxygen dissolved in the injected liquid, and does not represent a delay in the reduction of added sulfur.

It follows from these experiments that the difference in rates of H_2S production between thiosulfate-grown and hydrogen-grown bacteria is due to the accumulation of sulfur in the former. The source of sulfur for the much lower production of H_2S from hydrogen-grown bacteria proved to be traces of colloidal sulfur arising from oxidation by air of the 0.01 per cent $Na_2S \cdot 9H_2O$ added routinely during the harvesting procedure and to the wash water. If 0.01 per cent cysteine is substituted for the sulfide as reductant, the H_2S production falls to the extremely low level indicated by experiment 4, table 2.

An interesting aspect of the H_2S production induced by added sulfur is that the rate is higher per cell volume than that observed in cells naturally filled with sulfur granules by growth in thiosulfate medium. The initial rate of reaction after the addition of colloidal sulfur in the experiment was 2.0 cell volumes H_2S per hour, more than three times the highest rates observed in the absence of added sulfur. The effect of sulfur can always be duplicated by boiled, acid-extracted, sulfur-filled bacteria.

This result indicates that the availability of sulfur normally limits the rate of H_2S production even in thiosulfate-grown cells. It suggests that the intracellular sulfur is not as available for reduction as the extracellular sulfur, or that the surface area of one or two intracellular sulfur granules is simply not sufficient for maximum rates of reaction. The particle size of the added

sulfur proved to be important since a hydrophilic suspension of sulfur particles above colloidal size was found to give a lower rate of H_2S production than colloidal sulfur. The frequency of bombardment of the cell surface by the sulfur particles may be a factor in determining the rate of reaction.

Given an excess of colloidal sulfur, the bacteria may produce up to three cell volumes of H_2S in a 4-hour period. To reduce this amount of sulfur some reductant must be initially present in the cells at a concentration of at least 0.27 N. The reductant was not identified but the evidence indicates that only a small part of it could be sulfhydryl compounds. If this were not the case then one would expect the iodine titer of the cells to show a large decrease during incubation in the dark, equivalent to the H_2S production. Figure 2, however, shows that the changes in cell titer are small compared to the H_2S changes. In some experiments the equivalent H_2S production was found to be four times larger than the initial acid iodine titer of the cells.

The utilization of added pyruvate or malate for sulfur reduction is suggested by the fact that the addition of a mixture of these substances was found to increase H_2S production from bacteria plus colloidal sulfur by 25 per cent. The experiments of Gaffron (1935) and Van Niel (1936) also indicate a stimulation of H_2S production by added malate.

Growth experiment. Although the couple H_2S/S has a low potential at pH 7 (Latimer, 1938), near that of DPNH/DPN$^+$ (Rodkey, 1954), it is energetically possible that the oxidation of such a substrate as pyruvate by sulfur might furnish enough energy for coupled formation of high energy phosphate bonds and growth. It was decided to test the possibility of anaerobic growth in the dark with sulfur as oxidant although Kohlmiller and Gest (1951) had failed to demonstrate growth of a related organism, *Rhodospirillum rubrum*, using $K_3Fe(CN)_6$ as oxidant. Sulfur is a much poorer oxidant than $K_3Fe(CN)_6$ but is a more natural substrate for purple bacteria. Accordingly, strain D was inoculated into an anaerobic medium containing malate, pyruvate, yeast extract and 0.5 per cent colloidal sulfur. The H_2S formed was continuously removed. No growth occurred, however, until the culture was exposed to light after a week of incubation in the dark.

Identification of fermentation acids. Gaffron (1934) and Roelofsen (1935) observed the production of considerable and varying amounts of unidentified acids, which decreased the bicarbonate concentration during endogenous fermentation in the dark. In the present experiments, 70 to 100 per cent of the acid produced by both hydrogen-grown and thiosulfate-grown bacteria could be recovered as steam-volatile acid.

Paper chromatography and a Duclaux distillation indicated that more than 90 per cent of the steam-volatile acid is acetic acid. Further confirmation came from the fact that treatment of the volatile acids with dichromate-H_2SO_4 oxidizing mixture did not decrease the volatile acid titer. Kohlmiller and Gest (1951) have reported endogenous production of acetic acid at an unspecified rate from cells of *R. rubrum*, grown on organic medium. They found the acetic acid to be accompanied by varying proportions of CO_2, hydrogen, propionate, and higher fatty acids, depending on the pCO_2 and cultural conditions.

Production of hydrogen. No evidence could be obtained for the production of any gas in the dark other than CO_2 from cells grown on thiosulfate medium. From hydrogen-grown cells, however, which had been harvested using cysteine instead of Na_2S as reductant, the production of another gas at a rate of up to 0.11 cell volumes per hour could be observed. Since this gas was absorbed by palladium black but not by alkali, it was presumed to be hydrogen. These observations confirm those of Roelofsen (1935) who found hydrogen production from cells of strain D grown on peptone medium but not from cells grown on thiosulfate medium. When colloidal sulfur is added to hydrogen-grown cells, the production of hydrogen is inhibited (table 4, expt. 1) and any hydrogen in the gas phase will be actively absorbed down to a partial pressure as low as 0.1 to 0.2 per cent. Thus it again appears that the difference in endogenous metabolism between the thiosulfate- and hydrogen-grown bacteria may be explained by the presence of sulfur in the former.

It should be noted that hydrogen-grown bacteria carry on an endogenous fermentation in the absence of elemental sulfur but the rate of production of CO_2 and acetic acid, especially CO_2, is greatly stimulated by the addition of colloidal sulfur (table 4, expt. 1).

Other products of the endogenous fermentation.

TABLE 4

Endogenous fermentation products of Chromatium strain D

In each experiment, washed cells of Chromatium strain D, grown in inorganic medium containing either molecular hydrogen ("H_2-grown") or thiosulfate ("S-grown") as H donor, were suspended in a medium containing $NaHCO_3$, 0.005–0.008 M, and NaCl, 0.02–0.03 M and incubated in the dark at 34 C. The gas phase was 2 per cent CO_2 in nitrogen unless CO is indicated, in which case 2 per cent CO_2 in CO was used.

Experiment	Time of Incubation	Conditions	CO_2	H_2	H_2S	Total Acid	Volatile Acid (Acetic)
	hr		*μM*	*μM*	*μM*	*μEq*	*μEq*
1	1.0	H_2-grown	0.4	0.4	<0.01	2.0	
		H_2-grown + S*	2.8	0.0	4.1	3.6	
2	4.7	S-grown	3.5	0.0	5.1	2.1	2.0
3	2.5	S-grown	21		22	17	12
4	2.7	H_2-grown	1.0	1.3		4.7	
		H_2-grown in CO	0.5	0.0		6.1	
5	4.4	S-grown	6.6		7.2	5.2	5.5
		S-grown, in CO	4.2		8.0	6.5	6.7
6	1.9	S-grown	3.5		4.7	1.5	
		S-grown + DNP†	4.9		6.2	4.5	

* Colloidal sulfur added at the beginning of the incubation period.

† 2,4-dinitrophenol 3×10^{-4} M.

Besides CO_2, acetic acid, H_2S, or, in the case of hydrogen-grown bacteria, hydrogen, no other products of the endogenous fermentation were detected in quantities equivalent to more than $\frac{1}{10}$ of the acetic acid production. Analyses were made for the following substances: neutral volatile material oxidizable by a dichromate-H_2SO_4 mixture, lactate, glycerol, 2,3-butylene glycol, and acetoin. The analyses were made on bacterial suspensions cleared by $Zn(OH)_2$ precipitation, according to methods 1, 8, 17, 18, and 23, collected and modified by Neish (1952). Other acids besides the fatty acids were not looked for since in most experiments more than 90 per cent of the acid produced was steam-volatile.

Endogenous photoreduction. The H_2S and acetic acid produced by endogenous fermentation in the dark can be reutilized as hydrogen donors by the bacteria during a subsequent light period. This is evidently the cause of the phenomenon of endogenous photoreduction or "autoassimilation" observed by Gaffron (1934, 1935) and Roelofsen (1935) using strain D. Gaffron (1935) proposed H_2S as the substrate for the endogenous photoreduction, whereas Roelofsen (1935) implicated organic acids.

Effect of inhibitors. The rate of production of H_2S from colloidal sulfur is 90 per cent inhibited by heating anaerobically for 3 min at 70 C and more than 98 per cent inhibited by heating at 90 C, suggesting that enzyme-catalyzed reactions are involved.

Gaffron (1935) found that the presence of CO inhibits acid formation during the photometabolism of thiosulfate. The effect of CO on the endogenous fermentation, on the other hand, is to increase acid production and decrease CO_2 production from cells grown on either thiosulfate or hydrogen media (table 4, expts. 4 and 5). The production of hydrogen is completely inhibited by CO, but H_2S production is not. Another experiment demonstrated that the substitution of helium for nitrogen in the gas phase does not alter the proportions of fermentation products. These results suggest that heavy-metal enzymes are involved in the production of H_2 and CO_2 but not H_2S or acetic acid.

At certain concentrations 2,4-dinitrophenol significantly increases the rate of production of all fermentation products, especially fixed acid (table 4, expt. 6). According to prevailing theories of the mode of action of dinitrophenol (Simon, 1953) this suggests that the endogenous fermentation is normally coupled to phosphate esterification.

H_2S production from yeast. It has been known since the work of Dumas (1874) that yeast cells can reduce sulfur, but a search of the literature failed to reveal data useful for comparison with the results found here for Thiorhodaceae. Some experimental work was therefore necessary.

When colloidal sulfur was added to washed cells of fresh baker's yeast (Fleischmann), initial rates of H_2S production of up to 1.5 cell volumes per hour, and a total production up to 2.7 cell volumes, could be observed. In rate and magnitude these values fall in the range of those found previously for the purple sulfur bacteria.

As with the bacteria, the H_2S production may greatly exceed the initial iodine titer of the cells. Direct titration of live or boiled yeast cells with iodine at pH 1 gave values between 0.25 and 0.30 μEq per 10 μL cells, which is about $\frac{1}{8}$ of the equivalents of H_2S that may be evolved.

The production of H_2S is accompanied by the evolution of CO_2 at approximately the same rate. This rate of CO_2 production is about 10 times that of the endogenous rate without added sulfur. Tetrathionate reduction proceeds at approximately the same rate as sulfur reduction. The addition of H_2S, thiosulfate, sulfite, dithionate, or oxidized glutathione caused no detectable stimulation of the endogenous fermentation.

As with Chromatium, H_2S production from yeast cells is retarded more than 90 per cent by heat denaturation.

DISCUSSION

The reduction of sulfur by yeast would appear to be a rather unusual reaction for this organism, but the reduction of sulfur by Thiorhodaceae must be considered a normal physiological process, since these organisms are usually found in nature containing intracellular S droplets. The relatively high rate at which H_2S may be produced, up to $\frac{1}{2}$ cell volume per hour for a period of several hours, is suggestive of the endogenous respiration of green plants, with S instead of O_2 serving as oxidant. Although it apparently does not allow growth, such a "sulfur respiration" might nevertheless provide useful energy for flagellar movements and synthetic reactions required for survival in the dark.

While this paper was in preparation it was learned that Larsen (1953) observed the evolution of considerable amounts of a gas absorbable by a $HgCl_2$ solution, presumably H_2S, from suspensions of *Chlorobium thiosulfatophilum* incubated anaerobically in the dark. It thus seems probable that H_2S production is an important feature of the metabolic pattern in all photosynthetic bacteria capable of utilizing H_2S for growth in the light.

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SUMMARY

The endogenous fermentation of washed cells of a Chromatium species strain D, grown on an inorganic medium containing thiosulfate, results in the formation of varying proportions of H_2S, CO_2 and acetic acid. The formation of significant amounts of nonvolatile iodine-reducing material as previously reported (Van Niel, 1936) could not be confirmed. The evidence indicates that the source of the H_2S is elemental sulfur which is enzymatically reduced in a reaction coupled with the oxidation of reserve organic materials. Reduction of sulfate, measured with the radioactive isotope S^{35}, was found to be negligible, at least with cells containing traces of stored sulfur.

When the cells are grown on an inorganic medium containing molecular hydrogen as H-donor, the endogenous fermentation results in the production of CO_2, acetic acid, and hydrogen, but not H_2S unless colloidal sulfur is present. The addition of colloidal sulfur to cells devoid of stored sulfur was also found to stimulate the endogenous production of CO_2 and acetic acid, but anaerobic growth in the dark on an organic medium with sulfur as oxidant could not be demonstrated.

The endogenous production of CO_2 by baker's yeast is stimulated about tenfold by the addition of colloidal sulfur. For equal cell volumes, the rate and magnitude of the production of H_2S approximates that observable in Chromatium strain D, with added colloidal sulfur.

A continuous culture method has been described which presents advantages over previous methods for cultivation of the Thiorhodaceae.

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