JOURNAL OF BACTERIOLOGY, Apr. 1978, p. 310-317
0021-9193/78/0134-0310$02.00/0

Vol. 134, No. 1
*Printed in U.S.A.*

# Bacterial Leaching Patterns on Pyrite Crystal Surfaces

J. C. BENNETT* AND H. TRIBUTSCH
*Fritz-Haber-Institut der Max-Planck-Gesellschaft, Berlin 33, Germany*

Received for publication 22 December 1977

Selected pyrite crystals were placed as a bacterial energy source into stationary cultures of *Thiobacillus ferrooxidans*. Scanning electron microscope studies performed after a period of 2 years on these crystals revealed bacterial etching pits in characteristic patterns; they include pit arrangements in loose statistical disorder, in pairs, in clusters, and most remarkably in pearl-string-like chains. It has previously been confirmed that the chemical processes of bacterial leaching occur mainly in the region of contact between bacteria and the sulfide surface. The evidence presented in this experiment strongly suggests that the observed bacterial distributions are critically dependent on crystal structure and on deviations in the crystal order (fracture lines, dislocations) of the leachable substrate.

Because of the growing economic importance of ore leaching by chemoautotrophic bacteria of the species *Thiobacillus ferrooxidans*, there has been considerable research effort to determine the role played by this microorganism in the oxidation of various metal sulfides. The main nutritional requirements, environmental conditions, and other biochemical and physiological characteristics of the species have been reviewed previously (4).

The requirement of contact between bacteria and insoluble sulfides has been postulated to be a key factor in the microbiological leaching of sulfides. There are several facts that support this hypothesis. The lag period of the bacterial activity is shortened by the addition of surface-active agents (3). *T. ferrooxidans* culture solutions show appreciable concentration of surface-active agents produced by the bacterial activity (7). Interposing a semipermeable membrane between a bacterial suspension of the related species *T. thiooxidans* and sulfur has an inhibitory effect (2), and, in actively growing cultures of *T. ferrooxidans* on metal sulfides, the bacterial cells are mainly attached to the sulfide particles (5).

The erosion of sulfur crystal surfaces by *T. ferrooxidans* has been observed with carbon replica and transmission electron microscopy (6) and with scanning electron microscopy on colloidal sulfur particles (1).

Apparently, the first scanning microscope observations of *T. ferrooxidans* growing on metal sulfide crystals were published by one of us (H.T.) (9). Surfaces of inoculated galena (PbS) crystals showed characteristic pits, some with bacteria sitting in them. The observed characteristics made it possible to draw conclusions about the mechanism of bacterial leaching activity. Direct evidence of the required contact between the bacteria and the sulfide surfaces, of the possible involvement of a chemical carrier in the bacterial dissolution process, and of the influence of crystalline imperfections on the distribution of the bacterial corrosion pits could be given. However, the continuous precipitation of $PbSO_4$ on the PbS surfaces seriously interfered with the formation of clear corrosion pit patterns. To overcome this problem in long-term experiments, efforts have been concentrated on a more suitable substrate, pyrite ($FeS_2$).

In this study we want to present more information on this subject, based on scanning electron microscopic observations of $FeS_2$ crystals that were exposed to the action of *T. ferrooxidans* for long periods.

## MATERIALS AND METHODS

Natural crystals of $FeS_2$ were cleaved, and some pieces that were previously not directly in contact with air were immediately introduced into actively growing *T. ferrooxidans* cultures prepared according to a procedure described by Silverman and Lundgren (8). However, the $FeSO_4 \cdot 7H_2O$ solution was replaced by water, and the pH was adjusted to 2.5 with $H_2SO_4$. As an energy source, 0.5 g of synthetic $FeS_2$ (E. Merck AG, Darmstadt) was added. A 2-ml sample of an identically prepared, actively growing culture was used as inoculum. To avoid damage to the crystal surfaces, the culture flasks were not stirred but only kept at room temperature in a dark place. Volume changes due to evaporation were compensated for by the addition of distilled water. Crystal samples for the scanning electron microscope were carefully rinsed with distilled water, allowed to dry at room temperature, and glued to specimen stubs with conductive epoxy resin. Samples were coated with gold-palladium and examined with a scanning electron microscope at 5 kV with the sample platform tilted at 30 or 50°.

## RESULTS

Excluding bacteria, the only components of the culture medium that might have interacted with $FeS_2$ and dissolved it were $H^+$, $O_2$, and $Fe^{3+}$. During prolonged interaction with $FeS_2$, they produced etching patterns reflecting the cubic structure of the crystal. How can bacterial activity be expected to affect crystal-surface structures? Experimental evidence from sulfur and lead sulfide surfaces shows that bacterial leaching occurs mainly in the region of contact between the solid substrate and bacteria. If this mechanism also proceeds on $FeS_2$ surfaces, prolonged bacterial activity should be reflected by three main characteristic surface features. (i) Holes resembling the bacteria in form with sizes approximately equal to or slightly larger than the bacteria should be present; (ii) the distribution patterns of these corrosion holes should exhibit the influence of the bacterial reproduction behavior or colony formation, or both; (iii) there should be some influence of crystal orientation and of deviations from ideal crystal structure on the development of bacterial corrosion patterns.

Five selected $FeS_2$ crystal pieces were kept in *T. ferrooxidans* cultures for a period of 2 years. The study made with the scanning electron microscope showed effects of bacterial activity on all five crystals; they were clearly visible, especially in certain restricted areas, and their general appearance did not differ from one crystal to another. The most remarkable characteristic observed on the $FeS_2$ crystals was the presence of both bacteria and corrosion pits, with a correspondence between the distribution patterns of bacteria and the corrosion pits. The bacteria appeared as slightly distorted rods with rounded ends, 1 to 1.5 μm long and 0.5 μm in diameter. Isolated corrosion pits appeared rounded or elongated, presenting sizes slightly larger than bacteria and penetrating into the crystal matrix to varying depths. They did not resemble any known chemical etching structure (Fig. 1a and 2a), and since there were also bacteria in some of the pits, they were apparently a consequence of the bacterial dissolution of the crystal. Isolated bacterial corrosion pits were often found either to have their main axis oriented in a well-defined direction or to be statistically oriented (Fig. 1c and d).

The distribution of the corrosion pits themselves was not always found to be statistical. It was possible to observe different distributions such as pairs, pearl-string-like chains, or accumulations of pits in an otherwise uncorroded crystal surface.

The most striking distribution was the pearl-string-like arrangement (Fig. 1e, 2, and 3); it appeared on all the crystals, and more than 20 arrays of this type were found on the samples. The pits forming the chains were found to vary between quasi-circular and elongated shapes. The main axis of the bacterial corrosion pits was found to be parallel to the chain direction in some cases and perpendicular in others (Fig. 1f, 2a, and 2b).

Some interesting details were observed. Sometimes there were gaps in the hole sequence of the chains (Fig. 1e, 2, and 3), and sometimes holes merged into a channel-like structure (Fig. 1e and 2b). Different development stages from

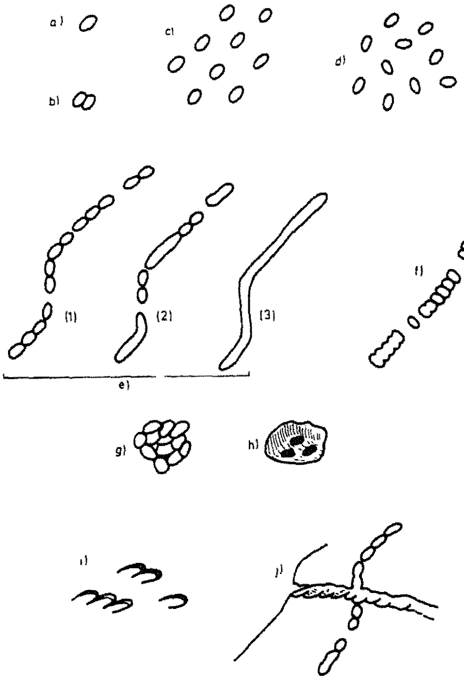

FIG. 1. *Schematic drawing of bacterial pits and most frequently observed distribution patterns. (a) Isolated bacterial corrosion pit; (b) pair of pits; (c) and (d) statistically distributed pits with parallel and irregular axial orientations, respectively; (e; 1, 2, and 3) different development stages of a pearl-string-like chain of pits into a channel-like corrosion structure; (f) pearl-string-like chain with pit axes perpendicular to the chain direction; (g) cluster of bacterial etching pits; (h) hole with several bacteria inside, probably developed from a cluster; (i) bacterial corrosion pits inclined with respect to the crystal surface; (j) bacterial corrosion chains intersecting each other at right angles, probably following tiny cracks in the cubic crystal.*

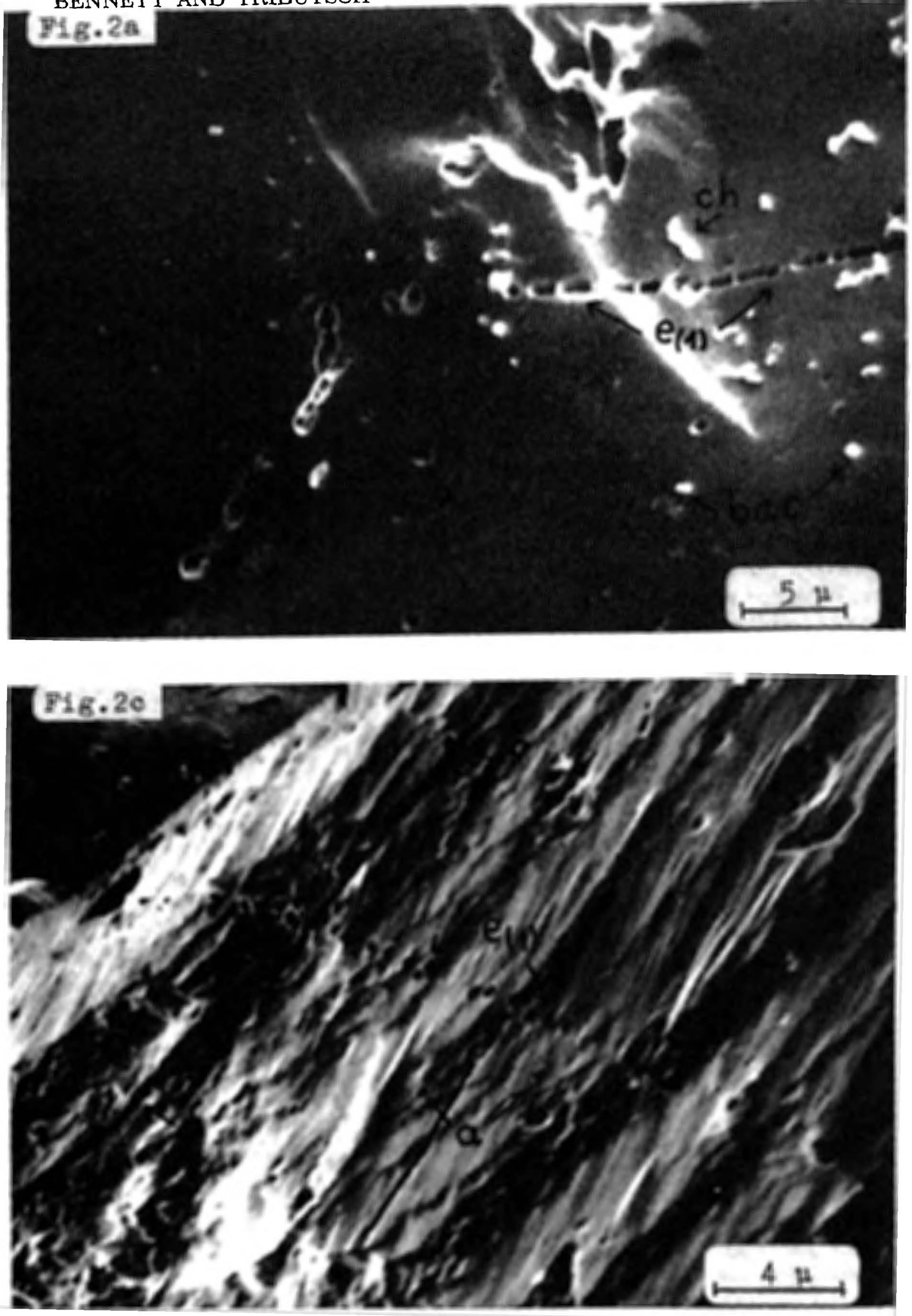

FIG. 2. *Scanning electron micrographs of inoculated* $FeS_2$ *crystals. Symbols: cl, cluster; bac, bacteria; ch, chain. Other symbols explained in legend to Fig. 1.*

the pearl-string-like chains to the channels were observed, and it was concluded that the channels were the result of an enhanced chemical corrosion along the chain (by $O_2$ and $Fe^{3+}$). Figure 2d shows a chain of bacterial corrosion pits exhibiting these different stages of development.

It is also possible that larger holes containing bacteria (Fig. 1h and 2d) were the result of enhanced corrosion in the region of bacterial clusters.

The distribution patterns of bacteria resemble closely those of corrosion pits. Bacteria are

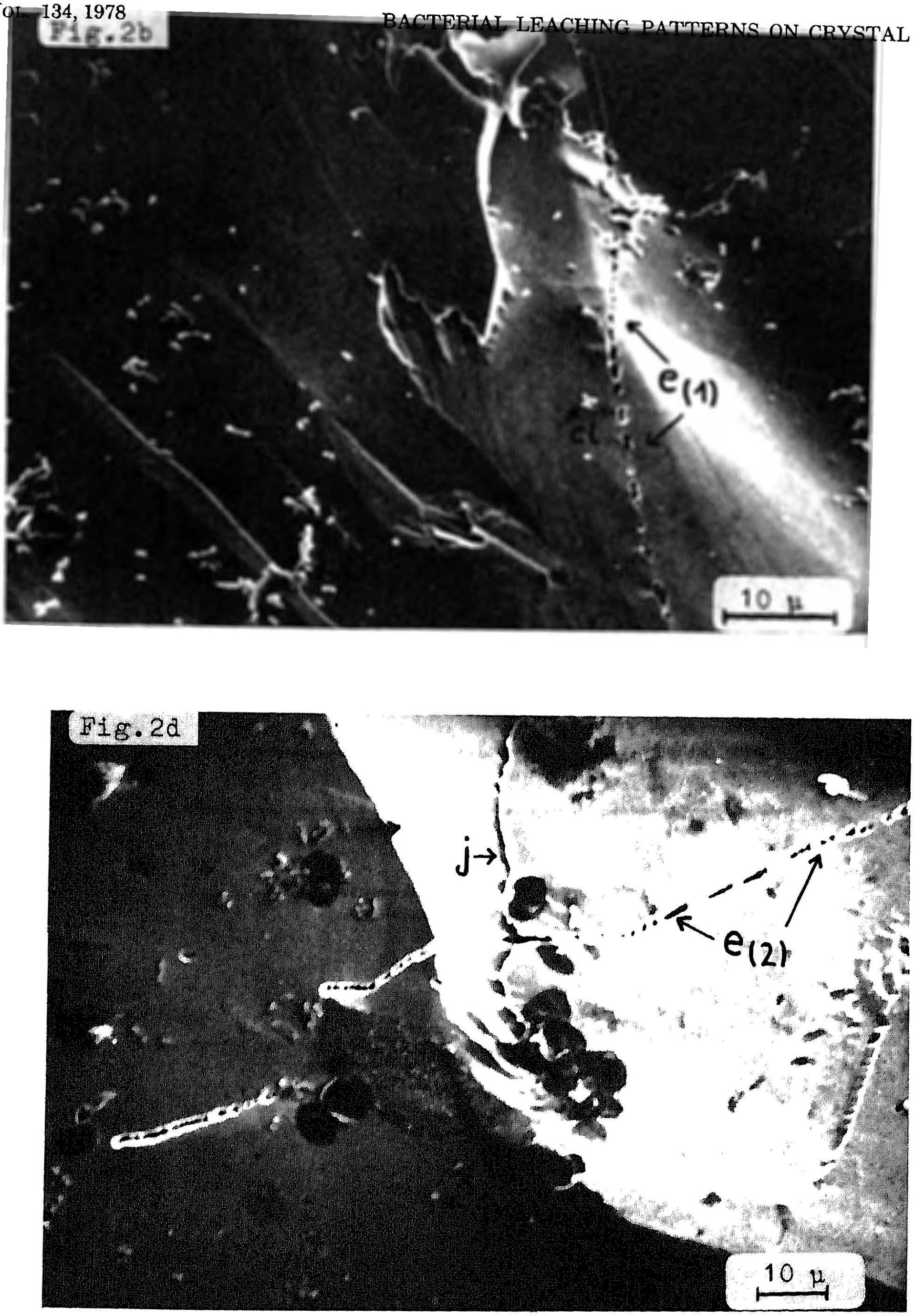

FIG. 2 b AND d

found either isolated, forming clusters, or arranged pearl strings (Fig. 3).

Some details in the distribution of corrosion pits suggested that they were a consequence not only of the bacterial reproduction behavior but also of the interaction of the bacteria with some structural properties of the solid substrate. The above-mentioned missing pits in the pearl-string-like chains indicated that the direction of a chain may be maintained without the bacteria being in intimate contact with each other. This observation meant that the information control-

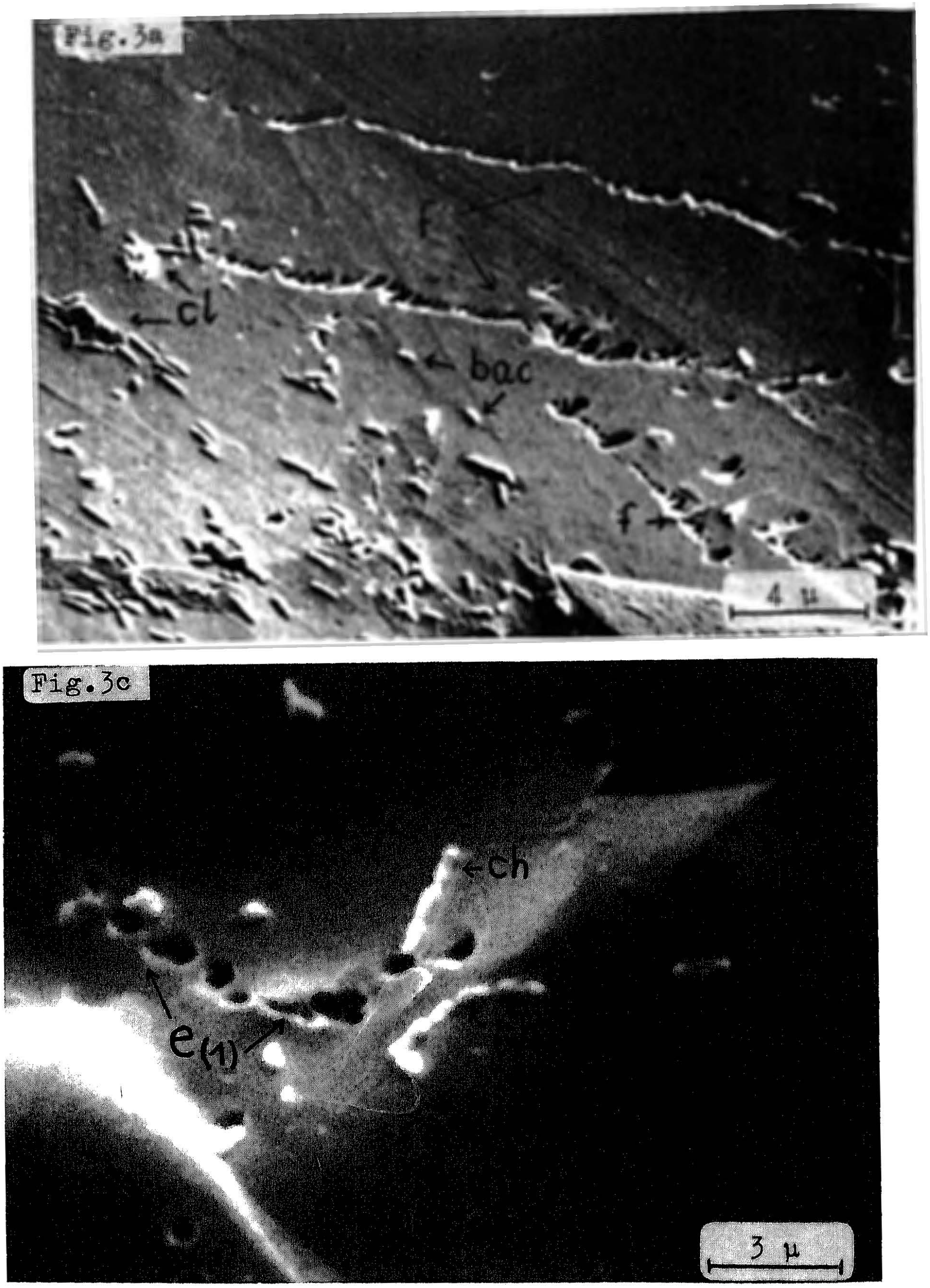

FIG. 3. *Scanning electron micrographs of inoculated* $FeS_2$ *crystals. Symbols as explained in legends to Fig. 1 and 2.*

ling the development of a chain of corrosion pits must be provided by the crystal itself in the form of a deviation from the ideal structure of the crystal, such as a dislocation, where the bacterial dissolution may proceed faster than in a crystallographically undisturbed area. Further evidence was the fact that chains were frequently found to intersect each other at angles of approximately 90° (Fig. 1j and 2d). In cubic crystals such as $FeS_2$ this may be the result of fracture lines that are perpendicular to each other. Another possible indication of an ordering

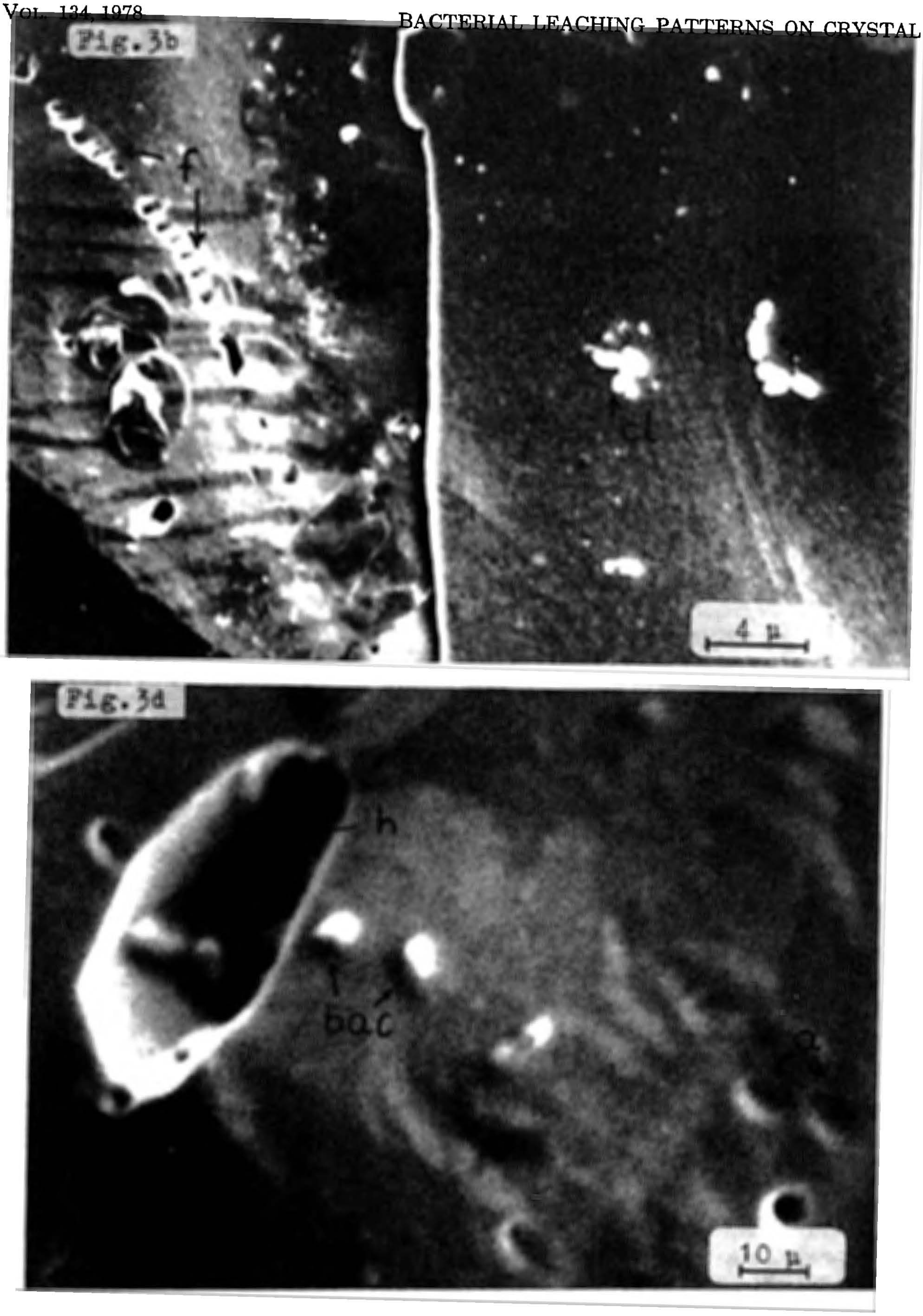

FIG. 3 b AND d

function of the substrate was that chains of corrosion pits continued in a straight line after passing over a step in the surface plane (Fig. 2d). The existence of corrosion pits with principal axes inclined toward the surface (Fig. 1i) also supported the conclusion that the structural properties of the substrate affect the distribution and leaching activities of bacteria on a sulfide surface.

## DISCUSSION

In spite of considerable interest in the mechanism of bacterial leaching of sulfide minerals, it has not yet been possible to resolve basic details of the physical-chemical processes involved. The main reason for the difficulties encountered in the chemistry of bacterial attack is that it is a heterogeneous reaction occurring at an interface between a semiconducting solid and an aqueous medium. The experimental methods that have been applied until now (leaching studies in shaker flasks and percolators, analysis of reaction products, control of environmental parameters) do not appear to be adequate for the characterization of the nature of reactions that involve semiconductor electrochemistry, interface phenomena, and heterogeneous reactions.

Scanning electron microscope studies in combination with electrochemical investigations of semiconducting metal sulfides are more promising tools to determine the reaction parameters involved in the bacterial attack on sulfides.

An attempt in this direction was undertaken, and it was actually possible, to some extent, to confirm the kind of mechanism *T. ferrooxidans* employs in the attack of metal sulfides. The demonstration of pit formation on the surface of a metal sulfide in contact with bacterial cells shows that bacteria generate a chemical agent to increase the dissolution rate of the metal sulfide in localized areas. The bacterial agent that is apparently involved in the enhanced sulfide dissolution may, in principle, act in three different ways: by oxidation of $Fe^{2+}$ to $Fe^{3+}$, by solubilizing molecular sulfur on the surfaces, or by a direct attack on crystallized metal sulfide surfaces. The experimental observations that $Fe^{3+}$ as well as $O_2$ efficiently oxidizes many sulfides producing molecular sulfur and that traces of bacterial attack remained in iron-free lead sulfide cultures (9) suggest that the thickness of a surface layer of molecular sulfur may, under most conditions, critically control the dissolution rate of sulfides. It is therefore possible that bacteria form the pits by feeding on the sulfur layer that may be rapidly formed after sulfide particles are placed in an acidified solution containing $O_2$. Additional arguments for such a conclusion may be derived from energetic and kinetic considerations. In a metal sulfide like $FeS_2$ only two or three electrons have to be extracted to liberate two atoms of sulfur. If the bacteria feeds mainly on $Fe^{2+}$, sulfur would accumulate until dissolution is stopped. There would be little energy available to support the bacteria further, especially since the oxidation of iron only liberates approximately 4.5% of the energy available from the equally probable oxidation of sulfur to sulfate. These considerations support the conclusion that the pits formed by bacterial activity result from the breakdown of the sulfur layer that limits the chemical dissolution of metal sulfides.

At the moment it cannot be decided whether the agent is capable of interacting directly with the metal sulfide as well. The formation of pits would be understandable if bacteria were merely feeding on molecular sulfur.

The existence of characteristic pit patterns on metal sulfide surfaces makes it possible to draw certain conclusions with respect to the reproduction and feeding behavior of *T. ferrooxidans* on the surface of its energy source. Scanning electron microscope studies have revealed bacteria individually distributed, organized in groups, or arranged in pearl-string-like chains. These patterns were equally evident in the distribution of bacterial corrosion pits. It is not possible to determine to what extent undetermined statistical factors are responsible for these characteristic distributions. However, there is unequivocal evidence that the substrate provides parameters that affect bacterial organization under certain circumstances. The pearl-string organization of bacteria colonies could be responsible for the formation of lines of pits. However, this cannot explain why bacterial pits are found arranged in a line that is not a continuous array of pits but is frequently interrupted by gaps where no bacterial attack is observed. Only the characteristic properties of a cubic crystal, providing tiny cracks along splitting planes that are perpendicular to each other, can account for the chains of bacterial dips occasionally found intersecting each other at right angles. The observation that the holes formed by bacterial activity are sometimes inclined with respect to the surface on which they are found also indicates an influence of the substrate. These observations suggest that bacteria can distinguish between surface areas that mechanically or chemically deviate from ideally crystallized sulfide substrates. This suggests that it is more favorable for the bacteria to obtain their energy from solid surfaces that are characterized either by weaker chemical bonding or by an increased surface area for chemical interaction along cracks or polycrystalline regions. It has to be concluded that *T. ferrooxidans* is able to distinguish between favorable and less favorable sites for energy extraction, selecting the site of attack according to the availability of nutrient. Such selectivity of bacterial attack is additional evidence for the involvement of a chemical carrier in the leaching mechanism. It is known that chemical etching proceeds faster in regions of distortion of crystal periodicity because of the availability of additional free bonds for chemical interactions and because of the increased surface area. It is difficult to imagine that bacteria with sizes of approximately 1 $\mu$m would be able to distinguish between crystalline sites of different atomic order using a mechanism involving a direct interaction between the bacterial cell and irregular submicroscopic crystalline imperfections.

This investigation shows that bacterial leaching of metal sulfides proceeds not only by means of an increase in iron oxidation, if iron is present in the mineral, but also by means of a localized chemical attack characterized by typical dissolution patterns.

To investigate the detailed mechanism of bacterial leaching activity further, it would be necessary to concentrate future investigations mainly on electrochemical properties of semiconducting metal sulfides as well as on the identification of the chemical compound that bacteria use to solubilize the energy-supplying molecular (or, possibly, crystal-bound) sulfur.

## ACKNOWLEDGMENTS

The work was supported by a grant from the Deutsche Forschungsgemeinschaft.

We thank H. Büttner for his skillful and patient work on the scanning electron microscope and C. Lober for valuable technical assistance. We gratefully acknowledge the constructive criticism and support of H. Gerischer.

## LITERATURE CITED

1. **Baldensperger, J., L. J. Guarraia, and W. J. Humphreys.** 1974. Scanning electron microscopy of thiobacilli grown on colloidal sulfur. Arch. Microbiol. **99**:323–329.
2. **Cook, T. M.** 1964. Growth of *Thiobacillus thiooxidans* in shaken culture. J. Bacteriol. **88**:620–623.
3. **Duncan, D. W.** 1967. Microbiological leaching of sulfide minerals. Aust. Min. **59**:21–25.
4. **Lundgren, D. G., J. R. Vestal, and F. R. Tabita.** 1972. The microbiology of mine drainage pollution, p. 69–88. *In* R. Mitchell (ed.), Water pollution microbiology. Wiley Interscience, New York.
5. **Razzell, W. E.** 1962. Bacterial leaching of metallic sulphides. Trans. Can. Inst. Min. Metall. **65**:136–137.
6. **Schaeffer, W. I., P. E. Holbert, and W. W. Umbreit.** 1963. Attachment of *Thiobacillus thiooxidans* to sulfur crystals. J. Bacteriol. **85**:137–140.
7. **Schaeffer, W. I., and W. W. Umbreit.** 1963. Phosphotidylinositol as a wetting agent in sulfur oxidation by *Thiobacillus thiooxidans*. J. Bacteriol. **85**:492–493.
8. **Silverman, M. P., and D. G. Lundgren.** 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. **77**:642–647.
9. **Tributsch, H.** 1976. The oxidative disintegration of sulfide crystals by *Thiobacillus ferrooxidans*. Naturwissenschaften **63**:88.