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Energy Supply for the Chemoautotroph *Ferrobacillus ferrooxidans*

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ABSTRACT

DUGAN, PATRICK R. (Syracuse University, Syracuse, N.Y.), AND DONALD G. LUNDGREN. Energy supply for the chemoautotroph *Ferrobacillus ferrooxidans*. J. Bacteriol. **89**:825–834. 1965.—A working model is proposed to explain dissimilatory ferrous iron oxidation by *Ferrobacillus ferrooxidans*, that is, oxidation linked to an energy source. The model is supported by experimental evidence reported here as well as in the literature. Polarographic assays of the culture medium demonstrated an iron "complex" involving oxygen. The initial "complex" would be oxygenated, but not oxidized because no electron transport has taken place. The "complex" is formed in solution or on the cell surface and is somehow reacted with iron oxidase (or oxygenase), resulting in the release of an electron. Either sulfate or a flavoprotein is suggested as involved in the initial electron-transfer link between iron and the cell. The electron is transported in the cell through a typical electron-transport system involving coenzyme Q_6, cytochrome *c*, and cytochrome *a*; oxygen is the final electron acceptor. Electron micrographs of intact and sectioned cells are included to show structural detail in support of the model.

Several bacterial isolates have been reported which have the ability to catalyze the oxidation of ferrous iron and utilize the associated energy for carbon reduction (Beck and Elsden, 1958; Colmer, Temple, and Hinkle, 1950; Leathen, Kinsel, and Braley, 1956). The oxidation of ferrous to ferric ion is accompanied by production of acid, and in all reported instances the H^+ cations are associated with $SO_4^{=}$ anions, yielding sulfuric acid (Dugan and Lundgren, 1964*a*). This investigation is directed toward the elucidation of the mechanisms involved in dissimilatory ferrous iron oxidation by *Ferrobacillus ferrooxidans*—iron oxidation which supplies energy rather than assimilatory utilization. A preliminary report of this work has been published (Dugan and Lundgren, 1964*b*).

Growth of ferrobacilli in the experimental culture system resulted in the oxidation of approximately 9,000 μg of ferrous iron per milliliter of medium by 96 hr at 25 C, and yielded approximately 2×10^8 cells per milliliter of medium. The cells turn over large amounts of iron as a consequence of the low energy yield from the oxidation (11.3 kcal per gram atom; Temple and Colmer, 1951). To couple the energy released from ferrous iron in the form of electrons to carbon reduction mechanisms within the cell, the ferrous ion must either enter or be attached to binding sites at the surface of the cell. Further, any postulated scheme for coupling iron oxidation to cell metabolism must also account for an oxygen requirement and for the sulfate requirement reported by Lazaroff (1963). A polarographic assay was employed to detect reaction products formed when iron, oxygen, and cells were reacted in the growth medium. Radioactive iron and sulfate were employed to follow uptake of these ions involved in iron oxidation.

MATERIALS AND METHODS

Culture conditions. The organism, medium, and culture growth system used throughout this study were those previously described by Silverman and Lundgren (1959*a*). Cells were harvested after 4 to 5 days of incubation at ambient temperature (25 ± 2 C), and the precipitate in the medium was allowed to settle for 12 hr before the cleared supernatant liquid was centrifuged at 45,000 rev/min (50,000 × *g*) in a Sharples Supercentrifuge. Approximately 1.0 g of cell paste (wet weight) was obtained per 16 liters of medium. The cells were suspended in sterile distilled water, adjusted to *p*H 3.0 with H_2SO_4 (dilute H_2SO_4), washed three times with dilute acid, and stored until needed.

Polarographic assays. All of the constituent cations present in the growth medium, and also oxygen, were detected by their reductive half-

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wave potentials by use of a Sargent Polarograph, model XV (E. H. Sargent and Co., Chicago, Ill.).

Each cation of the growth medium (9K medium) was examined by preparing individual solutions in dilute H_2SO_4. This was necessary to demonstrate that their half-wave potentials did not overlap or otherwise interfere with detection of iron or oxygen in solution. The salts mixture used in the medium, without added $FeSO_4$, was also examined. The $FeSO_4$ in the medium had to be diluted 1:50 with dilute H_2SO_4 prior to analysis. Dilution of those cations present in lower concentrations, coupled with instrumental attenuation of sensitivity, allowed iron detection with a negligible response from the other medium constituents. Current sensitivity and potential range adjustments were made repeatedly during the course of the investigation. Polarograms were recorded on 4-ml samples in a Sargent (S29381) "rapid deaerating" electrolysis vessel. Triton X-100 (0.1 ml of a 0.1% aqueous solution) was added as a maximal suppressor (Meites, 1955). The salts solutions were always maintained at *p*H 3.0 in dilute H_2SO_4, and the spent culture medium was diluted with *p*H 3.0 H_2SO_4 before assaying.

Uptake of radioactive iron and sulfur by resting cells. Commercial radioactive ferric iron ($Fe^{59}Cl_3$) was reduced to the ferrous state prior to use by the method of Erdey and Suehla (1963). A 4-ml amount of a $Fe^{59}Cl_3$ solution containing 0.116 mg of Fe was treated with 0.2 mg of ascorbic acid plus 1.0 ml of dilute H_2SO_4, and the solution was stored for 2 hr prior to use. Analysis for ferrous ion was performed on 0.1-ml samples with *o*-phenanthroline as the reagent to insure that all ferric ion had been reduced (Harvey, Smart, and Amis, 1955). The specific activity of the ferrous iron solution was 50 μc/ml. S^{35} was employed in the form of $H_2S^{35}O_4$ in HCl.

Labeled Cell Pellet

Extract for 5 min in water at 85 C and centrifuge → (supernatant fluid) Adjust to volume and count *Hot water fraction*
(residue) ↓

Extract for 30 min in 5% trichloroacetic acid at 5 C and centrifuge → (supernatant fluid) Adjust to volume and count *Cold trichloroacetic acid fraction*
(residue) ↓

Extract for 30 min in 75% ethyl alcohol at 45 C and centrifuge → (supernatant fluid) Adjust to volume and count *Ethyl alcohol-soluble fraction*
(residue) ↓

Extract for 15 min in ether-ethyl alcohol (1:1) at 45 C and centrifuge → (supernatant fluid) Adjust to volume and count *Ether-ethyl alcohol fraction*
(residue) ↓

Extract for 30 min in 5% trichloroacetic acid (boiling) and centrifuge → (supernatant fluid) Adjust to volume and count *Hot trichloroacetic acid fraction*
↓

Cell residue → Suspend in proper volume of water and count *Cell residue*

FIG. 1. *Chemical fractionation procedure for Fe^{59}-labeled cells of Ferrobacillus ferrooxidans.*

An attempt was made to study the movement of ferrous and sulfate ions in ferrobacilli by determining the water-free space (WFS) of the cells with the use of the isotopic procedure of Roberts et al. (1955). The WFS is defined as that part of the total volume of a cell which has the same concentration of diffusible material as that found in the surrounding medium:

$$\text{WFS} = \frac{\text{counts per ml of cells per min}}{\text{counts per ml of medium per min}}$$

Experimental determinations were made by exposing 0.2 g (wet weight) of washed cells in a 10-ml glass centrifuge tube to solutions containing either of the isotopes as specified in the footnote to Tables 1 and 2.

Fe^{59} was detected by measuring γ emissions directly in glass centrifuge tubes with a well-type scintillation counter. All counts were corrected for background. S^{35} was counted in a gas-flow Geiger-Mueller chamber. Sample (1 ml) was pipetted into an aluminum planchet, dried, and counted. All counts were corrected for background and self-absorption.

The distribution of labeled iron in radioactive cells was checked by chemical fractionation. *F. ferrooxidans* cells were labeled with Fe^{59} by suspending the cells in dilute H_2SO_4 containing radioactive iron-59 for 48 hr at 25 C. The cells were centrifuged, the supernatant liquid was decanted, and the cell pellet was washed twice with dilute H_2SO_4. The washed pellet (0.2 g, wet weight) was chemically fractionated by use of the procedure of Roberts et al. (1955) as outlined in Fig. 1. Each fraction was adjusted to a 6-ml volume prior to counting in the well-type scintillation counter.

RESULTS

Figure 2 is a polarogram showing the oxygen wave in the salts medium without added iron and also showing the absence of the wave after the same solution was deaerated. Figure 3 shows the oxygen wave of a solution similar to that shown in Fig. 2, but with 2.0 ml of a washed *F. ferrooxidans* suspension added; this polarogram was recorded after an elapsed time of 15 min. The oxygen wave shown in Fig. 4 covers a period of 10 min after the addition of $FeSO_4$ (400 μg/ml in dilute H_2SO_4) to the salts solution plus cells. Each unit (1, 2, 3, and 4) represents an elapsed time of about 2.5 min.

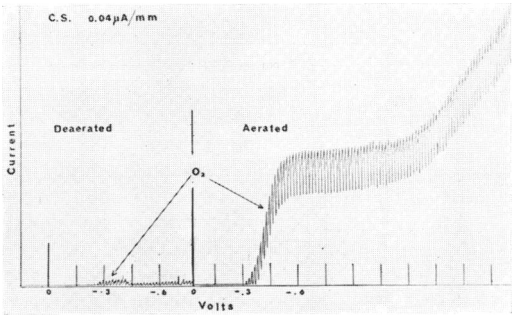


FIG. 2. *Polarogram showing the oxygen wave in deaerated and aerated culture salts solution without ferrous sulfate. C.S. = current sensitivity.*

The curve in Fig. 4 (unit 1) appears to be composed of two waves. The first has a half-wave potential of about −0.1 v, and this half-wave potential shifts toward a less negative potential with time (Fig. 4, units 2, 3, and 4). The second wave remains constant with respect to potential at about −0.4 v, which is the approximate potential shown for oxygen in Fig. 2. This second wave displays a decrease in diffusion current with respect to time, and we interpret this behavior as a conversion of oxygen to a complex which has a more positive reductive half-wave potential. The complex may go through chemical transitions, which would explain the atypical pattern of the waves illustrated in Fig. 4 and 5. These do not appear to be polarographic maxima. The diffusion current of this wave (Fig. 4) continued to diminish with time beyond that which is shown. Figure 5 shows a polarogram of the complete growth medium taken from a 43-hr culture, after the cells were removed by centrifugation and the medium was diluted 1:50 before being purged with N_2 for 5 min. The waves in the 0- to −0.9-v range (Fig. 5) were not removed by the nitrogen purge and were not due to oxygen or ferrous or ferric ions; the latter two ions have established half-wave potentials of about −1.8 and −2.5 v, respectively. The 0- to −0.9-v waves disappeared by 96 hr, as evidenced by assays of spent medium. The presence of the wave(s) in the 0- to −0.9-v region has been interpreted as due to a ferrous iron-oxygen complex in solution (or on the cell), although chemical identification has not been made. The importance of the waves as evidence of a complex entity involved in iron oxidation may be that this form has a lower oxidation-reduction potential than the $Fe^{++} \rightleftarrows Fe^{+++}$ system; the latter is more compatible with biological electron acceptors. This has been suggested by Blaylock and Nason (1963).

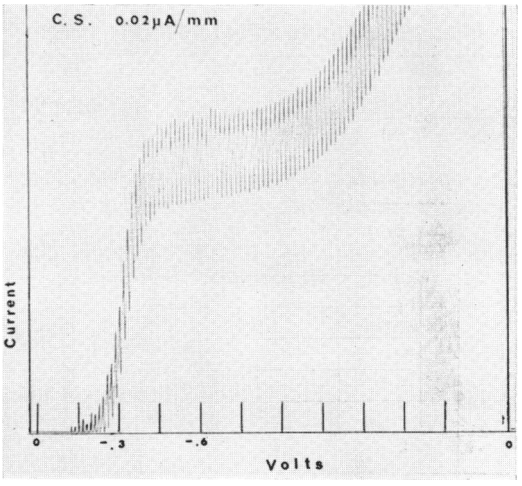


FIG. 3. *Polarogram showing the oxygen wave in culture salts solution containing Ferrobacillus ferrooxidans but without ferrous sulfate.*

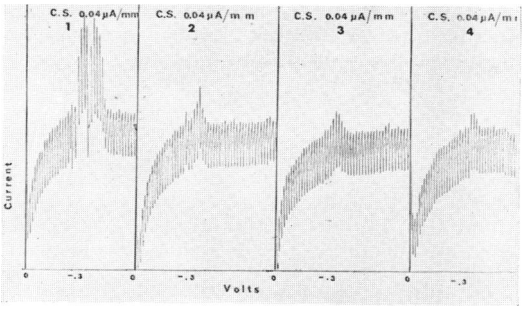


FIG. 4. *Polarograms showing a quantitative change in the diffusion current and a shift of the oxygen half-wave potential towards zero voltage over a total elapsed time of approximately 10 min in the culture medium containing cells and ferrous sulfate.*

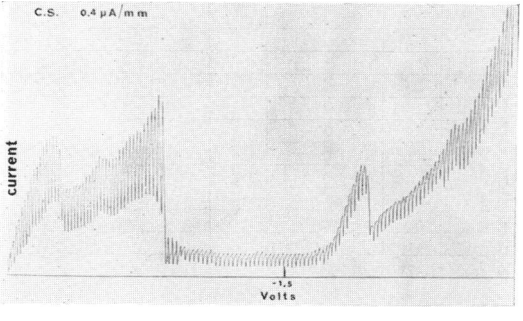


FIG. 5. *Polarogram of deaerated culture medium taken from an active culture of ferrobacilli after 43 hr.*

TABLE 1. *Water-free space (WFS) determinations by use of uptake of Fe^{59}*

Tube no.*	System	Test condition	Counts per ml per min	Calculated per cent of total activity with cells	Calculated per cent WFS†
1	Medium	0 C, 12 min	317,341	—	—
1	Cells	—	126,655	28.3	199.5
2	Medium	0 C, 12 min	301,831	—	—
2	Cells	—	125,190	29.2	207.5
3	Medium	0 C, 12 min	143,609	—	—
3	Cells	—	68,600	32.0	238.5
4	Medium	37 C, 9 min	296,300	—	—
4	Cells	—	644,530	68.3	1,085
5	Medium	37 C, 2 hr	240,193	—	—
5	Cells	—	87,680	26.4	183.5

* Tube 1 contained 1.0 ml of $Fe^{59}SO_4$ solution (50 μc/ml) plus 4.0 ml of cold $FeSO_4$ (1,000 ppm) and 1.0 ml of dilute H_2SO_4. All solutions were precooled to 0 C in an ice bath. The cell suspension was mixed with a Vortex mixer and centrifuged in the cold; the supernatant liquid was saved for counting. A total elapsed time of 12 min was recorded for the time it took to obtain the first supernatant fluid. The tube was rinsed with 6 ml of dilute H_2SO_4 (0 C) without disturbing the cell pellet, and the rinse was saved for counting. The cells were then suspended and washed with 6.0 ml of cold dilute H_2SO_4 and again centrifuged. This second supernatant solution was also saved for counting, as was the second quick rinse with 6.0 ml of dilute H_2SO_4. The counts from the supernatant fluids and rinses were combined to give one value for the medium. The cells were suspended in 6.0 ml of dilute H_2SO_4 for counting. Tube 2 was a duplicate of tube 1. Tube 3 contained one-half the amount of Fe^{59} and cold $FeSO_4$ used in tube 1, plus additional dilute H_2SO_4 to maintain a 6.0-ml volume. Tube 4 was identical to tube 1 except the contents were aerated for 9 min at 37 C with air expelled through a small capillary tube. The tubes were then handled in an identical manner as above. Iron is oxidized by *Ferrobacillus ferrooxidans* at 37 C, but the cells do not multiply (Silverman and Lundgren, 1959*b*). Tube 5 contained 9,000 ppm of cold $FeSO_4$ in H_2SO_4, and the tubes were aerated for 2 hr at 37 C. Otherwise the treatment was identical to tube 1.

† A 0.2-g amount of cells was dispersed in a 6.0-ml volume. Activity in 0.2 g was counted; therefore, cell counts per milliliter per minute were multiplied by 5 to give counts per milliliter per minute per gram of cells, assuming 1 g of cells was equal to 1 ml of cells.

Iron-59 and sulfur-35 uptake. Table 1 shows the calculated values for the water-free space of the cells as determined by the isotope method; it is apparent that these values are not valid, i.e., they exceed 100%. This means that iron was concentrated from solution by the nonmetabolizing cells and that the absorbed iron was held rather tightly. The per cent activity of labeled cells remained unchanged after exposure to various chemical solutions (Table 3). The labeled ion found in or on the cells exchanged readily with nonlabeled iron when present either alone or in combination with individual salts of the growth medium or with alkyl benzene sulfate.

Comparable experiments with $S^{35}O_4$ gave similar results where a concentration effect was evident (Table 2), resulting in nonvalid WFS values. This concentration effect was independent of temperature and the amount of isotope used. Exchange of adsorbed $S^{35}O_4$ with each of the test solutions shown in Table 3 was much greater than for iron, presumably because, in each instance, cold carrier sulfate in the form of

TABLE 2. *Water-free space (WFS) determinations by use of uptake of $S^{35}O_4$*

Tube no.*	System	Test condition	Counts per ml per min	Calculated per cent of activity/ml with cells	Calculated per cent WFS†
1	Medium	0 C, 8 min	87,416	45.9	1,698
1	Cells	—	74,236	—	—
2	Medium	0 C, 9 min	70,982	49.0	1,900
2	Cells	—	68,132	—	—
3	Medium	0 C, 8 min	33,220	46.8	1,760
3	Cells	—	29,288	—	—
4	Medium	0 C, 8 min	50,896	46.5	1,740
4	Cells	—	44,302	—	—
5	Medium	25 C, 15 min	79,260	46.5	1,730
5	Cells	—	68,204	—	—

* Tube 1 contained 0.2 g (wet weight) of washed cells in 1 ml of dilute H_2SO_4 held in a 10-ml glass centrifuge tube plus 0.5 ml of $H_2S^{35}O_4$ solution (100 μc/ml) and 2.5 ml of dilute cold H_2SO_4, giving a total volume of 4 ml. The tube was maintained at 0 C, and all solutions were previously chilled. The tube was mixed, centrifuged, and washed as described for the Fe^{59} experiment. Counts of supernatant liquids plus rinses were combined to give a single value in counts per milliliter per minute for the medium. Tube 2 was a duplicate of tube 1. Tube 3 was a duplicate of tube 1 except it contained one-half as much radioactivity. Tube 4 was a duplicate of tube 3. Tube 5 was a duplicate of tube 1 except the tube was held at 25 C for 7 min instead of at 0 C.

† A 0.2-g amount of cells was dispersed in a 4.0-ml volume, and only 1 ml (0.05 g) was counted; therefore, cell counts per milliliter per minute were multiplied by 20 to give counts per milliliter per minute per gram of cells, assuming 1.0 g of cells was equal to 1 ml of cells.

H_2SO_4 was present. All uptake experiments were repeated three times and found to be reproducible.

The cell residue fraction, the principal structural protein of the cell, which remained after chemical fractionation contained the highest percentage of the original activity, suggesting the involvement of these structures in concentrating the iron (Table 4). Even when resting cells were exposed to radioactivity for only 1 hr prior to chemical fractionation, roughly 50% of the activity was found in this fraction. A higher percentage of iron was extracted by cold trichloroacetic acid in the experiments with a short time of exposure, representing more loosely bound or pooled iron. As expected, the hot trichloroacetic acid fraction had some activity, and is probably related to iron chelation by components such as guanine. The latter observation has been repeated many times. Electron micrographs of the residual fraction revealed distorted cells, but some structural features of the cell were still evident (Fig. 6); the cell envelope was removed by the treatment, leaving a protoplasmic mass. For comparison, normal cells with the envelope of the cell intact are shown in Fig. 7.

TABLE 3. *Loss of Fe^{59} from resting cells of Ferrobacillus ferrooxidans in the presence of various chemical solutions*

Chemical solution*	Counts/min in supernatant fluid	Counts/min in cells	Activity remaining with cells
			%
H_2SO_4, dilute	256	3,584	93.0
H_2SO_4, dilute	768	15,872	95.0
$FeSO_4$, 3%	2,355	1,587	46.0
$FeSO_4$, 3%	9,113	7,296	44.0
$FeSO_4$, 3.2%	36,864	14,080	28.0
H_2SO_4, dilute	2,048	46,080	96.0
$(NH_4)_2SO_4$, 0.24%	1,024	50,588	98.5
$MgSO_4$, 0.08%	1,016	51,968	98.7
KH_2PO_4, 0.04%	108	45,321	99.9
ABS, 80 ppm†	1,050	46,592	98.3
$FeSO_4$, 2.7%	8,832	9,267	51.2
$FeSO_4$, 2.7%	10,368	10,780	51.1
$FeSO_4$, 2.7% + KH_2PO_4, 0.04%	11,622	11,904	50.6
$FeSO_4$, 2.7% + KH_2PO_4, 0.08%	12,108	10,240	46.0
$FeSO_4$, 2.7% + KH_2PO_4, 0.16%	13,209	9,164	41.2
$FeSO_4$, 2.7% + $(NH_4)_2 \cdot SO_4$, 0.24%	10,060	11,059	52.3
$FeSO_4$, 2.7% + $(NH_4)_2 \cdot SO_4$, 0.48%	10,006	11,002	52.1
$FeSO_4$, 2.7% + ABS, 80 ppm	9,728	11,007	53.0
$FeSO_4$, 2.7% + ABS, 400 ppm	9,754	11,724	54.5
$FeSO_4$, 2.7% + KCl, 0.04%	10,740	10,680	49.9

* All chemicals were dissolved in dilute H_2SO_4. Cells were exposed to the chemical solutions for 11 hr.

† ABS = alkyl benzene sulfonate.

TABLE 4. *Fractionation and distribution of Fe^{59} activity*

Chemical fractionation	Counts per ml per min	Per cent of total activity
Hot water, 5 min	768	2.7
Cold trichloroacetic acid, 30 min	512	1.8
Ethyl alcohol, 30 min	240	0.1
Ether-ethyl alcohol (1:1), 15 min	68	—
Hot trichloroacetic acid	3,372	12.0
Cell residual	22,296	82.0
Cold trichloroacetic acid, 5%, 5 C, 30 min	587	25.0*
Ethyl alcohol, 75%, 45 C, 15 min	99	4.0
Ether-ethyl alcohol (1:1), 45 C, 15 min	46	2.0
Hot trichloroacetic acid, 5%, boil 30 min	488	21.0
Cell residual	1,122	48.0

* Cells exposed to Fe^{59} for 1 hr prior to fractionation.

DISCUSSION

Dissimilatory iron oxidation by chemoautotrophs is related to several questions. How does the mechanism operate? How does the mechanism account for both the relatively low yield of energy per gram atom of iron (necessitating the oxidation of large amounts of iron for growth)? How is the ferric iron by-product handled so that little energy waste occurs? Allied to these questions are the important questions of utilization of energy and of the source of reducing power, presumably ferrous iron, to reduce carbon dioxide. These latter questions will not be considered here, but suggestions as to their mechanisms have been advanced (Blaylock and Nason, 1963; Aleem and Lees, 1963).

Enough experimental data are now available to construct a working model for a mechanism to explain ferrous iron oxidation. The model is offered as an aid to the designing of experiments to test assumptions, and will undoubtedly be subject to change as more information becomes

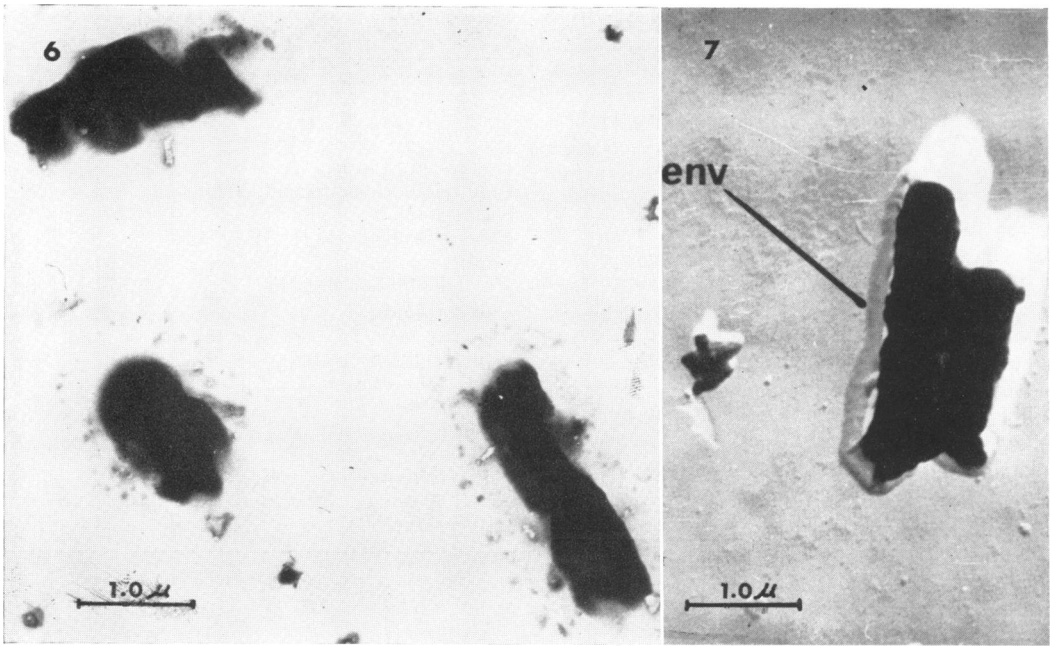


FIG. 6. *Electron micrograph of chemically fractionated Ferrobacillus ferrooxidans. Treated cells were placed on a carbon-coated grid, dried, and shadowed with germanium. 45,000 ×.*

FIG. 7. *Electron micrograph of Ferrobacillus ferrooxidans shadowed with germanium and showing the cell envelope (env). 45,000 ×.*

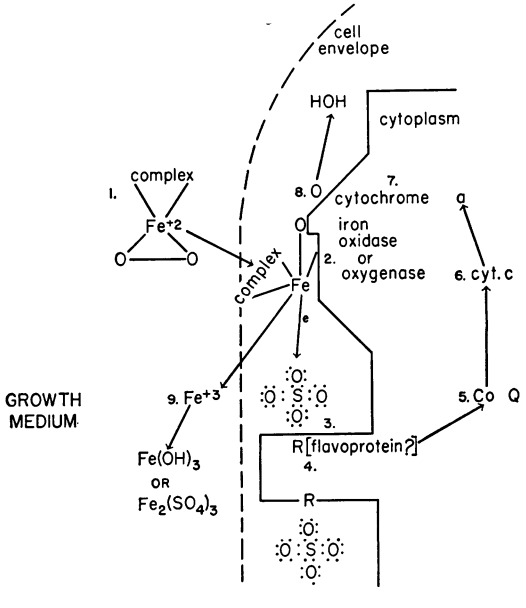


FIG. 8. *Schematic drawing of a model of the postulated mechanism of iron oxidation by Ferrobacillus ferrooxidans.*

available. Progress in this area of autotrophy has been slow, largely because of the difficulties in obtaining masses of cells for investigational purposes.

Figure 8 depicts how iron oxidation might occur in ferrobacilli. The model is supported by experimental work reported here as well as by data from other investigators.

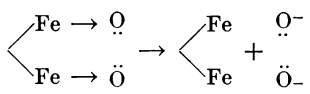
Ferrous ion forms a "complex" in the growth medium (1) or at the cell surface. Polarograms reveal two waves in the range of 0 to −0.9 v for spent culture medium of active cultures of ferrobacilli. These waves were not due to dissolved oxygen, ferrous or ferric ion, or other "pure" constituents of the growth medium, and it has been concluded that they are not due to reaction products in the medium and that they probably involve iron ions and oxygen.

Several authors have indicated that either ferrous or ferric ion will complex with oxygen as well as with phosphate and sulfate ions (Stumm and Lee, 1961; Matoo, 1960; Subrahmanya, 1956; King and Davidson, 1958). Complexes of iron and sulfate have been identified in acid solutions by Beaukenkemp and Harrington (1960). It is suggested that the iron reaches the

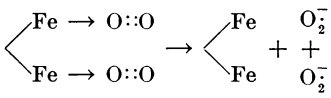
cell in the form of a complex containing an altered or "activated" form of oxygen which could be in the form of a peroxide or transient free radical (Kemula and Kublik, 1956; Stumm and Lee, 1961). The "complexed" ferrous ion would be oxygenated, but not oxidized because no electron transport has taken place. In a study of mechanisms involved in the polarographic reduction of oxygen, Hacobian (1953) reported that oxygen has reduced to H_2O_2 and that H_2O_2 had a half-wave potential of 0.1 v in his system. We suggest that the wave which forms in this voltage region in our system may be due to a complex which has peroxidic properties.

The "complex" is somehow bound to the cell wall or membrane, or both, at a site (2 in Fig. 8) where iron oxidase (or oxygenase) causes the release of an electron. Blaylock and Nason (1963) reported the separation from *F. ferrooxidans* of an iron oxidase which was functional in cell-free extracts. The iron oxidase in extracts has also been demonstrated manometrically in our laboratory (*unpublished data*). Sulfate is a requirement for iron oxidation (Lazaroff, 1963), and could be the initial transfer link between iron and the cell; in the present work, radioactive sulfate has been shown to be bound to the cell. The electron could be transferred to an electron-deficient sulfate group (3) which is bound to the cell surface at a site adjacent to the enzyme. The sulfate is shown bound to a group (R) on the cell (4) which influences the monovalent sulfate. The R group would be an integral part of the electron-transport system. Alternatively, the ferrous iron electron may transfer directly to a flavoprotein (4), as suggested by Blaylock and Nason (1963); the R group would then be a flavoprotein. The electron is transported in the cell through a typical electron-transport system. We recently reported a quinone of the coenzyme Q_6 type in *F. ferrooxidans* extracts; this quinone probably functions in the electron-transport system (Dugan and Lundgren, 1964*c*). Coenzyme Q functions in electron transport between flavoprotein and cytochrome *c*. Cytochrome *c* (6) was found in extracts of *F. ferrooxidans* (Vernon et al., 1960; Blaylock and Nason, 1963). Cytochrome *a* (7) has been suggested as the component which transfers the electron to oxygen (Vernon et al., 1960; Blaylock and Nason, 1963). The oxygen molecule (8), shown in a "complexed" form, would be split by the oxygenase enzyme (2), and one atom would act as the terminal electron acceptor and be converted to water; the second oxygen atom could react with oxidized substrate, that is, ferric iron, to form ferric oxide or hydroxide (9). If, however, an iron oxidase were the functional enzyme, then both atoms of the oxygen molecule presumably act as terminal electron acceptors, and the subsequent reaction of ferric ions to form ferric oxide or hydroxide would probably not involve an enzyme. The ferric ions would diffuse away from the cell after oxidation and would react with water and sulfate to form the hydroxides and sulfates (9).

This initial reaction (1) shown in this model appears to be consistent with the recent postulation of Handler, Rajagopalan, and Aleman (1964). They suggested that reduced oxygen is caused by an autoxidizable iron flavoprotein by the following type of mechanism:



The flavoprotein enzymes (aldehyde oxidase, dihydroorotic acid dehydrogenase, and rabbit liver aldehyde oxidase) have pairs of iron atoms so situated as to permit simultaneous binding of both atoms of a molecule of oxygen to the metal pair. Transfer of one electron from each iron atom to the oxygen molecule would result in peroxide formation. At higher oxygen tensions



configurations occur where transfer of a single electron would result in an oxygen-free radical (the superoxide anion) ($O_2^{\cdot-}$) which could then react with sulfite or cytochrome *c*. We suggest, in the case of iron oxidase or oxygenase, that a complex between oxygen and iron forms first and the complex is bound to active sites on the cell, and that oxidation of sulfite by oxygen radicals in the system of Handler et al. (1964) is compatible with our postulated involvement of a sulfate complex in electron transfer in our system. Different sites on the ferrobacilli may act to trigger the original complex formation in solution.

Results of WFS determinations indicate that both ferrous iron and sulfate were absorbed strongly by nonmetabolizing cells (0 C) in a matter of minutes. Further, when cells were labeled with iron and chemically fractionated, much iron remained attached to the residual protein structure. Little else can be inferred from results of such tests.

A study of fine structure of ferrobacilli has recently been undertaken in the hope that some of this information will aid in the interpretation of the mechanism of iron oxidation (Mahoney and Lundgren, *unpublished data*). The fine struc-

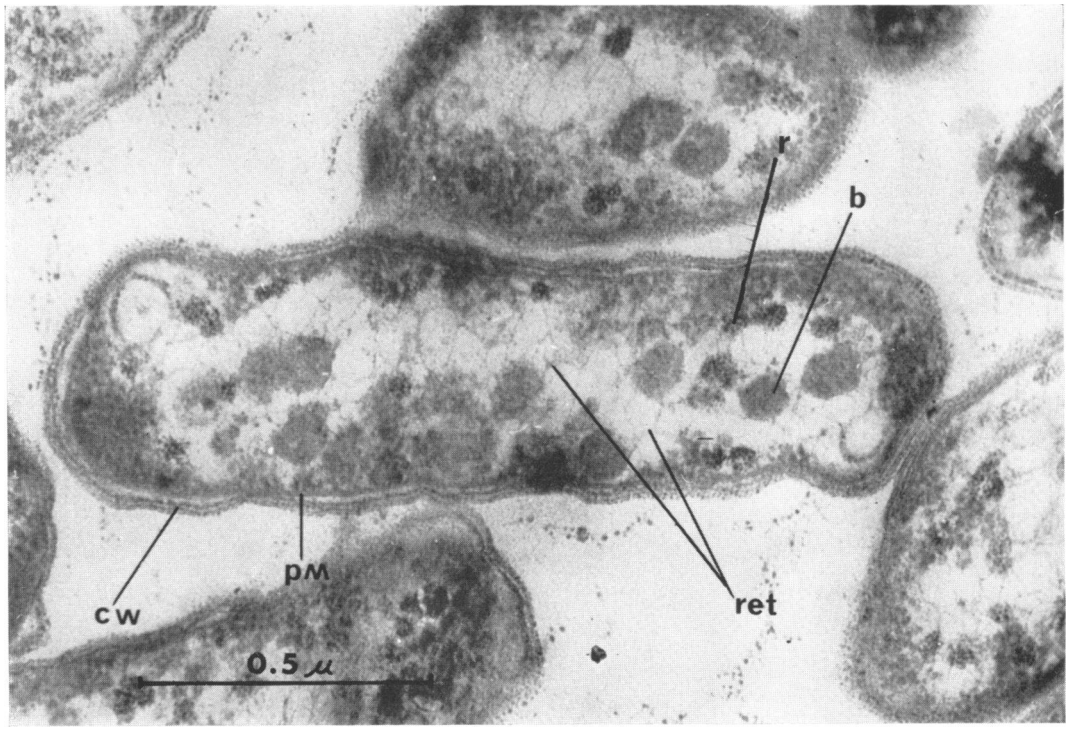


FIG. 9. *Electron micrograph of sectioned Ferrobacillus ferrooxidans. The specimen was fixed and handled following the procedure of Kellenberger, Ryter, and Séchaud (1958). Epon 812 was the embedding polymer, and sections were cut with glass knives and examined in an RCA EMU-2D electron microscope. CW, layered cell wall; pm, cytoplasmic membrane; b, dense membranous bodies believed to be mesosomes; r, ribosomal cluster; ret, irregular reticulum-like network in the transparent region is the nucleoplasm of the cell. 70,000 ×.*

ture of *F. ferrooxidans* (Fig. 9) shows a complex type of envelope structure (cell wall plus cytoplasmic membrane) somewhat typical of gram-negative organisms. The cell wall appears to be multilayered, and the cytoplasmic membrane is of the "unit" membrane type. Such an envelope assembly undoubtedly assists the organism in living in an acidic environment (optimal growth *p*H, 3.0), in addition to restricting the availability of organic substrates. The envelope probably functions in iron oxidation, but at present its exact role is unknown. We visualize (Fig. 10) a lattice of sulfate, phosphate, and iron ions several ions thick accumulating in the cell envelope and allowing electrons to migrate to receptor sites, in a manner analogous to that of a dry-cell battery where sulfate (or other ions) concentrated from the medium serve as the electrolyte. Organic compounds, if present, would tend to line up or become attached in a layer surrounding the envelope, and would block further transfer of sulfate and iron ions. This may explain the

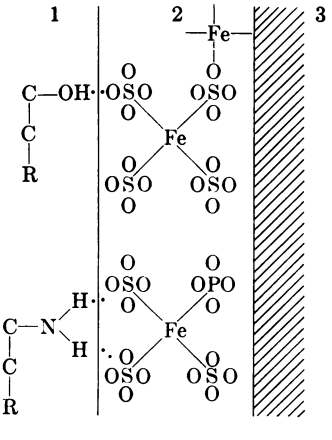


FIG. 10. *Simplified model showing possible role of organic compounds interfering with iron transfer. (1) Organic layer; (2) envelope layer; (3) cell interior.*

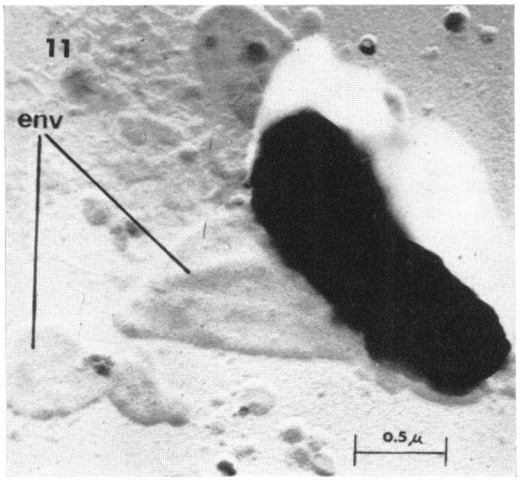


FIG. 11. *Electron micrograph of Ferrobacillus ferrooxiaans treated with sonic energy to remove the cell envelope (env). The envelope pieces are seen along with the remaining cell mass. 36,000 ×.*

organism's inability to grow on most organic compounds; this is the general case for autotrophs, which are inhibited by the presence of many compounds ordinarily considered nutrients. If the organic nutrients were able to penetrate to the active surface of the cell (cytoplasmic membrane), it is conceivable that they would be metabolized heterotrophically. This concept is consistent with the observations of Remsen and Lundgren (1963), who found that cells cultured in an iron sulfate medium are inhibited by most organic compounds, but could in time adapt to glucose as an energy source. Brief exposure of iron-grown cells to sonic energy for the purpose of removing envelope material (Fig. 11) left cells capable of growth on glucose but not on iron.

Our working model fits the available evidence, and we believe it will be helpful in devising future experiments directed towards the elucidation of the mechanism of iron oxidation. The concepts discussed in this paper, as developed from data originating in our laboratory as well as from the reports of others, lead us to conclude that autotrophic bacteria are special cases of heterotrophic bacteria, a consideration which has considerable bearing upon the evolutionary position of autotrophic organisms.

From the electron micrograph of *F. ferrooxidans*, it is apparent that fine structure in this autotroph is similar to that of heterotrophs. This subject will be treated in a separate publication.

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