# AN ULTRASTRUCTURAL STUDY OF SELECTED CHEMOAUTOTROPHIC BACTERIA

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To My Father and Mother

## ABSTRACT

The ultrastructure of five Thiobacillus species was studied. The structure of their cell envelopes, the exception being autotrophic T. novellus, was similar to other Gram-negative bacteria. Autotrophic T. novellus either did not have a middle electron dense layer or had such a diffuse middle layer that it did not appear in thin section studies. The cell envelope of heterotrophically grown T. novellus was typical of normal heterotrophic Gram-negative bacteria. The cytokinetic properties of each species was studied and found unique in a number of cases.

The cytoplasm of actively growing thiobacilli contained ribosomal and nuclear material arranged in much the same manner throughout the species. T. denitrificans and T. ferrooxidans contained mesosome-like structures. The cytoplasm of heterotrophic T. novellus contained large electron transparent globules which were found to be polysaccharide by specific staining methods and by chemical analysis.

The ultrastructure of Ferrobacillus ferrooxidans was compared to that of Thiobacillus ferrooxidans and was found to be identical in all respects. The ultrastructures of autotrophic and heterotrophic Hydrogenomonas facilis

were compared and found identical except in the rate of deposition of poly-β-hydroxybutyric acid in the cytoplasm.

Ultrathin sections of the nitrifying bacteria Nitrosomonas europaea and Nitrobacter agilis were compared. The cell envelope of N. agilis was not typical of normal Gram-negative bacteria while that of N. europaea was typical. The cytoplasmic membrane of both organisms pinched off membranous organelles which aligned themselves along the cell envelope inside the cytoplasm or in the case of N. agilis, between the cytoplasmic membrane and the remainder of the cell wall. The cytoplasm of N. agilis also contained a fibrillar whorl of unknown function.

The effects of hydroxylamine and magnesium ion were studied with reference to their effect on ammonia oxidation and accompanied morphological change of N. europaea spheroplasts. The presence of hydroxylamine or pre-incubation with magnesium ion caused a regaining of ability to oxidize ammonia which had been lost during lysozyme treatment. When studied in thin section, the interior of inactive spheroplasts was homogeneous with relatively little disruption. When activated with hydroxylamine the spheroplasts contained membrane bound inclusions with regular, dense matrices. Spheroplasts pre-incubated with magnesium ion showed the same type of inclusion.

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# ABBREVIATIONS

| | |
|---|---|
| EDTA | ethylenediamine tetracetic acid |
| ATP | adenosine triphosphate |
| ADP | adenosine diphosphate |
| A.T.C.C. | American Type Culture Collection |
| C.M. | cytoplasmic membrane |
| $g_1$ and $g_2$ | globular cell envelope layers |
| L | outermost cell envelope layer |
| M | cell envelope layer adjacent to cytoplasmic membrane |
| R | aggregate of ribosomal material not necessarily a polysome |

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# INTRODUCTION

# INTRODUCTION

Since the introduction of the electron microscope as a tool for the study of bacterial structure (Marton, 1941), the vast majority of work has dealt with heterotrophic organisms. In the past ten years the autotrophic bacteria have come under close scrutiny because it was thought that their structure would in some way mirror the unique way they derive energy from inorganic substrates.

As a group, the thiobacilli were first to arouse the interest of cytologists but early work (Umbreit and Anderson, 1942) revealed little. With the aid of improved ultratomes and fixatives (Pease, 1964), the architecture of the thiobacilli has been studied with more success. To date, seven species have been subjected to ultrastructural study. T. thiooxidans (Mahoney and Edwards, 1966), T. novellus (Kocur, Martinec and Mazenec, 1968), Thiobacillus A2 (Taylor and Hoare, 1969), T. denitrificans, T. intermedius, T. neapolitanus and T. thioparus (Shively, Decker and Greenawalt, 1970) had cell envelopes similar to other Gram-negative bacteria. The cytoplasm showed some irregular structure characteristic in each species and varied in some cases depending on the phase of growth from which samples were taken.

Selected species of the thiobacilli which use sulfur or thiosulfate as energy sources may exhibit different structural arrangement. For example, T. thioparus oxidizes thiosulfate aerobically while T. denitrificans oxidizes it anaerobically and could therefore show structural difference because of physiological difference. T. thiooxidans uses elemental sulfur as an energy source and could therefore appear structurally different from the thiosulfate oxidizing species. The study of the detailed cell structure and cytokinetic properties of several representative thiobacilli was undertaken with these points in mind.

The iron-oxidizing bacterium, Ferrobacillus ferrooxidans, was analysed chemically and studied in thin section (Lundgren, Anderson, Remsen and Mahoney, 1964) but no dramatic difference from normal Gram-negative bacteria was seen in its structure. It did contain a larger amount of phospholipid than heterotrophic cells (Korczynski, Agate and Lundgren, 1967), but this was evident only through chemical analysis and specific staining with light microscopy.

The two species T. ferrooxidans and F. ferrooxidans have been accepted as separate species largely because the original isolate of F. ferrooxidans could not grow with sulfur as the sole energy source (Colmer, Temple and Hinkle, 1949). Since that time there have been arguments

put forth that the two species are one and the same (Unz and Lundgren, 1961; Margalith, Silver and Lundgren, 1966). It seemed that since F. ferrooxidans is undoubtedly closely related to the thiobacilli an ultrastructural comparison of the two species might lend support to one view or the other.

The hydrogen bacterium, Hydrogenomonas facilis, has not undergone detailed ultrastructural study, however, the deposition of poly-β-hydroxybutyric acid in the cytoplasm of this organism was illustrated in thin section and identified chemically (Schlegel, Gottschalk and Von Bartha, 1961). Both H. facilis and T. novellus are easily grown in either the autotrophic or heterotrophic mode. T. novellus was studied in both modes (Kocur et al, 1968) and was reportedly the same in either case. H. facilis has not been studied in this respect. The comparison of the two growth modes in these species seemed appropriate in this study.

The nitrifying bacteria, Nitosomonas europaea and Nitrobacter agilis, were studied in relation to Nitrosocystis (Murray and Watson, 1965). These organisms showed structural difference to the heterotrophic bacteria in that they contained membranous structures in their cytoplasm which were thought to be sites of ammonia or nitrite oxidation depending on the organism. Later work with gently ruptured cells (Tsien, Lambert and Laudelout, 1968; Tsien and Laudelout, 1968) supported this theory.

The use of ruptured spheroplasts of N. europaea has proved to be a fruitful method in studying the localization of cytochromes in this organism (Rees and Nason, 1965). If the oxidation of ammonia is associated with the membrane parts of this organism (Lees, 1960) as in the case of N. agilis (Tsien et al, 1968), a structural change in these membranes could alter their ability to carry out the oxidation. Intact spheroplasts which oxidize ammonia only after specific treatment (Suzuki and Kwok, 1969) seemed to offer an ideal system for studying the relationship between membrane structure and ammonia oxidizing activity.

HISTORICAL

# HISTORICAL

In 1924, deBroglie was able to assign a wave length to electron particles and so completed the physical theory which permitted the design of the electron microscope (Wischnitzer, 1962). In 1934, Ruska and von Borries designed the first version of the transmission electron microscope which indicated this type of microscopy would far exceed the resolution of light microscopy (Wischnitzer, 1962). Since that time the design of electron microscopes has improved until the latest machines approach the theoretical limit of resolution for electron particle wave length. The development of electromagnetic lenses has to this point prevented the resolution from actually reaching this point. Individual machine design largely controls the resolution attainable at a given time.

Biologists were quick to grasp the fact that electron microscopy would be a useful tool in the study of bacteria (Marton, 1941) and in 1941, the first paper on bacterial ultrastructure was published (Mudd, Polevitsky, Anderson and Chambers, 1941). Accepted methods of light microscopy were used in this study and as a result, little more information was gathered from it than from previous studies.

Histological techniques at that time were inadequate for use in electron microscopy. The possibility of sectioning the bacterial cell and viewing it in profile had tempted a number of scientists to prepare ultrathin sections as early as 1939 (von Ardenne, 1939). Wedge-shaped sections were prepared on an ordinary microtome in hope that the very thin part would yield sectioned cells. This first attempt at ultramicrotomy met with very little success. A theory was proposed that very high sectioning speeds were required to produce very thin sections. In 1943 an ultratome was devised which was actually a razor blade attached to a centrifuge rotor (Obrien and McKinley, 1943). Such an ultratome was actually marketed in 1948 with the suggestion that a buyer could use it both as an ultratome and as an ultracentrifuge (Pease, 1964). Pease discovered that ultrathin sectioning could be done at normal speed by using hard embedding material. With the introduction of methacrylate as embedding material in 1949 (Pease, 1964) the major problems of ultramicrotomy were solved. In 1950, the fractured edges of glass were used as knives and greatly improved section quality (Latta and Hartmann, 1950).

Fixatives used in electron microscopy did not undergo so dramatic an evolution as did the other major components of the science. Histologists had known for many decades that osmic acid was an excellent tissue

fixative and it was natural that it should be among the first fixatives used on electron microscope specimens. A method for the preservation of cytoplasmic constituents was devised by Kellenberger and Ryter. It employs osmic acid in combination with versene buffer and uranyl acetate. This method has become known as the RK method and is considered a classic technique (Kellenberger, Ryter and Séchaud, 1958). The introduction of aldehydes in combination with osmic acid represents the only improvement in tissue fixation since the first electron microscope specimens were prepared (Pease, 1964). The solving of the major problems mentioned above allowed electron microscopy to be used in relating of ultrastructure to biochemical function.

From 1950 to 1960 the study of bacterial ultrastructure was limited almost exclusively to heterotrophs. The possibility that autotrophic bacteria would have ultrastructural differences with respect to heterotrophs eventually led to the study of their structure. The autotrophic bacteria and their unusual ability to capture energy from inorganic substances had already undergone extensive biochemical study. The reader is directed to several excellent reviews in this area (Van Neil, 1953; Lees, 1954, 1960; Vishniac and Santer, 1957; Mulder, 1964; Peck, 1968 and Wallace and Nicholas, 1969). The present discussion will be limited to ultrastructural studies

while biochemical properties of the selected organisms will be mentioned primarily to differentiate species.

Thiobacillus thiooxidans

The existence of a sulfur oxidizing organism which acidified its growth medium was first reported in 1914 (Lockett, 1914). A bacterium which had all the characteristics of this organism was isolated from the soil by Waksman and Joffe in 1922 and was named Thiobacillus thiooxidans (Waksman and Joffe, 1922). The organism prefers a pH range between 1 and 4 but it grows well at 0 pH and has been known to produce a negative pH in its environment (Harmsen, 1938). Optimal growth occurs in a temperature range between 20 and 25°. It is different from the other thiobacilli because of its ability to oxidize elemental sulfur much faster than any of the other species. The organism can be found in a number of habitats such as soil from which the original isolate was taken (Waksman et al, 1922), fresh water mud (Lockett, 1914) or from marine areas (Tilton, Stewart and Jones, 1967; Adair and Gunderson, 1969). The added requirement of a 2 to 15% sodium chloride concentration in the growth medium of marine isolates was the only significant difference between the isolates.

Thiobacillus thioparus

Thiobacillus thioparus was originally isolated by

Nathansohn in 1902 (Nathansohn, 1902) but was formally named two years later by Beijerinck (Beijerinck, 1904). It is an aerobic bacterium which grows well with thiosulfate as the sole energy source and with ammonium salts as the nitrogen source. As it grows, molecular sulfur is deposited in the medium. Optimal growth occurs at a pH near 7 and at a temperature of 30°. The organism can be isolated from fresh water, soil and marine habitats (Vishniac and Santer, 1957). It is actively motile when isolated from any source. Large amounts of cytochrome c are present in the organism shown by an intense red color seen in concentrated masses of cells. T. thioparus is the type species of the genus Thiobacillus largely because it was isolated first and because it embodies the characteristics of thiobacilli as a whole. Thiobacillus thiocyanoxidans, isolated in 1954 (Happold, Johnstone, Rogers and Youatt, 1954) and Thiobacillus neapolitanus were later assigned to the species T. thioparus because they were able to oxidize the same sulfur compounds (DeKruyff, Van der Walt and Schwartz, 1957; Happold, Jones and Pratt, 1958; Johnstone, Townshend and White, 1961). In 1965 T. thioparus and T. neapolitanus were once again evaluated by numerical taxonomic methods and were reported as two distinct species (Hutchinson, Johnstone and White, 1965).

Thiobacillus denitrificans

This organism was first isolated by Beijerinck in 1904 and differed from T. thioparus only in its ability to grow anaerobically using nitrate as a terminal electron acceptor (Beijerinck, 1904). It also grows well under aerobic conditions but temporarily loses its ability to reduce nitrate if kept in an aerobic state for any length of time (Vishniac et al, 1957). If this be the case, the organism should then become identical to T. thioparus. It requires ammonium salts for growth (Baalsrud and Baalsrud, 1954) as do all thiobacilli save Thiobacillus concretivorus (Parker, 1945). T. denitrificans grows optimally near neutral pH at a temperature of 30° (Vishniac et al, 1957). This species can be isolated from soil (Starkey, 1935), marine mud (Vishniac et al, 1957) and sewage (Hutchinson et al, 1965) with relative ease.

Thiobacillus ferrooxidans

Although the growth of bacteria with ferrous iron as the sole energy source was reported as early as 1888 (Winogradsky, 1888) the first pure culture isolate of an iron-oxidizer was reported in 1949 (Colmer et al, 1949). The organism was isolated from the acid drainage of a bituminous coal mine and was named Thiobacillus ferrooxidans because of its close relation to T. thiooxidans when viewed by light microscope. It was

dissimilar to T. thiooxidans in that it could not oxidize elemental sulfur rapidly. It differed from the other thiobacilli because it had the ability to use ferrous ions as electron donors. Allowing the organism to grow through several subcultures with ferrous ion as the sole energy source caused the organism to lose its ability to oxidize thiosulfate. Continued growth on thiosulfate caused no loss of ability to oxidize ferrous salts (Vishniac et al, 1957). The organism grew best in a pH between 3-4 and in a temperature range from 20-25°.

Thiobacillus novellus

Thiobacillus novellus was isolated by Starkey (1935) in 1935. The first isolate came from the soil and grew very well on media meant to support the growth of T. thioparus. It differed from T. thioparus in that it grew abundantly on organic substrate and was non-motile. Continued subculturing of this species on heterotrophic media causes it to become very sluggish in its ability to oxidize thiosulfate (Charles and Suzuki, 1965). The organism grows best in a pH range from neutrality to 8.5 and at a temperature of 30°. All isolates come from soil (Starkey, 1935) or marine habitats (Adair and Gunderson, 1968). In the case of marine isolates the organism is an obligate halophile.

Ferrobacillus ferrooxidans

Leathen et al (Leathen and Braley, 1954), in attempting to find the reason for sulfate and ferric-precipitate contamination of the Ohio river, isolated a bacterium which was unable to oxidize thiosulfate or sulfur but was able to grow very well by oxidizing ferrous ion to ferric ion. The organism was named Ferrobacillus ferrooxidans. Examination under a light microscope showed the organism was morphologically identical to T. ferrooxidans. Since that time a number of reports have suggested the two organisms are identical. For example, F. ferrooxidans has been grown with sulfur as the sole energy source (Unz et al, 1961; Margalith, et al, 1966), contrary to the reports on the original isolate. Lundgren et al (Lundgren, Anderson, Remsen and Mahoney, 1964) also showed by the analysis of dry cells to constituent amino acids that the two organisms were composed of an identical array of amino acids on a qualitative basis.

F. ferrooxidans grows at an optimum rate in a pH range from 3-4 and in a temperature range from 20-25°.

Hydrogenomonas facilis

Hydrogen oxidation in the soil was detected as early as 1839 (deSaussure, 1839) but the organisms that caused it were not isolated until 1906 (Kaserer, 1906; Niklewski, 1906). The first isolate was named Bacillus

pantotropha, and was later renamed Hydrogenomonas pantotropha. The hydrogenomonads found by both Kaserer and Niklewski were sensitive to a high oxygen content in the atmosphere. Several species can tolerate no more than 10% v/v oxygen (Repaske, 1966). Also, if the organisms were grown heterotrophically their ability to return to the autotrophic mode of growth was greatly reduced or absent. In 1952, Schatz et al (Schatz and Bovel, 1952) isolated a Hydrogenomonas species which would retain its autotrophic capacity even after several subcultures on heterotrophic medium. They named it Hydrogenomonas facilis because of this unique trait. The organism which was isolated from the soil grew best at a temperature near 30° and at a pH between 6.4 and 6.8. All recorded isolates have come from soil habitats.

There has been a question raised as to the validity of the Hydrogenomonas genus. Davis et al (Davis, Doudoroff, Stanier and Mandel, 1969) suggested that most of the hydrogen bacteria belong to the genus Pseudomonas since they are motile by polar flagella and are Gram-negative rods. Davis and his colleagues felt that hydrogen gas is not far removed from organic compounds and does not fulfill the requirements for autotrophism. In this case motile hydrogenomonads are simply considered pseudomonads with a unique substrate. The non-motile members would be placed in the genus Alkaligenes.

Nitrobacter agilis

Winogradsky in 1891, isolated a non-motile organism from the soil which oxidized nitrite to nitrate and named it Nitrobacter winogradsky (Winogradsky, 1891). In 1931, Nelson isolated a motile species with the same characteristics and named it Nitrobacter agile (Nelson, 1930). Both isolates were extremely sensitive to the presence of organic compounds in their growth medium, but grew very well in conjunction with several heterotrophic contaminants. Lees and Gould when reisolating the N. agilis in 1959, found it extremely difficult to separate Nitrobacter from its heterotrophic contaminants. They were eventually able to obtain pure cultures by using a complicated method of dilutions in combination with antibiotics. They found that the organism grew optimally in a temperature range of 30-36° at a pH of 8.5. Nitrobacter has been isolated from soil (Gould, 1959) and from marine habitats (Watson and Remsen, 1969).

Nitrosomonas europaea

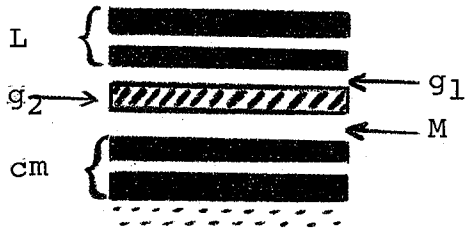
Winogradsky in 1891, was first able to isolate colonies of organisms which would oxidize ammonium ion to nitrite (Winogradsky, 1891). It had been known for approximately fifteen years previous to 1891 that biological organisms were responsible for the oxidation of ammonia in soil, but the extreme inhibitory effect of any

organic compound to the growth of the nitrifiers prevented their isolation. Winogradsky eventually isolated the organism responsible for the oxidation of ammonia to nitrite and named it Nitrosomonas. It is highly unlikely that Winogradsky's culture was a pure isolate. It is possible that Nitrosomonas has never been obtained in pure culture. Gould and Lees attempted a large number of cited methods as well as improvisations and were unable to isolate a pure culture of this organism (Gould, 1959). The organism grows best between 30 and 36° at a pH of 8.4. Nitrosomonas is motile with a single polar flagellum (Jensen, 1950).

## The Study of Bacterial Ultrastructure

Several characteristics of bacterial cells have aroused more than a passing interest in their structure. Firstly, they exist in a remarkably wide range of environments including extremes of temperature, salinity and acidity. Secondly, they maintain an integrity of structure as plant cells do but the vast majority if not all contain no cellulose in their cell walls. Thirdly, they are about the same size as a number of organelles which exist inside the plant and animal cells e.g. mitochondria and chloroplasts yet in most cases are free living with the ability to replicate and adjust to ecological conditions.

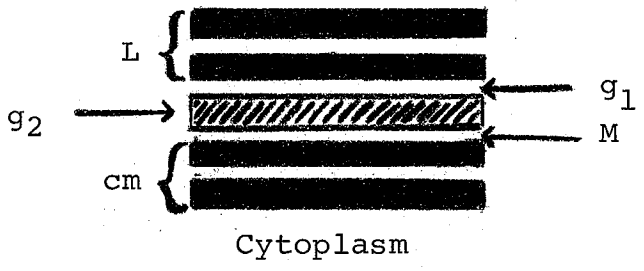
Important contributions to the study of the architecture of the bacterial cells have come from the study of heterotrophic organisms such as Escherichia coli (Lautrop, Reyn and Birch-Anderson, 1964; DePetris 1965, 1967; Frank and Dekegel, 1965; Murray, Steed and Elson, 1965; Pate and Ordal, 1965) Bacillus species (Chapman and Hillier, 1953; Glauert, Brieger and Allen, 1961; Godson, Hunter and Butler, 1961; Iterson, 1961) and a large number of others too numerous to mention. For a complete discussion of this area of ultrastructural research the reader is directed to an excellent symposium by Fuhs et al (Fuhs, Iterson, Cole and Shockman, 1965). The present discussion will deal for the most part with the study of autotrophic organisms. The system of labelling developed by DePetris (1967) in his comprehensive study of the cell wall of E. coli has been adopted for use throughout this study. It is demonstrated diagramatically below.



Umbreit and Anderson in 1942 published the first electron microscopical study of an autotrophic bacterium (Umbreit et al, 1942). They studied T. thiooxidans in a whole cell preparation. The cells were unstained and studied in one of the first RCA electron microscopes. They were able to observe that the cell contained a number of granules and vacuoles and had a cell wall distinct from the cytoplasm. The maximum magnification of the pictures was 19,000 x and yielded very little more than studies with the light microscope.

For the next sixteen years no work was published on the ultrastructure of autotrophs. In 1958 Fauré-Fremiet and Roullier studied the membrane structure in Thiovulum majus (Fauré-Fremier and Roullier, 1958). The organism was studied in thin section and was found to contain an elaboration of the cytoplasmic membrane which formed a system not unlike the endoplasmic reticulum of animal cells. It also contained a striated organelle in the cytoplasm which was unknown with respect to function, but was suspected of having some involvement in the peculiar motility of the organism. This organelle divided at the same time as the host cell with daughter organelles going to each of the daughter cells (Murray and Watson, 1963). The thiobacilli, between the years 1942 and 1966, were not studied with respect to ultrastructure. In 1966, Mahoney and Edwards re-examined T. thiooxidans in ultrathin section

(Mahoney and Edwards, 1966). They found that its cell envelope was identical to that described by DePetris (DePetris, 1967) in E. coli which was considered a typical Gram-negative species. The entire envelope was about 200 Å in thickness measured over a large number of micrographs. In profile it appeared as in the following diagram.




There were a large number of wrinkles in the cell envelope probably due to fixation and embedding procedures, however it could be seen that the cytoplasmic membrane was a smooth structure which invaginated into the cytoplasm on rare occasion. No organelles of a membranous nature could be seen.

The cytoplasm of the cells, especially distinct since the organism was stained by the Kellenberger and Ryter method (Kellenberger et al, 1958), showed a profusion of ribosomes, cytoplasmic fibrils and nuclear material. Also evident were round granules of unknown function and

origin. The organism's cytokinetic behaviour was typical of other Gram-negative bacteria such as E. coli (Conti and Gettner, 1962). No plate or septum appeared to precede the constrictive furrow.

T. novellus, an exception with respect to most thiobacilli in that it is a facultative organism, was studied by Kocur et al (Kocur, Martinec and Mazenec, 1968). The cell envelope was reported to be the same as that of the other Gram-negative organisms. A thick viscous capsule was easily demonstrated when the organism was grown on facultative medium. The cytoplasm contained a large vacuole which appeared to be enclosed in a triplet membrane. The content of the vacuole was not readily discernible. The cytoplasm also contained an electron-dense inclusion which was assumed to be made up of poly-metaphosphate.

In 1970, Shively and his colleagues (Shively, Decker and Greenawalt, 1970) prepared a comprehensive study of the ultrastructure of the thiobacilli. They found that the cell envelopes of five species were obviously like those of other Gram-negative organisms, while the middle layer of T. novellus was diffuse or missing. The middle layer of T. thioparus was extremely delicate relative to most of the species. A peculiar rippling or undulating contour was present in the majority of the species which the authors speculated was actually present in the living

cells.

Cultures of T. thioparus collected in maximal stationary phase contained a large number of polyhedral inclusions and on occasion a lamellar body which extended well into the cytoplasm. Actively dividing cells of this species had a more typical cytoplasmic structure showing typical ribosomal and nuclear structure with very few inclusions. Polyhedral inclusions were also found in T. neapolitans, T. intermedius and T. thiooxidans. T. denitrificans was the only species which contained mesosome-like structures. These were always attached to the cell envelope. Very electron dense granules, probably polymetaphosphate, were present in sections of T. denitrificans and T. novellus. The most striking inclusion was found in T. intermedius. It appeared to be crystalline in nature and ran transversely across the cell from wall to wall. The rare occurence of this inclusion prevented any speculation about its cause or nature.

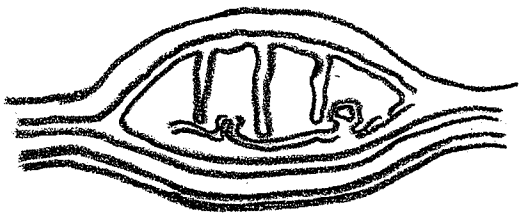
The iron-oxidizing bacterium F. ferrooxidans which is closely related to the thiobacilli, was first examined in thin section by Lundgren et al (Lundgren et al, 1964). They found that it had a cell envelope like that of typical Gram-negative bacteria. In the cytoplasm, beside the usual ribosomal particles, were aggregates of electron dense particles which were interpreted as polyribosomal clusters. Also present were organelles not unlike the

mesosomal inclusions of Gram-positive bacteria. The same organism was studied by Dugan and Lundgren (Dugan and Lundgren, 1965) with identical descriptions of findings.

Remsen and Lundgren (1966) using freeze etching technique were able to study the cell envelope of F. ferrooxidans with impressive results. The displacement of individual layers which occurred spontaneously during the production of replicas of frozen cells enabled the authors to describe in detail the structure of the cell envelope. The evidence concurred with previous findings, in addition however, fibrilar attachment of the outer cell wall became evident indicating the advantage of freeze etching techniques.

Hydrogenomonas was first studied in thin section in 1961 (Schlegel et al, 1961). The study was limited to the identification of large electron transparent inclusions which virtually filled the entire cell when the organism was allowed to grow to late maximal stationary phase. Chemical analysis showed that the inclusions were made up of poly-β-hydroxybutyrate. Pootjes et al, during the study of H. facilis by thin section, discovered the organism was susceptible to attack by a bacteriophage from soil (Pootjes, 1964; Pootjes, Mayhew and Korant, 1966). The phage was found when the organism was growing under heterotrophic conditions. This is the only autotroph to date which is known to be a host for phage particles.

Watson isolated a nitrifying organism from the ocean in 1960 and called it Nitrosocystis oceanus (Watson, 1962). In thin sections of this organism a number of irregular structures were observed (Murray and Watson, 1963). An organelle which lay between the cytoplasmic membrane and the cell wall of the bacterium was evident. It contained a number of distinct bars of material as diagrammed below:



Cytoplasm

It was apparent each cell had a number of these over its surface, however the function of the organelle was not found.

The same organism displayed a remarkable organelle in its cytoplasm (Murray and Watson, 1965). It consisted of a stack of highly oriented triplet membranes. They apparently originated from the cytoplasmic membrane and were oriented along the longitudinal axis of the cell. They were so closely stacked that obvious components of the cytoplasm were excluded from the structure. On the basis of Lees' (Lees, 1962) observation that energy-yielding

activity is often associated with membranes, Murray suggested that the membrane structure in Nitrosocystis was associated with ammonia oxidation.

Nitrobacter which was studied by Murray and Watson in conjunction with Nitrosocystis showed some exception to the structure of Gram-negative organisms. The cell wall was not obviously a triplet structure. The inner layer was electron dense and absorbed uranyl acetate to a large extent. An electron transparent layer and a cytoplasmic membrane which stained unusually dark in its innermost layer were also observed. The cytoplasmic membrane also invaginated into the cytoplasm and apparently pinched off into layer upon layer of membranous inclusions. These inclusions remained in close proximity to the cytoplasmic membrane and stacked so closely that constituents of the cytoplasm such as ribosomes were excluded from between them. They produced a bulge at one end of the cell which gave it the overall appearance of a club. The nuclear material, though quite electron transparent contained a large amount of finely whorled fibrils. An inclusion of moderate density which was called a chondroid was also observed in the cytoplasm.

Tsien and his colleagues (Tsien, Lambert and Laudelout, 1968) were able to gently rupture the Nitrobacter cell with a French press and in this way observe the membranous inclusions Murray and Watson had

described. The interior membranes when stained with phosphotungstate showed a regular array of structures which were concluded to be the site of nitrite oxidation in this organism. These findings agreed with the speculation of Murray (Murray and Watson, 1965) that the membranous organelles were in some way associated with the energy-yielding reactions of the cell.

Nitrosomonas, also studied by Murray and Watson (1965) displayed a cell envelope like that of the Gram-negative organisms. Membranes were also present in the cytoplasm. These membranes were not nearly as profuse as in Nitrosocystis and were more loosely spaced. They were also arranged more concentrically about the cell wall not more than one or two triplets deep. The similarity of Nitrosomonas cell walls to that of other Gram-negative organisms was also found through chemical analysis (Hofman, 1953; Hofman and Lees, 1953; Lewis, 1964).

The cytoplasm of Nitrosomonas contained inclusions which were presumed to be nutrient reserve. The configuration of the nuclear material was not unlike that of the other nitrifiers. It was not possible to detect ribosomal particles which were probably present.

## Ammonia Oxidation by Nitrosomonas

The ammonia oxidizing system of Nitrosomonas has been the object of research for some time (Lees, 1960).

The organism oxidized ammonia to nitrite with some facility in whole cell preparations (Gould, 1959) but in cell free preparations (Delwiche, Burge and Malavolta, 1961) the oxidation of ammonia did not occur. Hydroxylamine was oxidized however, with about 10% of it going to the production of nitrite (Rees, 1968) with concomitant production of phosphorylated compounds such as ATP and ADP (Burge, Malavolta and Delwiche, 1963).

The unusual capacity of nitrifiers to remain viable after long periods of storage was observed by Meikeljohn in 1954. It was noted that under storage conditions their ability to oxidize hydroxylamine persisted longer than their ability to oxidize ammonia (Lees, 1960). It was thought that the systems required for the oxidation of these compounds are on or near the cell surface with some membrane integrity required for their functioning. The work of Rees and Nason with ruptured spheroplasts of Nitrosomonas (Rees and Nason, 1965) provided evidence for association of cytochromes in membrane fractions. Oxidation of ammonia by spheroplasts of Nitrosomonas after hydroxylamine oxidation or magnesium ion treatment (Suzuki and Kwok, 1969) proved that cell walls were not necessary for ammonia oxidation. Recently, cell free ammonia oxidizing systems were isolated from Nitrosocystis (Watson, Asbell and Valois, 1970) and Nitrosomonas (Suzuki and Kwok, 1970).

# MATERIALS AND METHODS

# MATERIALS AND METHODS

## Organisms and Media

### 1. T. thiooxidans

Thiobacillus thiooxidans (A.T.C.C. 8085) was used throughout this study. It was grown on a medium devised by Starkey (Starkey, 1925) made up of the following:

| | |
|---|---|
| $(NH_4)_2SO_4$ | 0.3 g |
| $KH_2PO_4$ | 3.5 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| $CaCl_2$ | 0.25 g |
| $FeSO_4.7H_2O$ | 0.02 g |
| powdered sulfur (precipitated) | 50 g |
| distilled water | 1000 ml |

The $CaCl_2$ and $MgSO_4.7H_2O$ solutions were autoclaved separately and added to the growth flask after cooling. The final pH in the growth medium was 4.5.

Inocula and cells used in the study were grown in one liter batches in 2800 ml Fernbach flasks. The powdered sulfur was gently floated on top of the medium after the inoculum (10% v/v) had been added. Incubation was allowed to continue for 5 days at 28° with no agitation. The grown culture was passed through a Whatman #1 filter paper under pressure to remove elemental sulfur. The cells in the filtrate were harvested by centrifugation

and were embedded in 2% agar.

2. T. thioparus

Thiobacillus thioparus (A.T.C.C. 8158) was grown in Starkey's medium No. 2 (Starkey, 1934). The composition of this medium is as follows:

| | |
|---|---|
| $Na_2S_2O_3.5H_2O$ | 10.0 g |
| $KH_2PO_4$ | 4.0 g |
| $K_2HPO_4$ | 4.0 g |
| $CaCl_2$ | 0.05 g |
| $(NH_4)_2SO_4$ | 0.3 g |
| $FeCl_3.6H_2O$ | 0.02 g |
| $MnSO_4.2H_2O$ | 0.02 g |
| Phenol red (2%) | 0.3 g |
| distilled water | 1000 ml |

The pH was adjusted to 7.0 - 7.5 with 10% $K_2CO_3$ before inoculation and periodically while the culture was growing until the desired concentration of cells was reached. The medium used for the growth of stock cultures and inocula, minus thiosulfate, was sterilized at 121° for twenty minutes. Thiosulfate, in a 10% solution, was sterilized separately by filtration and added to the cooled medium. Stock cultures and inocula were grown in 500 ml Erlenmeyer flasks containing 200 ml sterilized medium. Cells used in this study were grown in 10 liter batches using unsterilized medium. When the culture had

reached the desired level of growth forced aeration was stopped and the culture was allowed to stand for one hour to allow inorganic precipitates to settle. That part of the culture relatively free of precipitate was decanted and centrifuged. The cells which were extremely delicate and prone to lysis were placed immediately in primary fixative and were embedded in 2% agar after primary fixation was complete.

3. T. denitrificans

The organism used in this study was Thiobacillus denitrificans (A.T.C.C. 13711). It was grown on medium described by Vishniac and Santer (Vishniac et al, 1957) which was prepared as follows:

Solution A

| | |
|---|---|
| $Na_2S_2O_3.5H_2O$ | 5.0 g |
| $KNO_3$ | 2.0 g |
| $NH_4Cl$ | 0.5 g |
| $MgCl_2.6H_2O$ | 0.5 g |
| distilled water | 800 ml |
| phenol red (2%) | 0.3 ml |

Solution B

| | |
|---|---|
| $KH_2PO_4$ | 20.0 g |
| distilled water | 100 ml |

Solution C

| | |
|---|---|
| $NaHCO_3$ | 10.0 g |

| | | |
|---|---|---|
| distilled water | 100 | ml |

Solution D

| | | |
|---|---|---|
| $FeSO_4.7H_2O$ | 2.0 | g |
| 0.1 N HCl | 100 | ml |

Each solution was sterilized independently. Solution C was saturated with $CO_2$ by aseptically bubbling compressed $CO_2$ through the mixture after it was sterilized. To the flask containing Solution A, which acted as the growth flask, was added 0.5 ml. of Solution D and a combination of Solutions B and C such that the final pH in the growth flask was 6.9. After inoculation, the atmosphere above the solution in the growth flask was aseptically replaced with helium. As growth progressed the pH was readjusted to 6.9 with samples of Solution C as required.

Stock cultures, inocula and cells for use in this study were grown in 2 liter Erlenmeyer flasks containing 800 - 850 ml medium. After the desired growth level was reached, cells to be used in electron microscope studies were harvested by centrifugation and embedded in 2% agar.

T. denitrificans was also grown aerobically in the same medium as above minus $KNO_3$. In this case filtered air was bubbled through the medium.

4. T. ferrooxidans

The organism used in this study was Thiobacillus

ferrooxidans (A.T.C.C. 19859). It was grown in medium devised by Colmer et al (Colmer et al, 1949) which was prepared as follows:

Solution A

| | |
|---|---|
| $(NH_4)_2SO_4$ | 0.8 g |
| $KH_2PO_4$ | 0.4 g |
| $MgSO_4.7H_2O$ | 0.16 g |
| distilled water | 800 ml |

Solution B

| | |
|---|---|
| $FeSO_4.7H_2O$ | 20.0 g |
| 1 N $H_2SO_4$ | 2.0 g |
| distilled water | 200 ml |

Solution A was sterilized at 121° for twenty minutes and Solution B was sterilized by filtration. When Solution A had cooled the two were mixed. The final pH was near 2.8.

Stock cultures and inocula were kept in 500 ml Erlenmeyer flasks containing 200 ml of the above medium. Cells used in this study were grown in 10 liter amounts of the same medium with forced aeration. Growth was followed by the deposit of ferric hydroxide on the sides and bottom of the carboy. When a satisfactory level of growth was acquired the aeration was stopped and the culture was allowed to stand for 12 hours at 4°. One half to three-quarters of the medium which was relatively free of ferric hydroxide was decanted and the cells in it were

harvested by centrifugation. The separation of cells from ferric hydroxide precipitate was satisfactory enough to enable cells to be isolated and embedded directly into 2% agar.

5. F. ferrooxidans

Ferrobacillus ferrooxidans (A.T.C.C. 13661) used in this study was grown and maintained on the same medium as T. ferrooxidans. The condition of its growth and harvesting were also kept as near as possible to those described for T. ferrooxidans.

6. T. novellus

(a) Growth and Preparation of Autotrophic Cells

Autotrophic Thiobacillus novellus (A.T.C.C. 8093) used in this study was grown on Starkey's medium (Starkey, 1934). The composition of this medium is as follows:

| | |
|---|---|
| $Na_2S_2O_3.5H_2O$ | 10.0 g |
| $K_2HPO_4$ | 6.0 g |
| $CaCl_2$ | 0.01 g |
| $MgSO_4.7H_2O$ | 0.1 g |
| $(NH_4)_2SO_4$ | 0.1 g |
| $FeCl_3.6H_2O$ | 0.02 g |
| $MnSO_4.2H_2O$ | 0.02 g |
| distilled water | 1000 ml |
| phenol red (2%) | 0.3 ml |

Stock cultures and inocula were maintained in

500 ml Erlenmeyer flasks containing 200 ml of the above medium. Cells used in this study were grown at room temperature in 10 liter batches aerated by passing compressed air through a cotton filter and two sintered glass spargers into the bottom of the medium. Growth was allowed to proceed until the pH of the culture medium dropped to 6.5; at this point aeration was stopped and the culture was allowed to stand for one hour. The bulk of the organic precipitate which was present settled to the bottom and was discarded. The cells in the decanted portion were harvested by centrifugation and were washed twice in deionized water. Entrained colloidal sulfur was removed by differential centrifugation after each washing. Cells used in electron microscopy were embedded in 2% Noble agar (Difco Laboratories, Detroit, Michigan). Cells used for chemical analysis were dried at 80° before analyses were carried out.

(b) Growth and Preparation of Heterotrophic Cells

Heterotrophic cells were grown in the same medium as autotrophic cells except that thiosulfate was replaced by 1% glucose. The cells were harvested and prepared as described above; no differential centrifugation was required, however, as no colloidal sulfur was present.

7. H. facilis

(a) Growth and Preparation of Autotrophic Cells

Hydrogenomonas facilis (A.T.C.C. 17695) was grown in a medium described by DeCicco et al (DeCicco and Stukus, 1968) which is prepared as follows:

Solution A

| | | |
|---|---|---|
| $(NH_4)_2SO_4$ | 10.0 | g |
| $MgSO_4.7H_2O$ | 1.0 | g |
| 0.2 M phosphate buffer pH 6.65 | 1000 | ml |

Solution B

| | | |
|---|---|---|
| $Fe(NH_4)_2(SO_4)_2.6H_2O$ | 1.0 | g |
| distilled water | 10 | ml |

Solution A was sterilized at 121° for twenty minutes while Solution B was sterilized by filtration. After Solution A was cooled, 1 ml of Solution B was added to it. An atmosphere of 70% hydrogen, 20% oxygen and 10% carbon dioxide was prepared by the displacement of water from a 5 gallon carboy. This atmosphere was bubbled through the growth medium after being passed through a cotton filter and was then returned to the carboy containing the gas mixture in a closed circuit arrangement powered by a peristaltic pump. A carboy containing fresh gas mixture was placed in the circuit twice during the growth of the organism.

Stock cultures, inocula and cells used in the following study were grown in this manner. When turbidity reached a desired level the cells were harvested by centrifugation and embedded in 2% agar.

(b) Growth and Preparation of Heterotrophic Cells

Heterotrophic H. facilis was grown in the same medium as the autotrophic form except that 20 g glutamate was added per liter of medium. This culture was grown at room temperature in a one liter Erlenmeyer flask containing 500 ml medium. No aeration except that which diffused through the cotton stopper was supplied. The organisms were harvested and embedded in the same way as the autotrophic form.

8. N. agilis

Nitrobacter agilis (A.T.C.C. 14123) was grown on Gould's medium (Gould, 1959) which was prepared according to the following recipe:

| | |
|---|---|
| $KH_2PO_4$ | 75.0 g |
| $CaCO_3$ | 2.5 g |
| $MgCl_2.6H_2O$ | 1.0 g |
| $MnSO_4.4H_2O$ | 0.1 g |
| $FeCl_3$ | 0.1 g |
| deionized water | 500 ml |

The pH of the above solution was adjusted to 7.7 with NaOH and the volume was made up to one liter with deionized water. The precipitate that was formed was removed by filtration through a 0.45 μ Millipore filter. The filtrate was adjusted to a volume of 2.5 liters with deionized water.

Medium for the growth of N. agilis consisted of 10 ml of the above medium diluted to 1000 ml with deionized water. It was then sterilized at 121° for an appropriate time. Nitrite in the form of a concentrated sodium nitrite solution was prepared, sterilized separately and added to this medium such that the final concentration of nitrite nitrogen was 20 mg/ml.

Stock cultures were kept in 50 ml Erlenmeyer flasks with 10 ml of the above medium. When they had oxidized 1200 mg/ml of nitrite nitrogen they were immediately subcultured in order to keep the cultures as active as possible. A large number (15 - 20) of stock cultures were continually cultivated since the incidence of contamination was very high. Inocula were prepared in 500 ml Erlenmeyer flasks containing 100 ml medium. Those stock cultures that were pure were inoculated into these flasks regardless of requirements so that a large number were available on demand. The cells used in this study were grown in 10 liter batches on a New Brunswick Microferm fermentor. After they had oxidized 2000 mg/ml nitrite nitrogen they were harvested by centrifugation and embedded directly into 2% agar.

9. N. europaea

The Nitrosomonas culture (Schmidt strain) (A.T.C.C. 19718) used throughout this study was kindly supplied by

A. B. Hooper, University of Minnesota. The medium used to cultivate the organism was A.T.C.C. medium number 221 consisting of the following:

| | |
|---|---|
| $(NH_4)_2SO_4$ | 3.0 g |
| $K_2HPO_4$ | 0.5 g |
| $MgSO_4$ | 0.05 g |
| $CaCl_2$ | 0.004g |
| chelated iron | 0.1 mg iron |
| cresol red | 0.05 g |
| distilled water | 1000 ml |

Chelated iron was made by preparing solutions with 0.29 mg $FeCl_3$/10 ml water and 0.6 mg EDTA/10 ml water and mixing them. $CaCl_2$ and $MgSO_4$ solutions were sterilized by heating independent of the growth medium. Chelated iron was sterilized by filtration. All solutions were mixed aseptically before inoculation.

Cells used in this study were grown in 5 gallon carboys containing 15 liters of the above medium. They were aerated by forcing compressed air through a cotton filter and glass spargers to the bottom of the medium. Cells used in electron microscope studies were harvested in the late logarithmic phase and embedded directly into 2% agar. Cells used in the preparation of spheroplasts were harvested in the maximum stationary phase, were washed three times in 0.1 M phosphate buffer pH 7.5 and stored in the same buffer at 4° until used.

## Preparation of Cells for Electron Microscopy

### Fixation Procedure

Hess method (Hess, 1966).

(a) Specimens are fixed for two hours at room temperature in a solution consisting of the following:

| | |
|---|---|
| 0.2 M cacodylate buffer pH 7.4 | 5.0 ml |
| distilled water | 3.5 ml |
| 25% glutaraldehyde | 1.2 ml |
| acrolein | 0.3 ml |

(b) Wash 4 times, 15 minutes each in 0.1 M cacodylate buffer pH 7.4.

(c) Fix for 2-4 hours in 1% $OsO_4$ in 0.1 M cacodylate buffer pH 7.4 at 0°.

(d) Wash 4 times, 15 minutes each in 0.1 M cacodylate buffer pH 7.4.

(e) Place in 0.5% aqueous uranyl acetate for 12 - 16 hours.

(f) Wash once in water for 15 minutes, dehydrate and embed.

### Dehydration

Specimens were allowed to stand in 50%, 70% and 90% ethanol for 15 minutes in each concentration and absolute alcohol for 30 minutes. The absolute ethanol was then decanted and replaced with a fresh volume and left for 30 minutes more.

Preparation for Embedding

When the last washing of absolute ethanol was decanted the tiny cubes of agar containing the bacterial cells were each placed in a gelatin capsule with several drops of the embedding mixture. These were allowed to stand for 6 hours. The capsules were then filled, the specimens were centered in the capsule and polymerization was carried out.

Embedding Material

The embedding material which was ultimately used consisted of the following (Kay, 1965):

| | |
|---|---|
| Methyl methacrylate | 15.0 ml |
| Butyl methacrylate | 85.0 ml |
| 5% divinyl benzene | 5.0 ml |
| Benzoyl peroxide | 1.0 gm |

The above chemicals were mixed and allowed to age for 24 hours at 4° before use. Polymerization was satisfactory after heating the filled capsules in a vacuum oven at 48 - 50° for 16 - 24 hours.

Staining Procedures

The stain which proved most satisfactory was lead citrate prepared according to Reynolds (Reynolds, 1953).

| | |
|---|---|
| Lead nitrate | 1.33 g |
| Sodium citrate | 1.76 g |
| distilled water | 30.0 ml |

This mixture was shaken vigorously for 1 minute and intermittently for another 30 minutes. The remaining precipitate was dissolved by the addition of 8.0 ml of 1 N NaOH and the solution was made up to 50 ml with distilled water. This solution, if stored in the dark at 4°, remains stable for about 6 months.

Sections were cut on an LKB Type 4801A Ultratome (LKB-Producter, Stockholm-Bromma) using a glass knife. They were post-stained in lead citrate under a nitrogen atmosphere for varying lengths of time, were washed in 0.01 N NaOH and distilled water and were allowed to dry before examination in the electron microscope.

For the electron microscopical detection of polysaccharide, whole cells were stained in 0.01% iodine solution for 40 minutes, washed in water for 20 minutes and placed on grids. The grids were dried on filter paper and held in the electron microscope for 30 minutes before the beam voltage was turned on. The period during which the iodine-stained cells were subjected to the electron beam was kept to a minimum because of the possible damage to the tungsten filament by iodine vapor.

Electron microscopes used to examine specimens were Philips models 75 and 100 and an AEI model 6B.

## Chemical Analyses

1. Protein Determination:

A desired mass of dried cells was hydrolyzed in

1.0 N NaOH at 80° until a clear solution was obtained. Samples of this solution were assayed for protein by the method of Lowry et al (Lowry, Rosebrough, Farr and Randall, 1951) using serum albumin as a standard.

2. Phosphate Determination:

The hydrolysate of cells treated with 1.0 N HCl at 100° for 10 minutes was assayed for the presence of phosphates by the method of Waygood (Waygood, 1948) as follows. An aliquot of the above solution containing approximately 500 mg phosphorus was placed in a small Kjeldahl flask containing 5 ml 60% perchloric acid and was heated in a fume hood until colourless. It was then cooled and transferred quantitatively to a 50 ml volumetric flask. To this flask 40 ml 1.0 N sodium acetate-acetic acid buffer pH 4.0 was added and the solution was made to 50 ml with distilled water. A suitable sample of this solution was placed in a test tube. To it was added 2.0 ml 1.0 N sodium acetate-acetic acid buffer pH 4.0 and 0.5 ml 5% aqueous ammonium molybdate. The tube was shaken and 1 ml 1% ascorbic acid in sodium acetate-acetic acid buffer was added. This solution was made to 10.0 ml with distilled water, was shaken and was allowed to stand for more than five minutes. Its optical density was measured on a Klett-Summerson spectrophotometer with a 660 mμ filter. The known standard used was potassium dihydrogen phosphate. This method is accurate in a range from 0 - 3 μg phosphorus.

3. Lipid Determination:

The total lipid content of cells was measured gravimetrically after extraction according to the method of Kates (Kates, Yengoyan and Sastry, 1965).

A known mass of dried cells was placed in a large centrifuge tube with 15 ml of a 2:1 mixture of methanol and chloroform and was agitated for 48 hours. The mixture was centrifuged at 10,000 x g for 20 minutes and the supernatant was poured into a vessel of known weight. The extraction was repeated with 15 ml of fresh solvent and this was added to the same vessel. The solvent was evaporated to dryness under nitrogen and the remaining residue was weighed.

4. Total Carbohydrates:

Cells were assayed for carbohydrate by the Anthrone method of Seifer et al (Seifter, Seymour, Novic and Muntwyler, 1950). A sample of hydrolysate prepared by hydrolyzing dried cells in 1.0 n HCl at 100° for 10 minutes was placed in a test tube and made to 3.0 ml total volume with $H_2O$. To this was added 6 ml 0.2% anthrone in 95% $H_2SO_4$. This mixture was placed in a boiling water bath for 3 minutes and while hot, its optical density was determined on a Klett-Summerson spectrophotometer using a 660 mμ filter. Assays were compared to glucose as a standard.

5. Poly-β-hydroxybutyrate Determination:

Poly-β-hydroxybutyrate was extracted by the method of Williamson and Wilkinson (Williamson and Wilkinson, 1958). A desired mass of dried cells was placed in 3 - 5 ml concentrated sodium hypochlorite solution. They were allowed to digest at 37° for 90 minutes. The mixture was centrifuged at 10,000 x g for 15 minutes and the supernatant was discarded. The pellet was extracted twice with dry ether and the ether was discarded. The remaining pellet was extracted with chloroform. The chloroform layer was evaporated to dryness.

The residue which remained was assayed for poly-β-hydroxybutyrate by the method of Slepecky and Law (Slepecky and Law, 1960). To the dried residue a measured amount of concentrated sulfuric acid was added. This was heated in a boiling water bath for 10 minutes and then cooled. The optical density of the resulting solution was measured at 235 mμ. From the molar extinction coefficient of $1.53 \times 10^4$ for poly-β-hydroxybutyrate as was published by Slepecky and Law the amount of poly-β-hydroxybutyrate in the unknown was calculated.

6. Nitrite Determination:

Nitrite was determined by the method of Bratton and Marshall (Bratton and Marshall, 1939) as follows:

Solution A

| | | |
|---|---|---|
| sulfanilic acid | 10.0 | g |
| concentrated HCl | 200 | ml |
| distilled water | 800 | ml |

Solution B

| | | |
|---|---|---|
| N-1-naphthylethylenediamine hydrochloride | 1.2 | g |
| distilled water | 1000 | ml |

A sample of unknown solution containing 0 - 2.0 µg nitrite nitrogen was made up to 6.5 ml with water. To this solution 0.5 ml of Solution A was added, the tube was shaken and 0.5 ml of Solution B was added. The mixture was allowed to stand for 10 minutes. Its optical density was then measured on a Klett-Summerson spectrophotometer using a 540 mµ filter. The unknown was compared to a sodium nitrite standard.

Preparation of Spheroplasts

Spheroplasts were prepared by the method of Rees and Nason (Rees and Nason, 1965) as described below. Washed Nitrosomonas cells were suspended in 0.25 M sucrose-0.1 M potassium phosphate buffer pH 7.5 and $2 \times 10^{-3}$ M EDTA containing 100 mg lysozyme (Sigma Chemical Co., St. Louis, Mo.) in a final volume of 40 ml per gram wet weight. The suspension was incubated for two hours at 28° with gentle swirling. Spheroplasts collected by

centrifugation at 2000 x g for twenty minutes were washed twice in 0.25 M sucrose-.1 M potassium phosphate buffer pH 7.5 and were stored in the same buffer. Spheroplasts used in electron microscope studies were placed directly into the primary fixative and embedded in 2% agar cubes after primary fixation was complete.

Measurement of Oxygen Utilization by Spheroplasts

The time course of oxygen utilization was measured on a Gilson Oxygraph fitted with a Clark Oxygen Electrode. The reaction mixture contained 0.1 ml spheroplasts suspension derived from 10 mg wet weight cells and 1.40 ml 0.25 M sucrose-0.1 M potassium phosphate buffer pH 7.5. Reactions were carried out at 20°.

# RESULTS

# RESULTS

## Ultrastructural Studies of Whole Cells

### 1. T. thiooxidans

The cell envelope of this organism (Figs. 1 and 2b) was relatively smooth with the exception of the L layer which appeared to be delicate and easily wrinkled. The overall thickness of the cell envelope was 200 Å calculated from a number of measurements from different sections. The L layer and the cytoplasmic membrane (Fig. 2b) were each 55 Å thick and the remaining three, the $g_1$, $g_2$, and M layers, were 90 - 100 Å thick. The M layer stained very intensely and because of its close proximity caused an overshadowing effect on the cytoplasmic membrane.

The cytoplasm of this organism was largely homogeneous showing no unusual inclusions. Ribosomes (Figs. 1 and 2a) were most intense near the cytoplasmic membrane. The nuclear material appeared dispersed throughout the entire cell but maintained a degree of separation from the cell envelope.

This species divided in a unique way. After the cell envelope had constricted as a unit to less than one quarter of the diameter of the cell, the cell wall components and the cytoplasmic membrane were built up to the

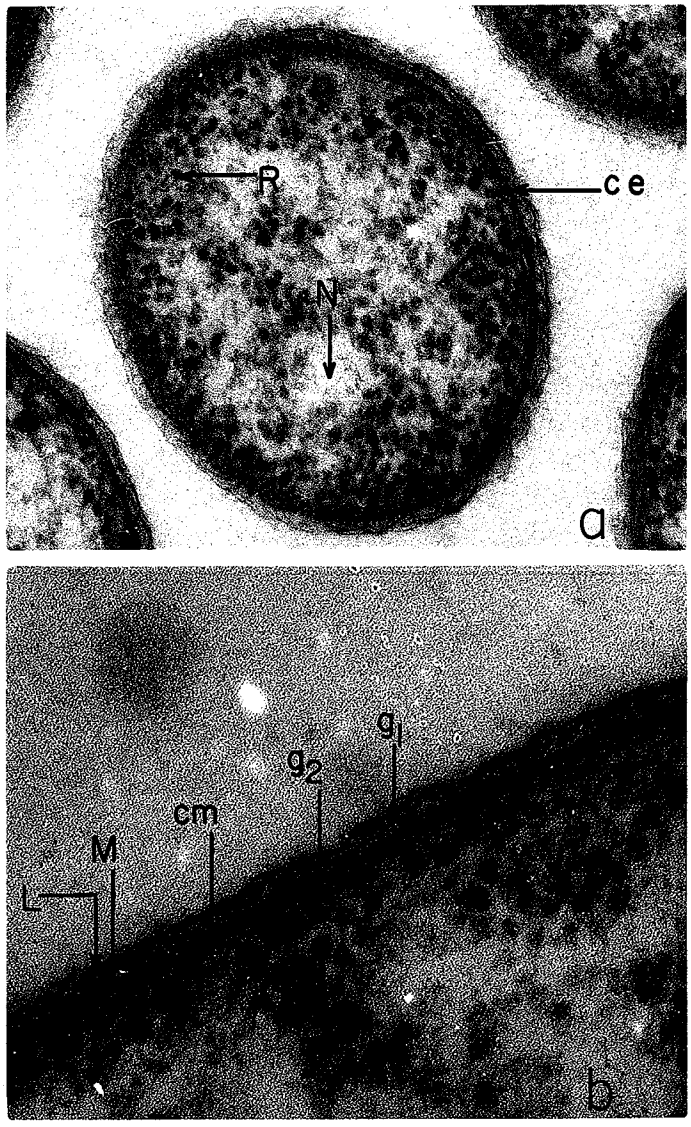
Fig. 1. Longitudinal section of T. thiooxidans showing general structure, ribosomal particle (R) and nuclear material (N). Cells were fixed by Hess' method (Hess, 1966), were embedded in cross-linked methacrylate and post-stained with lead citrate. X 190,000.


R
N

Fig. 2. Thin sections of T. thiooxidans. Cells were prepared as in Fig. 1.

(a) Cross section of cell showing ribosome (R), cell envelope (ce) and nuclear material (N). X 120,000.

(b) Section of cell envelope showing individual layers. X 266,000.


R
ce
N
a
g1
g2
cm
M
L
b

point where both daughter cells were complete (Figs. 3a, b and c). The constrictive furrow then proceeded. Despite this peculiar behavior, not unlike that of some Gram-positive organisms (Chapman and Hillier, 1953), no chains of cells were observed in light microscopical studies.

2. T. thioparus

This organism was extremely delicate in structure supported by the fact that it was very prone to autolysis. The cell envelope (Figs. 4 and 5b) showed a tendency to appear wrinkled or undulated probably due to fixation and embedding procedures. The L layer (Fig. 5b) was especially delicate and difficult to stain in methacrylate embedded specimens. In Epon preparations the cell wall wrinkled so badly that this method of embedding was discarded. Both the L layer and the cytoplasmic membrane (Fig. 5b) were 55 - 60 Å in thickness. The cytoplasmic membrane appeared as a smooth sac with no invaginations into the cytoplasm. The $g_2$ layer appeared as a discontinuous dark line (Fig. 5b) which became diffuse in most instances. The electron micrographs, by necessity, were developed at low contrast in order to retain small portions of the $g_2$ layer. The thickness of the cell envelope was calculated to be 190 - 200 Å.

The cytoplasm appeared homogeneous with only rare appearances of mesosome-like structures (Fig. 5a). For the

Fig. 3. Sections of dividing T. thiooxidans. Cells were prepared as in Fig. 1.

(a) Section of cell showing initial stage of division. X 175,000.

(b) Division having progressed showing incomplete cell walls (arrows). X 114,000.

(c) Division showing completed cell walls with constrictive furrow incomplete. X 104,000.

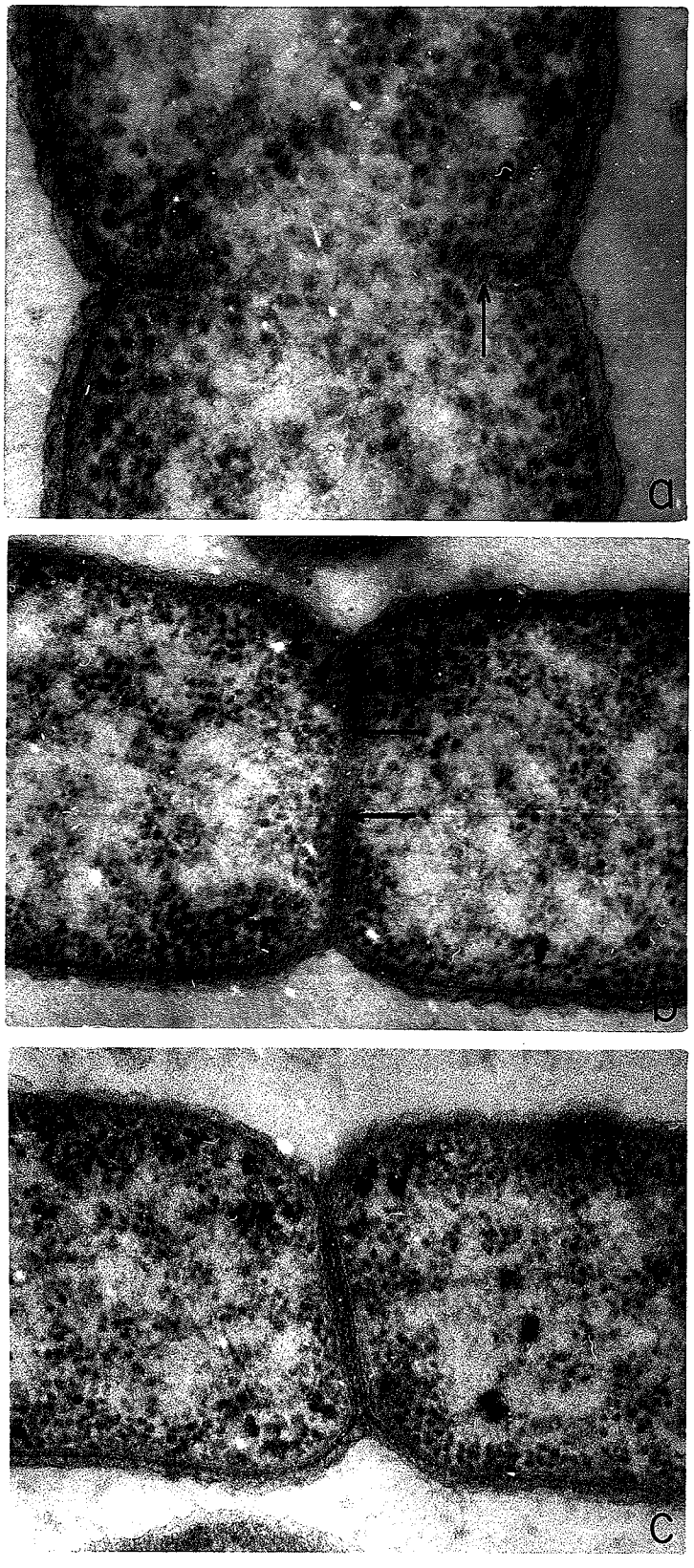

a
b
c

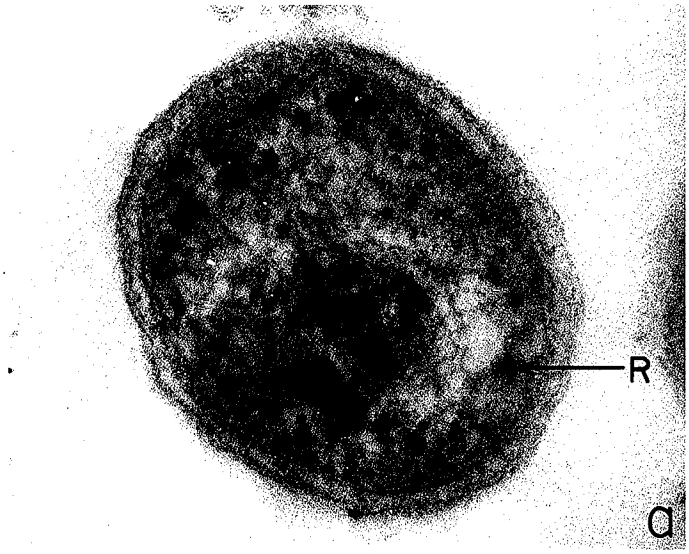
Fig. 4. Longitudinal section of T. thioparus showing general structure, ribosomal particle (R) and nuclear material (N). Cells were prepared as in Fig. 1. X 162,000.

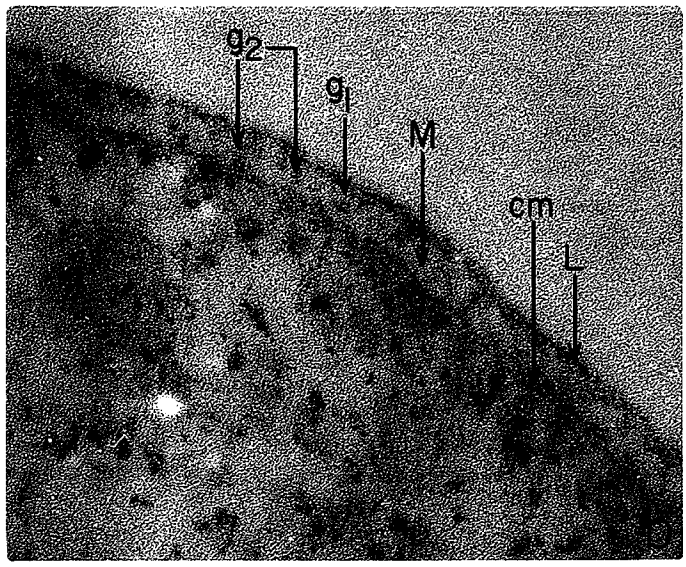

R
N

Fig. 5. Thin sections of T. thioparus. Cells were prepared as in Fig. 1.

(a) Cross-section of cell showing general structure and ribosome (R). X 200,000.

(b) Section of cell wall showing individual layers. Note discontinuity of $g_2$ layer.


R
a


g2
g1
M
cm
L

most part it was made up of ribosomal particles and nuclear material (Fig. 4a). Ribosomal particles were concentrated near the cytoplasmic membrane.

The cytokinetic behavior of this organism (Figs. 6a, b and c) was somewhat unusual. The cell constricted as is typical of Gram-negative bacteria, however, the cytoplasmic membrane formed in each daughter cell somewhat earlier in the division than expected (Fig. 6b). The constrictive furrow then carried on to completion preventing the buildup of chains of cells.

3. T. denitrificans

The cell envelope of T. denitrificans had an overall thickness of 250 - 270 Å (Figs. 7 and 8). The cell wall of the anaerobically grown organism appeared the same as that of the aerobically grown organism (Figs. 7 and 8). They were both typical of the Gram-negative bacterial cell envelope as described by DePetris (DePetris, 1967). The L layer appeared wrinkled in most preparations but this was probably caused by fixation and/or the embedding of cells in agar previous to fixation. The cytoplasmic membrane was 55 - 60 Å thick and was smooth without apparent invaginations into the cytoplasm although mesosome-like inclusions were present in the cytoplasm of both types of cells (Figs. 7 and 8) varying in size and stainable content from preparation to preparation. Ribosomes were undoubtedly present

Fig. 6. Thin sections of T. thioparus showing stages of division. Cells were prepared as in Fig. 1.

(a) Thin section of cell showing initial stages of division. X 120,000.

(b) Section of cell showing intermediate stage of division. Note partially completed cytoplasmic membrane (arrows). X 92,000.

(c) Section of cell showing near completion of division. X 160,000.

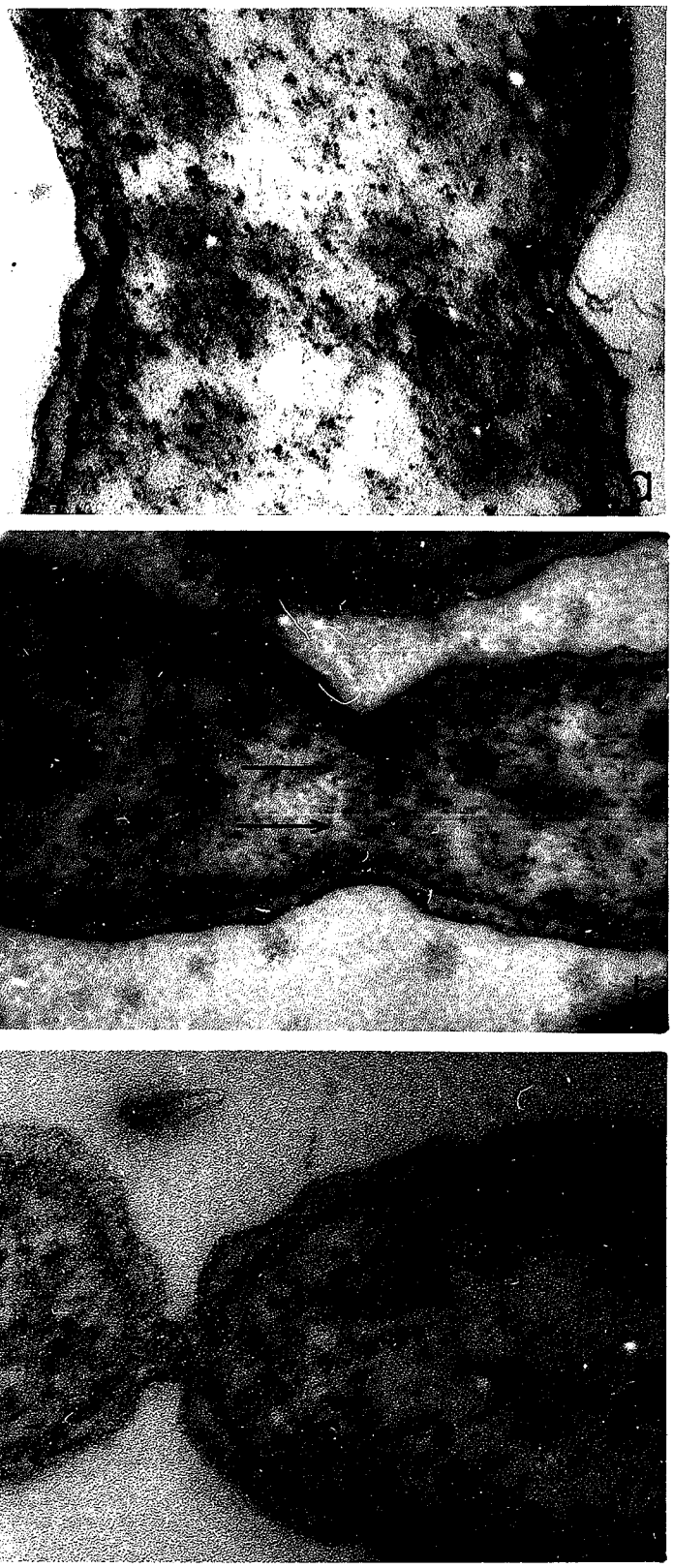


Fig. 7. Longitudinal section of T. denitrificans showing general structure, nuclear material (N) and mesosome (Me). Cells were prepared as in Fig. 1. X 198,000.

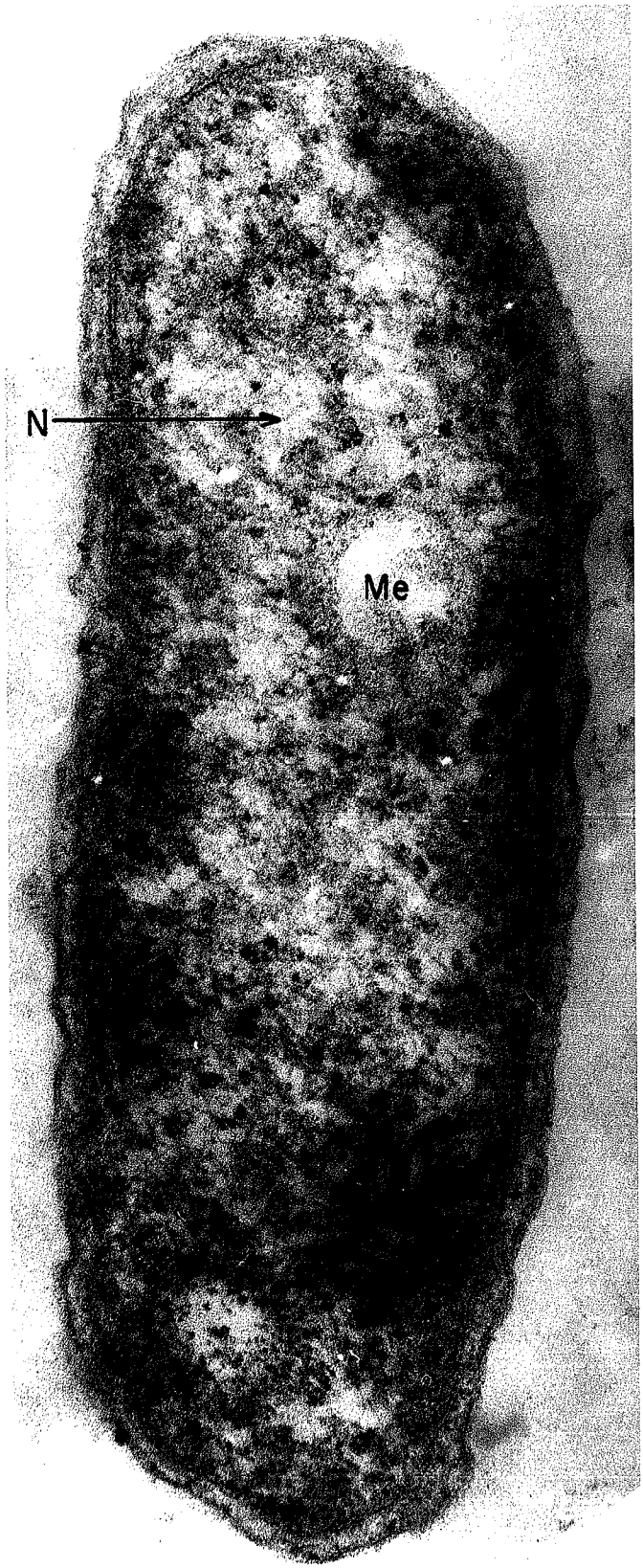

N
Me

Fig. 8. Longitudinal section of T. denitrificans showing general structure, nuclear material (N) and mesosome (Me). Note the presence of lead citrate stain (arrow). Cells were prepared as in Fig. 1. X 180,000.


N
Me

but were not demonstrable in every preparation unless very prolonged lead post-staining was used (Fig. 9b). Nuclear material was spread throughout the cell (Figs. 7 and 8) but for the most part maintained a distinct separation from the cell envelope.

The organism displayed a cytokinetic behavior not unlike T. thioparus (Figs. 10a, b and c). In rare instances very long cells were present but for the most part the normal length of pre-dividing cells was about one and one-half times the single cell length. The cell envelope would then constrict part way and the cytoplasmic membrane would form in both daughter cells (Fig. 10c) before the constrictive furrow would proceed. No chains of cells were observed.

4. T. ferrooxidans and F. ferrooxidans

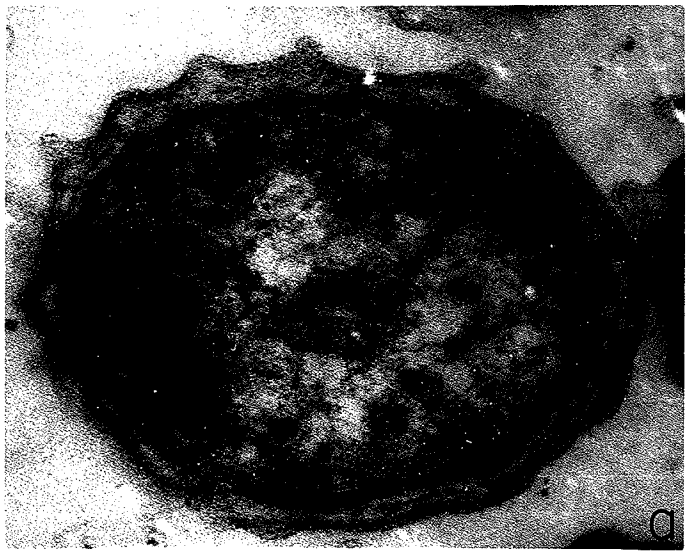
These organisms appeared identical in gross and detailed structure (Figs. 11 and 12). The cell envelopes of each type displayed the same "loose fitting" arrangement as previously described (Lundgren et al, 1964). The envelopes were both 260 - 270 Å thick and showed the same degree of undulation after having been subjected to the same fixation and embedding procedures (Figs. 11 and 12). The cytoplasmic membrane in either case was a typical three-layered membrane 55 - 60 Å thick.

The cytoplasm of these organisms contained several

Fig. 9. Thin sections of T. denitrificans. Cells were prepared as in Fig. 1.

(a) Cross section showing general structure. X 220,000.

(b) Section of cell envelope showing individual layers. X 300,000.


a

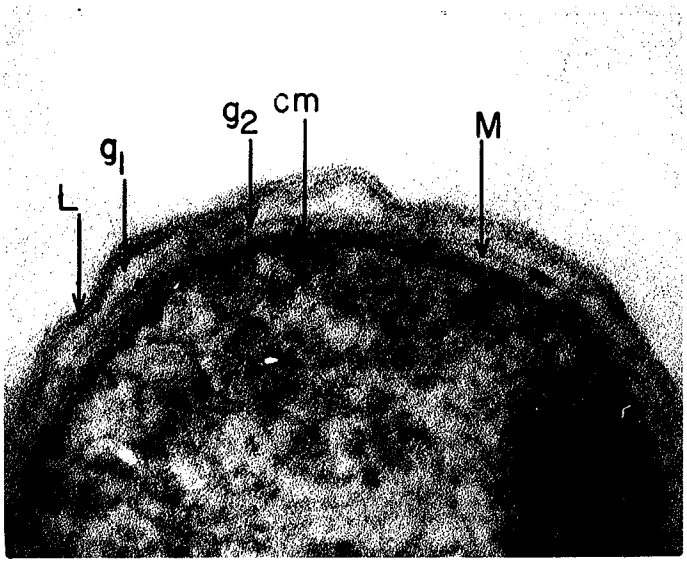
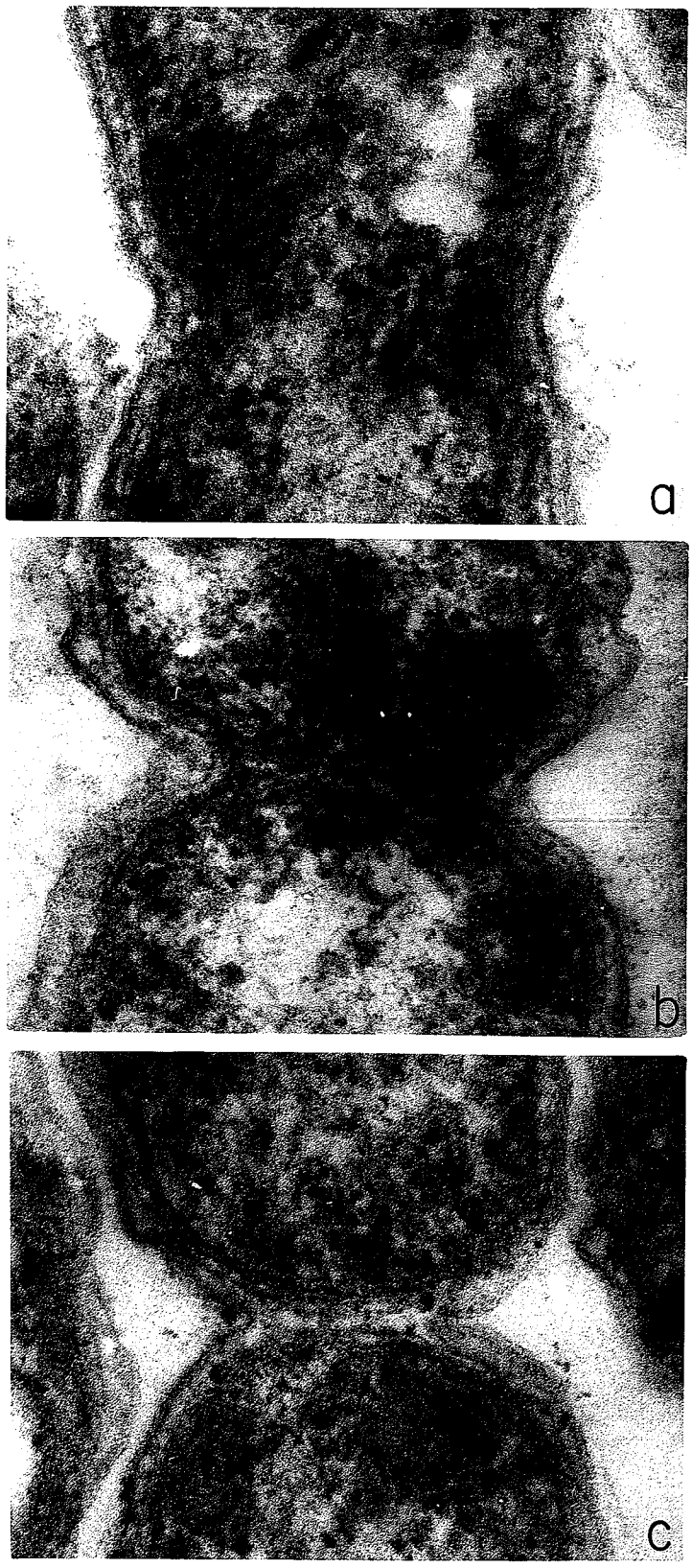

L
g1
g2
cm
M

Fig. 10. Thin sections of T. denitrificans showing stages of division. Cells were prepared as in Fig. 1.

(a) Section of cell in initial stage of division. X 200,000.

(b) Section of cell in intermediate stage of division. X 222,000.

(c) Section of cell in final stage of division. X 206,000.


a
b
c

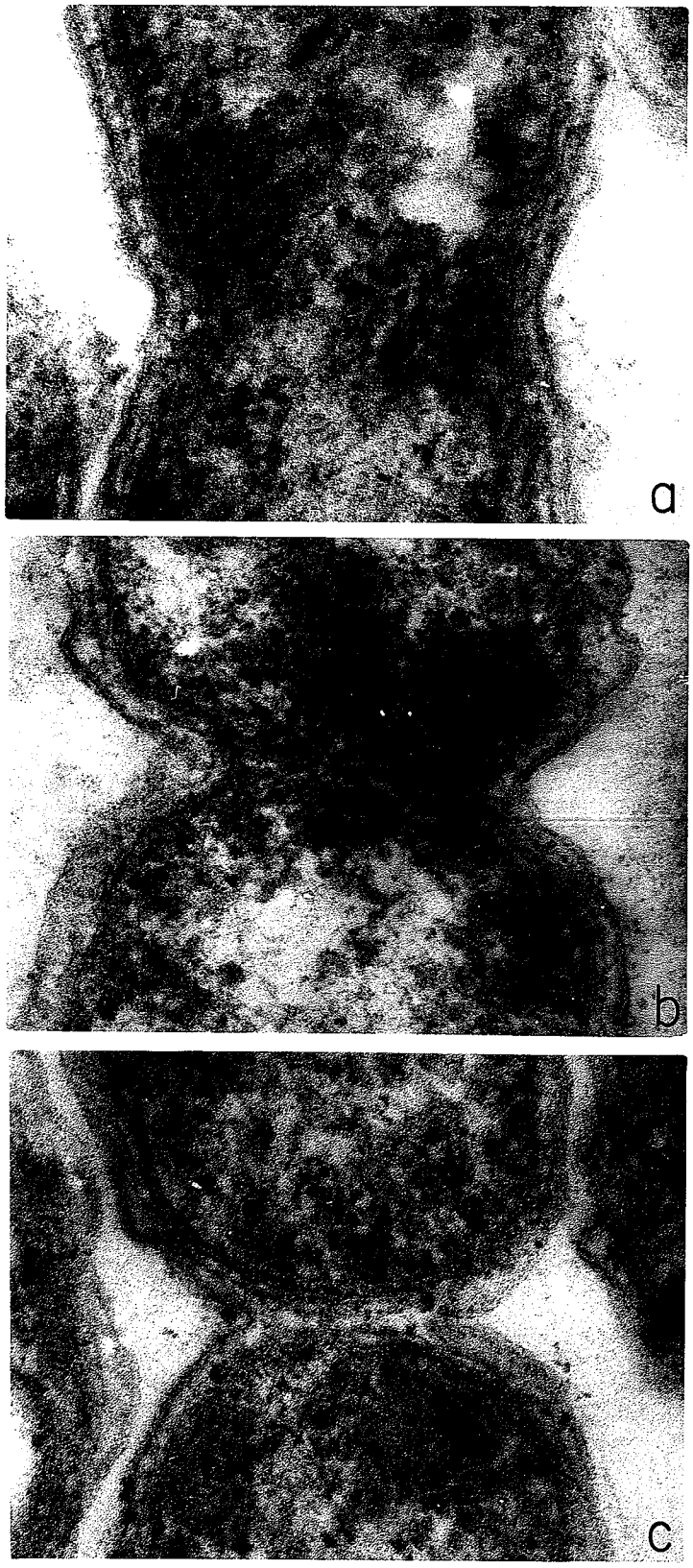

a
b
c

Fig. 11. Longitudinal section of T. ferrooxidans showing general structure, mesosomes (Me), ribosome (R), and nuclear material (N). Cells were prepared as in Fig. 1. X 190,000.

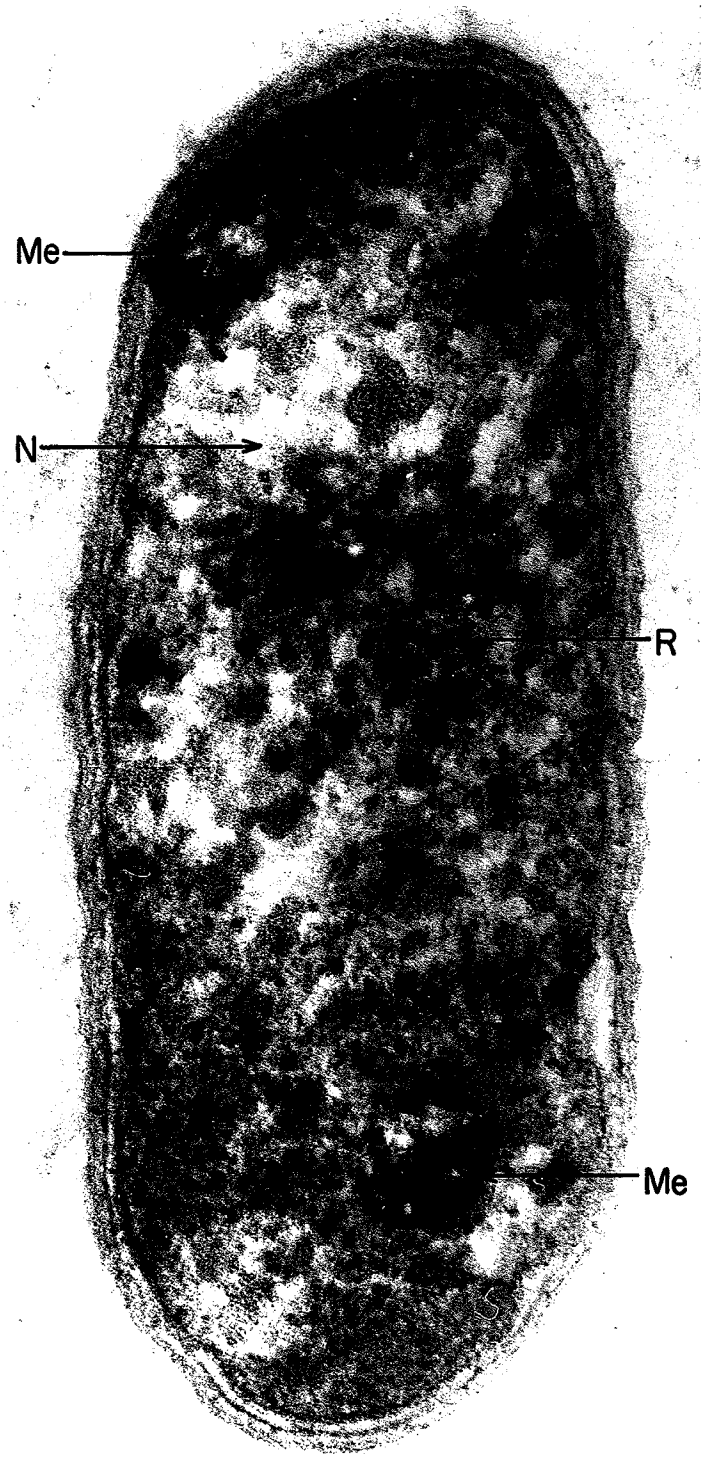

Me
N
R
Me

Fig. 12. Longitudinal section of F. ferrooxidans showing general structure, nuclear material (N), ribosome (R) and mesosome (Me). Cells were prepared as in Fig. 1. X 160,000.


N
R
Me


N
R
Me

inclusions. One of these appeared without fail in these two types but rarely in other types of bacteria. These exceptional inclusions appeared as masses of granular material (Figs. 11 and 12) which were thought to be polyribosomal aggregates by Lundgren *et al* (1964). Individual ribosomal particles were also present usually maintaining a close proximity to the cytoplasmic membrane. Mesosomal organelles were also observed. The nuclear material was also unique in these organisms since it dispersed throughout the cell maintaining no separation from the cell envelope.

The cytokinetic properties of the cell (Figs. 14a, b and c) were the same as those of *E. coli* (Conti *et al*, 1962). The entire cell envelope constricted and no element of it was laid down in advance of the constriction. No chains of organisms were seen in either case.

5. *T. novellus*

The cell envelope of the heterotrophic *T. novellus* (Fig. 17a) had a typical L, $g_1$, and $g_2$ layer, however the M layer was unusually thick compared to that of other Gram-negative bacteria. It is probable that the proximity of the $g_2$ to the L layer in this picture is not an artifact but the unusual thickness of the M layer may be attributed to the fixation and embedding damage. The cell envelope of the autotrophic *T. novellus* was atypical in that the $g_2$ layer was not visible between the $g_1$ and M layers (Fig.

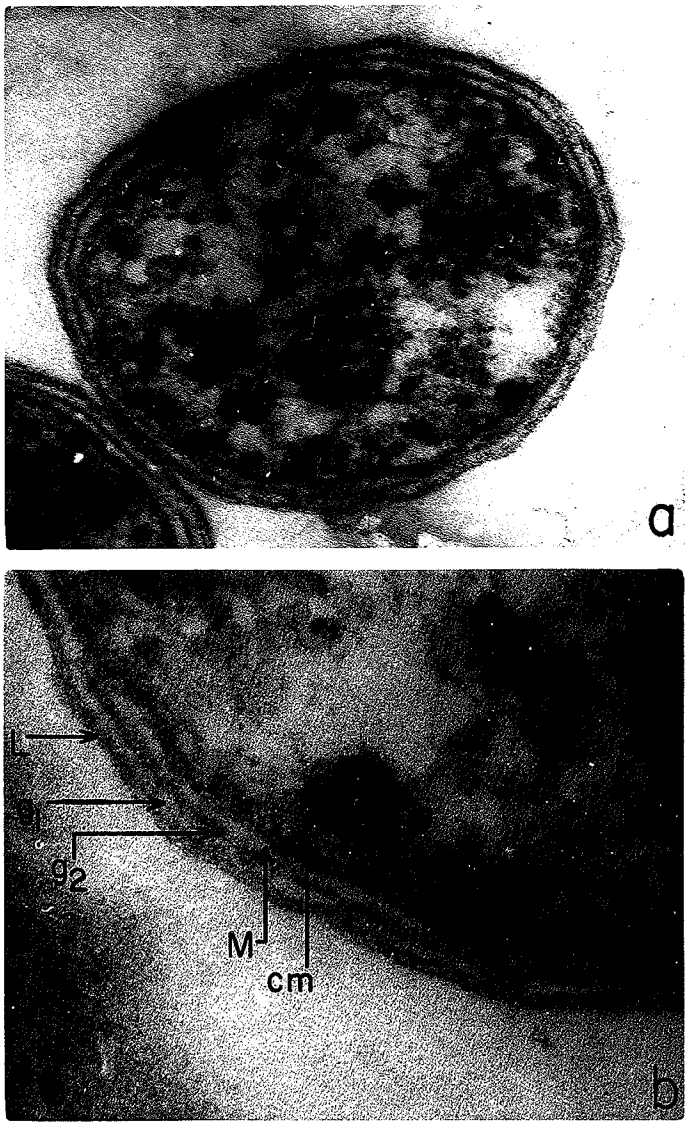

a
L
g1
g2
M
cm
b

Fig. 13. Thin sections of F. ferrooxidans. Cells were prepared as in Fig. 1.

(a) Cross section showing general structure. X 190,000.

(b) Section of cell wall showing individual layers. X 340,000.


a

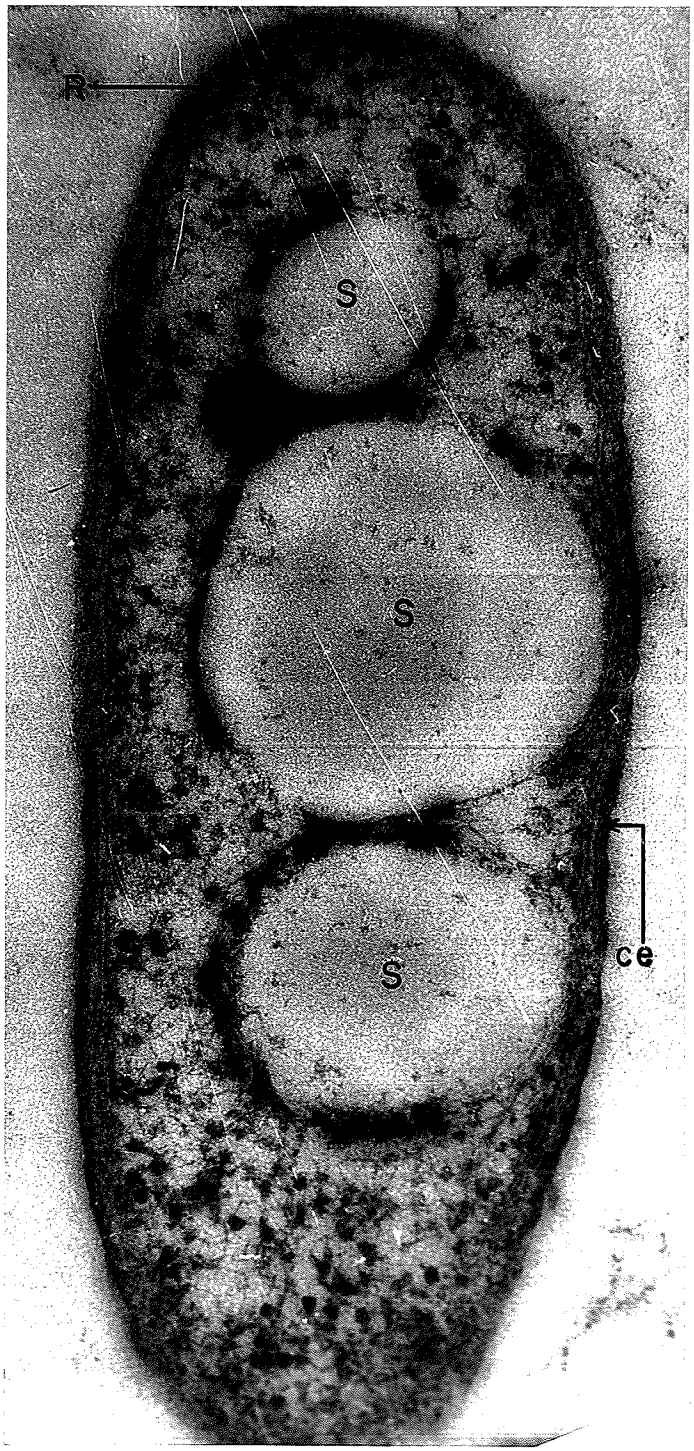
Fig. 14. Thin sections of dividing cells of F. ferrooxidans. Cells were prepared as in Fig. 1.

(a) Thin section showing initiation of division. X 116,000.

(b) Section showing intermediate stage of division. X 150,000.

(c) Section showing division to a point where the cell envelopes are almost pinched off. X 160,000.

Fig. 15. Longitudinal section of heterotrophically grown T. novellus showing general structure, ribosome (R), polysaccharide inclusion (S) and cell envelope (ce). Cells were prepared as in Fig. 1. X 120,000.


R
S
S
S
ce

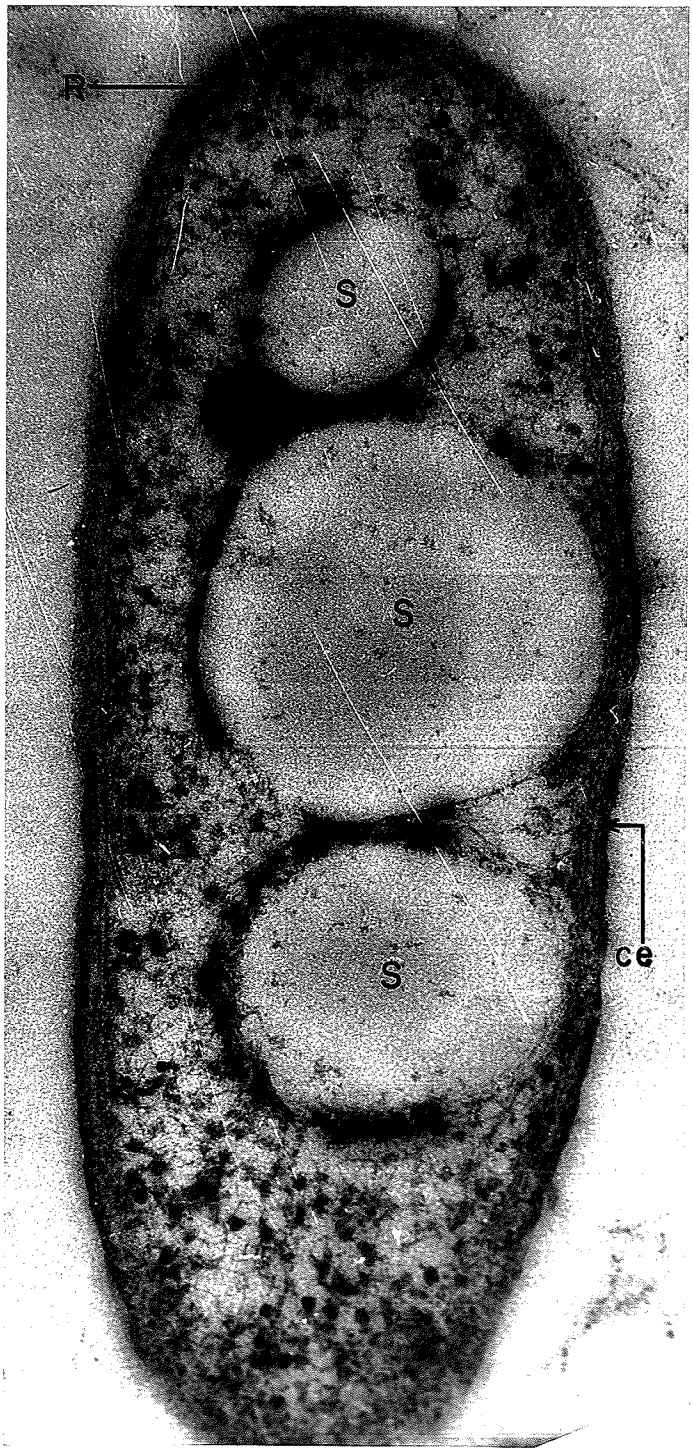
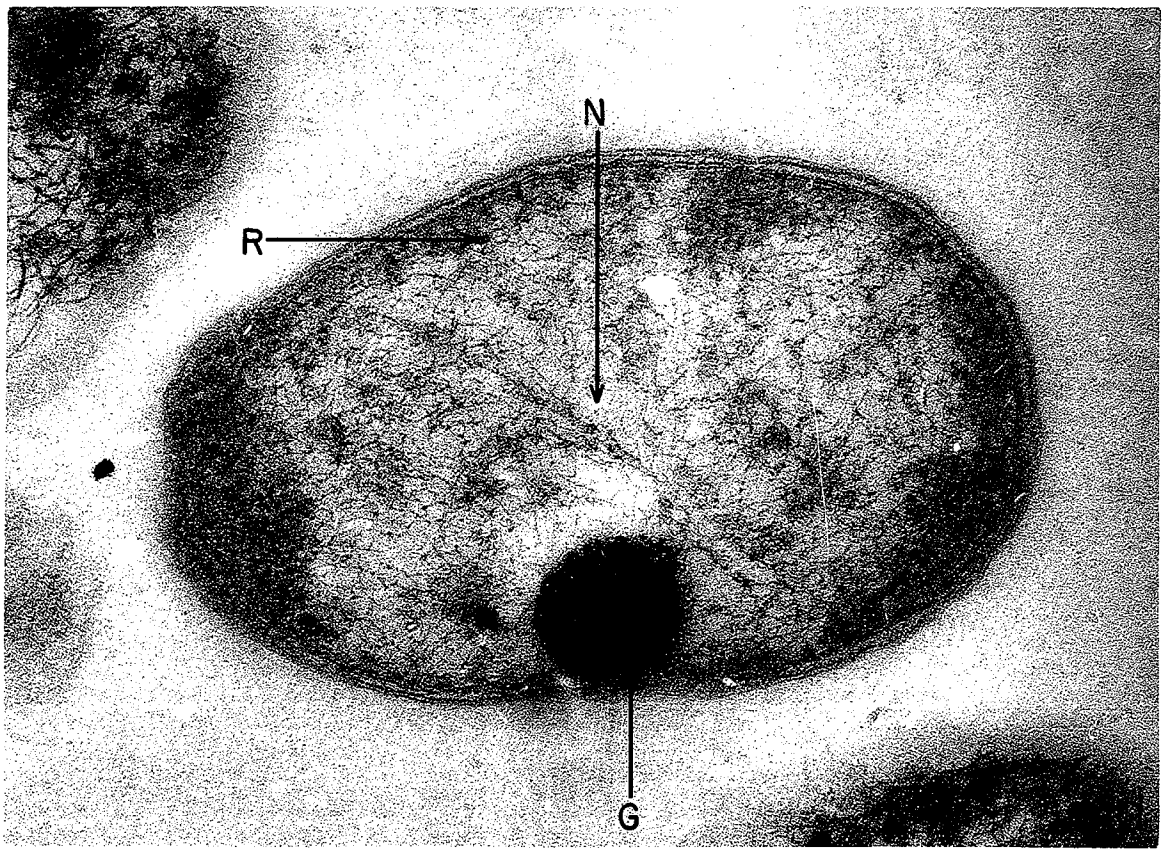
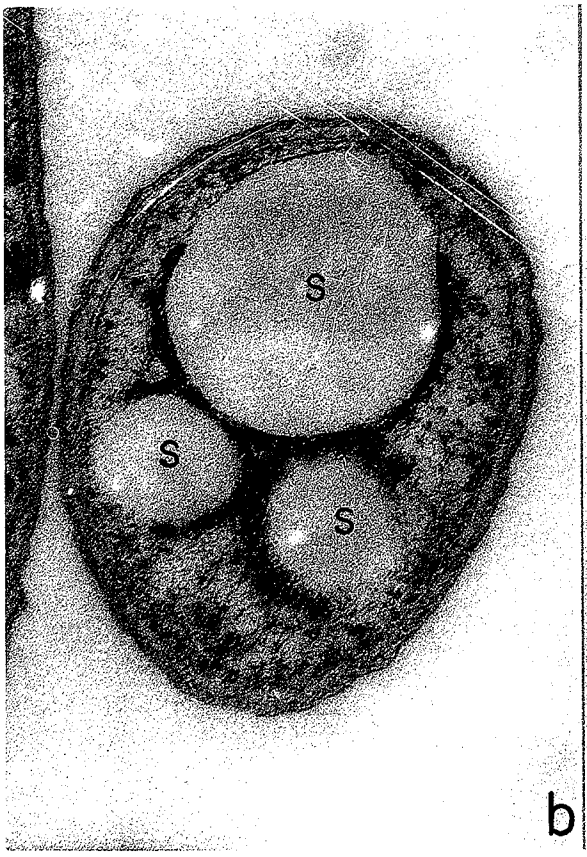

R
S
S
S
ce

Fig. 16. (a) Longitudinal section of autotrophically grown T. novellus showing general structure, ribosome (R), nuclear material (N) and granule (G). Cells were prepared as in Fig. 1. X 119,000.

(b) Cross section of heterotrophically grown T. novellus showing arrangement of polysaccharide globules (S). X 108,000.

(c) Whole cell mount of heterotrophically grown T. novellus showing polysaccharide globules (S) and background cytoplasm intensity (Cy). Cells were prepared as previously described (Materials and Methods, page 42). X 80,000.


N
R
G


S
S
S
b

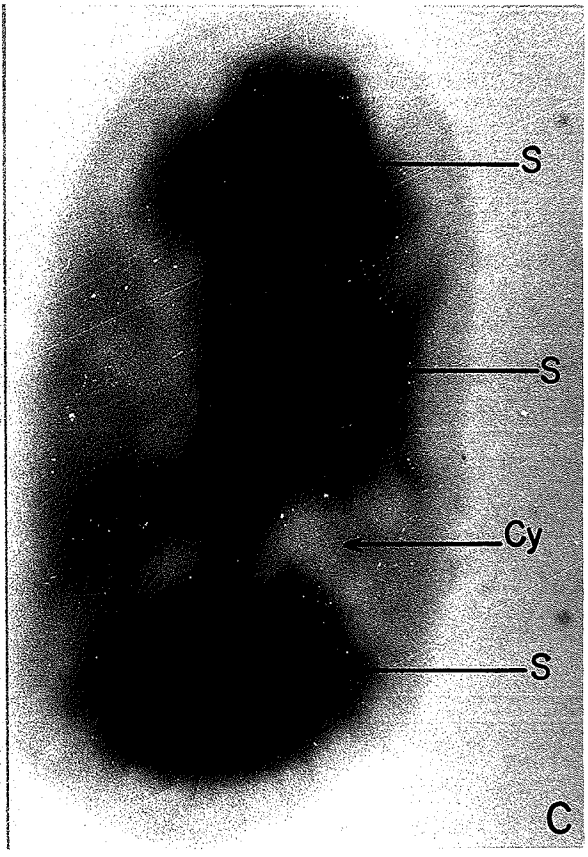
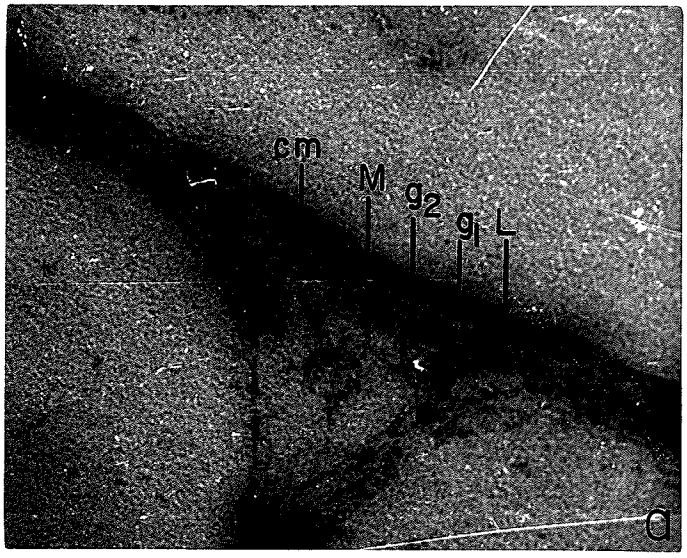
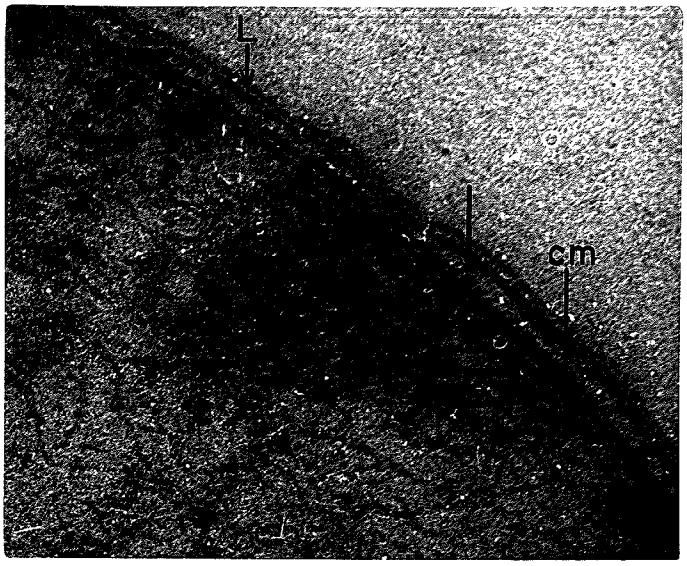

S
S
Cy
S
C

Fig. 17. (a) Section of cell envelope of heterotrophically grown T. novellus showing individual layers. Cells were prepared as in Fig. 1. X 260,000.

(b) Section of cell envelope of autotrophically grown T. novellus showing individual layers. Note diffuse nature or absence of $g_2$ layer (arrow). X 295,000.


cm
M
g2
g1
L
a


L
cm

17b). The cytoplasmic membrane of each cell was 55 - 65 Å thick. The overall thickness of the cell envelope of the heterotrophic cell was near 260 Å while that of the autotrophic cell was about 220 Å thick.

The cytoplasm of the autotrophic cell was very regular containing ribosomal particles near the cytoplasmic membrane and nuclear material throughout except at the tips of the cells where the cytoplasm appeared quite dense (Fig. 16a). There was also an extremely dense granule present in the vast majority of the cells (Fig. 16a). These may be aggregations of polymetaphosphate. The most striking feature of the heterotrophic cell was the presence of large electron transparent inclusions in the cytoplasm (Fig. 15, 16b and 16c). Chemical analysis suggested that these inclusions were made up of polysaccharide. The results of the analysis are shown in Table I.

It is evident from this table that only the polysaccharide content varied appreciably from one type of cell to the other. Specific staining with iodine caused the inclusions to turn electron dense (Fig. 16c) confirming the presence of polysaccharide. The cytoplasm of the heterotroph also contained electron dense granules (Fig. 15) similar in consistency to the granules found in autotrophic cells.

The cytokinetic behavior of both types of cells was

TABLE I

Analysis of dried whole cells of heterotrophically and autotrophically grown T. novellus

| Mode of growth | Protein | $PO_4$ | Lipid* | Polysaccharide | Total |
|---|---|---|---|---|---|
| Autotrophic | 44% | 5.2% | 24.2% | 6.2% | 79.6% |
| Heterotrophic | 43% | 8.7% | 19.1% | 17.4% | 88.2% |

*Assay of poly-β-hydroxybutyric acid showed that none was present in autotrophically grown cells while heterotrophic cells contained 4.6% by weight. This does not alter the above table since poly-β-hydroxybutyrate is also included in the general lipid assay.

identical. That of the autotrophic cell was chosen as an example (Fig. 18a, b and c). The cell envelope simply constricted as a unit with no laying down of single components before the constriction. It was also evident in these Figures that some degree of damage had occured in the cell wall area during fixation and embedding.

6. H. facilis

This organism was structurally the same in either the heterotrophic or autotrophic mode of growth. The only exception to this statement was the rate of deposition of poly-β-hydroxybutyric acid in the cytoplasm of each. The heterotroph (Figs. 20 and 21a) began to deposit this material at an earlier stage in the growth cycle than did the autotroph (Fig. 19). Each of these organisms was harvested slightly after the middle of the logarithmic phase of growth. Harvesting at later stages of growth resulted in cells virtually packed with poly-β-hydroxybutyrate. The globules then masked other cytoplasmic inclusions by squeezing them into a small area around the periphery of the cell.

The cell envelope, poorly defined in most sections, was the same in either growth mode (Figs. 21b and c). Its total thickness was 240 - 260 Å and the layers coincided with the typical Gram-negative cell envelope described by DePetris (1967). The nuclear material in actively growing

Fig. 18. Section of dividing cells of autotrophic *T. novellus*. Cells were prepared as in Fig. 1.

(a) Section showing initiation of cell division. X 160,000.

(b) Section showing division proceeded to approximately mid-way. X 155,000.

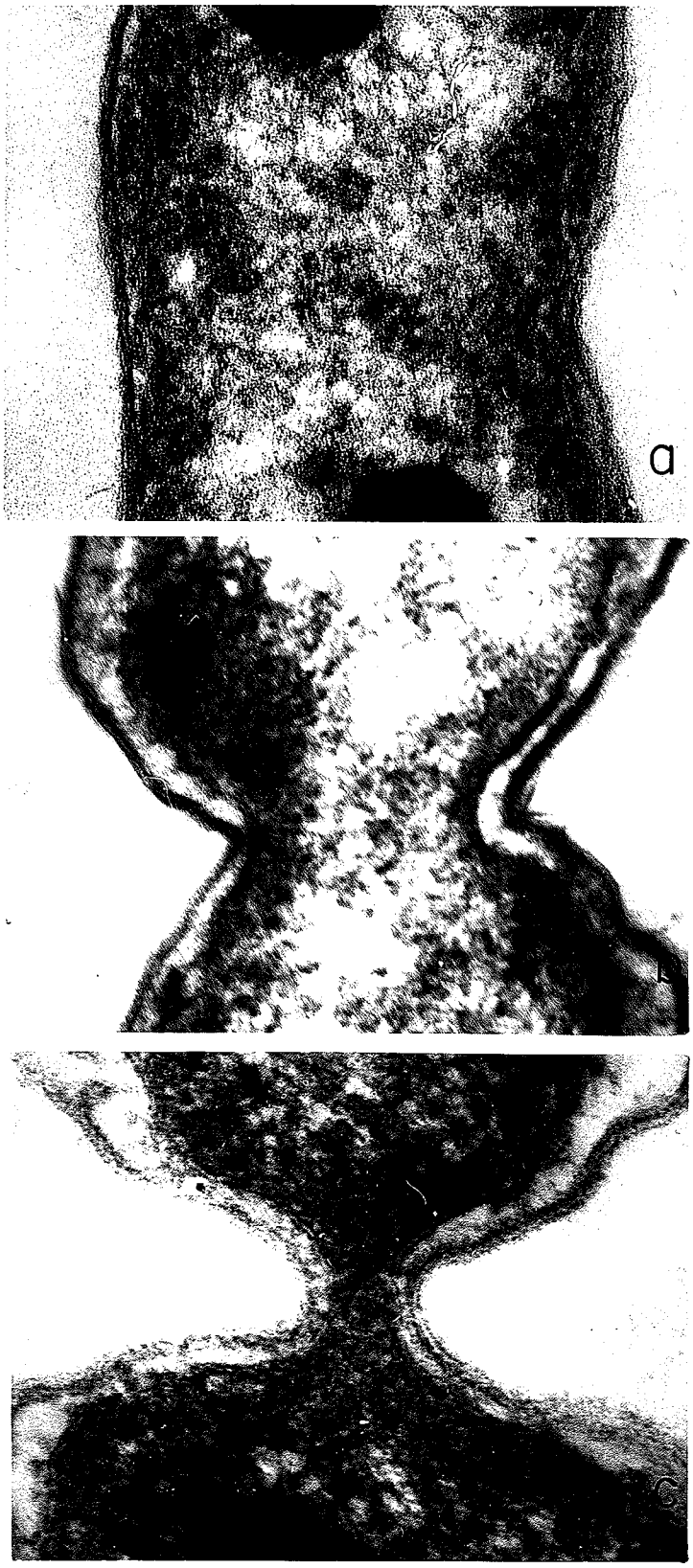
(c) Section showing division near completion. X 180,000.

Fig. 18. Section of dividing cells of autotrophic *T. novellus*. Cells were prepared as in Fig. 1.

(a) Section showing initiation of cell division. X 160,000.

(b) Section showing division proceeded to approximately mid-way. X 155,000.

(c) Section showing division near completion. X 180,000.


a

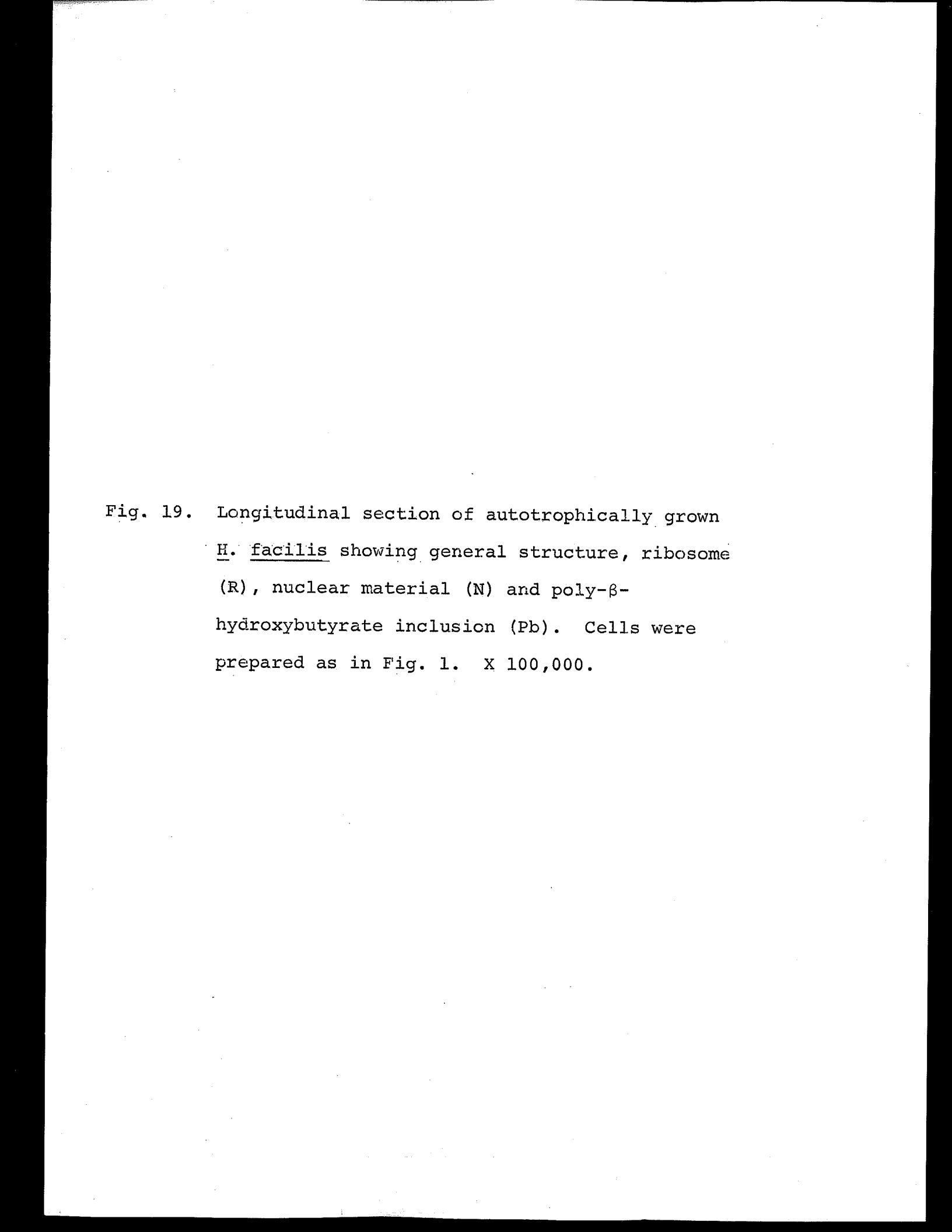


Fig. 19. Longitudinal section of autotrophically grown H. facilis showing general structure, ribosome (R), nuclear material (N) and poly-β-hydroxybutyrate inclusion (Pb). Cells were prepared as in Fig. 1. X 100,000.

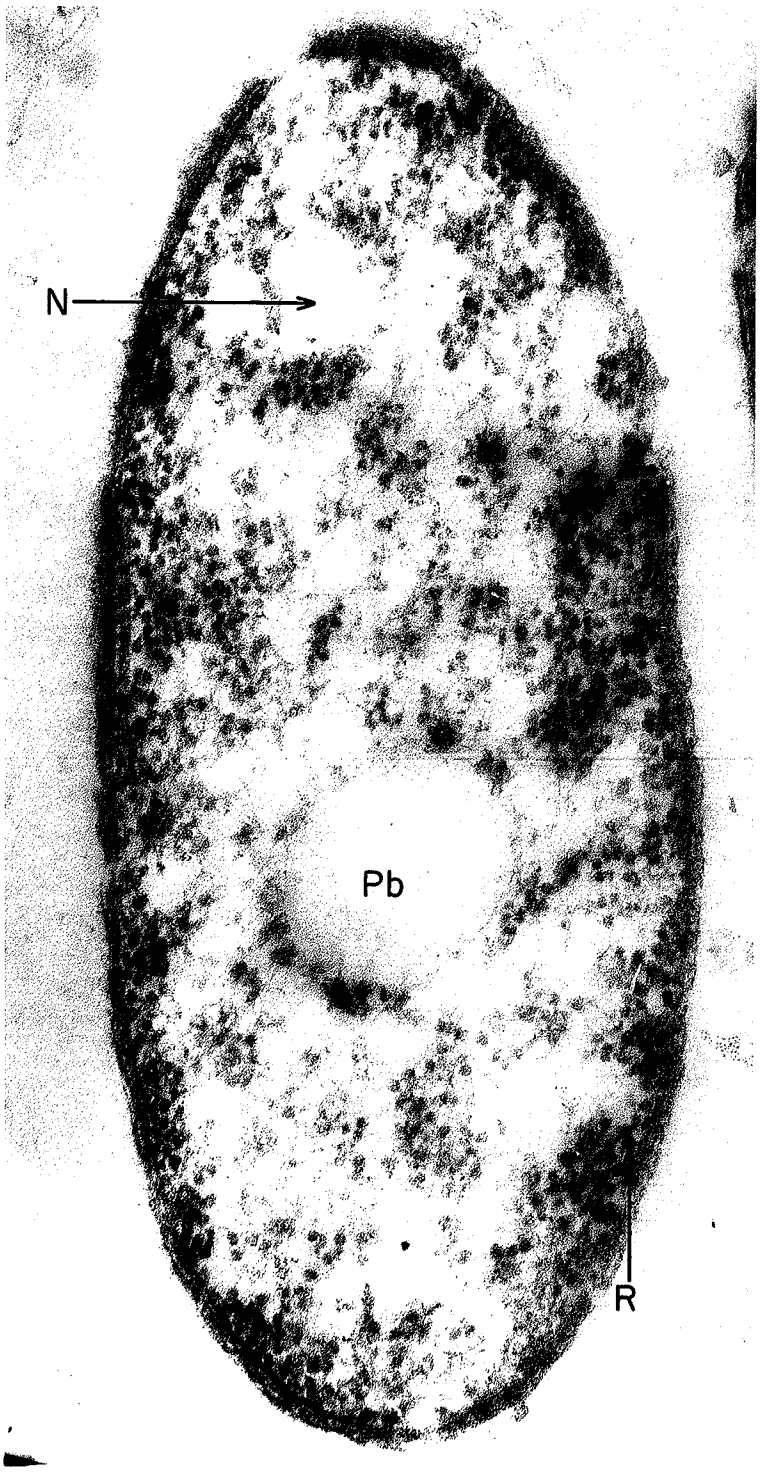

N
Pb
R

Fig. 20. Longitudinal section of heterotrophically grown H. facilis showing general structure, ribosome (R), nuclear material (N) and arrangement of poly-β-hydroxybutyrate globules (Pb). Cells were prepared as in Fig. 1. X 99,000.

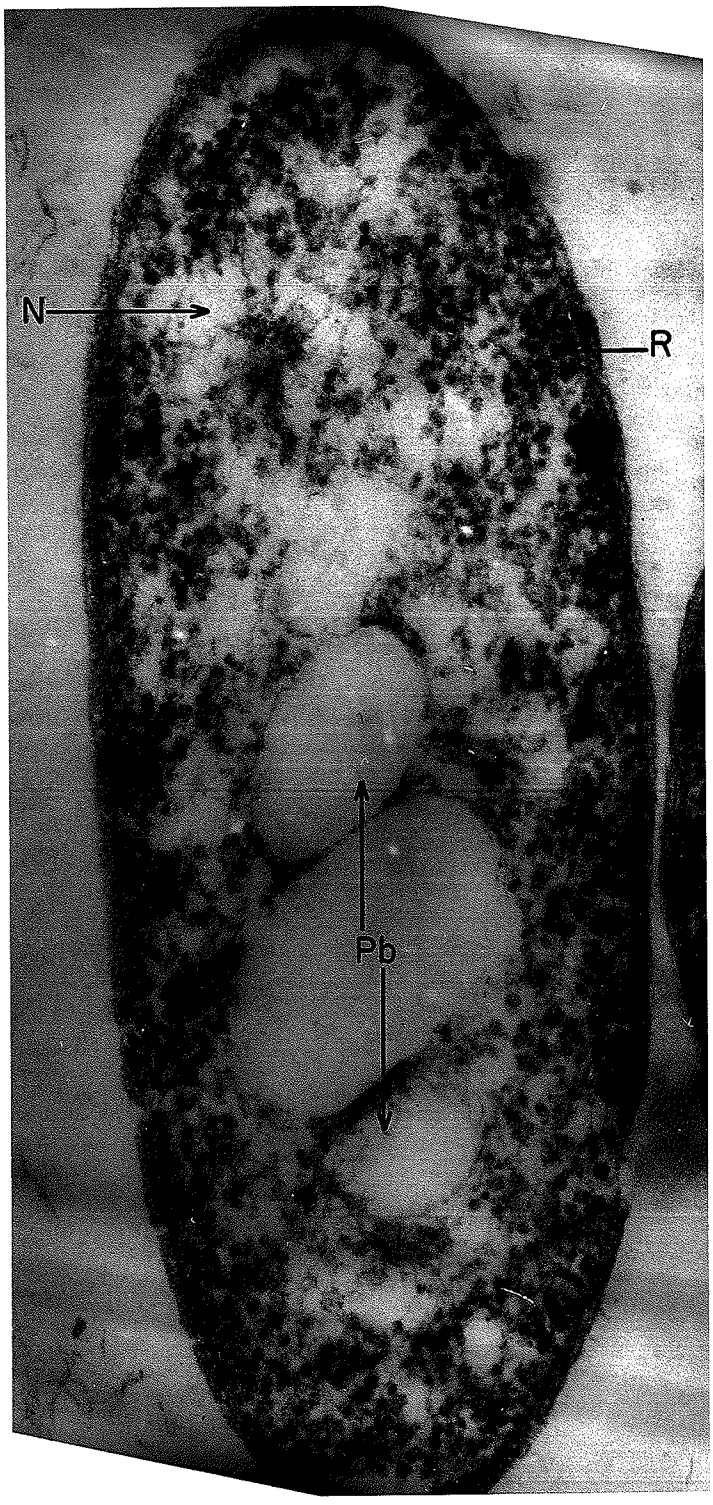
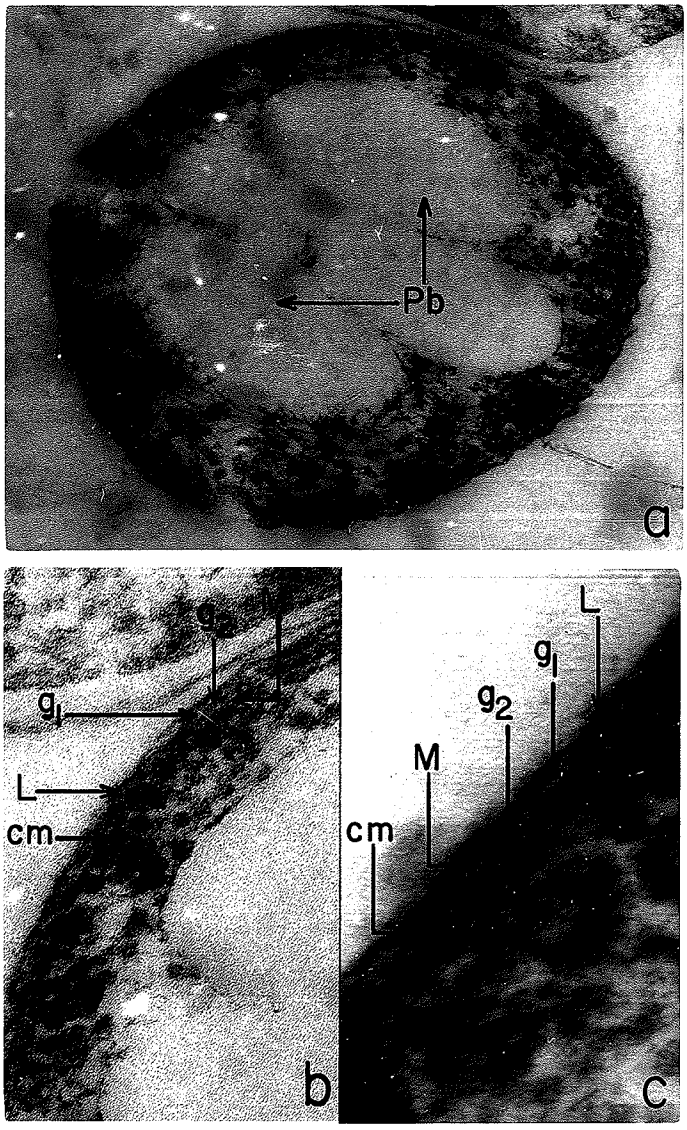

N
R
Pb

Fig. 21. Thin sections of H. facilis. Cells were prepared as in Fig. 1.

(a) Cross section of cell showing general structure and poly-β-hydroxybutyrate inclusions (Pb). X 80,000.

(b) Section of cell wall of heterotrophically grown H. facilis showing individual layers. X 150,000.

(c) Section of cell wall of autotrophically grown H. facilis showing individual layers. X 290,000.


Pb
a
g
g1
L
cm
b
L
g1
g2
M
cm
C

cells spread throughout the cytoplasm but was displaced toward the cell wall as cells aged (Fig. 21a). Ribosomal particles were dispersed throughout the cell in more abundance than in the thiobacilli. There was also a concentrated area of ribosomes near the cytoplasmic membrane.

H. facilis divided in the same way whether grown autotrophically or heterotrophically. The autotrophic cell division (Figs. 22a, b and c) which was chosen as representative was carried out in the same way as in other normal Gram-negative species (Conti et al, 1962). The cell envelope constricted as a unit and pinched off leaving single cells as opposed to chains of cells.

7. N. agilis

The cell envelope of N. agilis was distinctly different from that of normal Gram-negative bacteria (Fig. 24b). The equivalent of the L layer was 100 - 120 Å thick as compared to the normal 55 - 60 Å of Gram-negative cells. The inner dark layer of the triplet stained very heavily suggesting a heavy protein concentration in this area. The equivalent of the $g_1$, $g_2$ and M layers appeared as an electron transparent area 100 - 200 Å thick. It was prone to damage (Fig. 24a) as seen in the irregularity of the cross-sectioned cell. The cytoplasmic membrane (Fig. 24b) appeared typical with respect to its width and staining properties, however, as seems the case in all nitrifiers

Fig. 22. Thin sections of autotrophic H. facilis. Cells were prepared as in Fig. 1.

(a) Section of cell in initial stages of division. X 132,000.

(b) Section showing intermediate stage of division. X 120,000.

(c) Section of cell nearing completion of division. X 120,000.

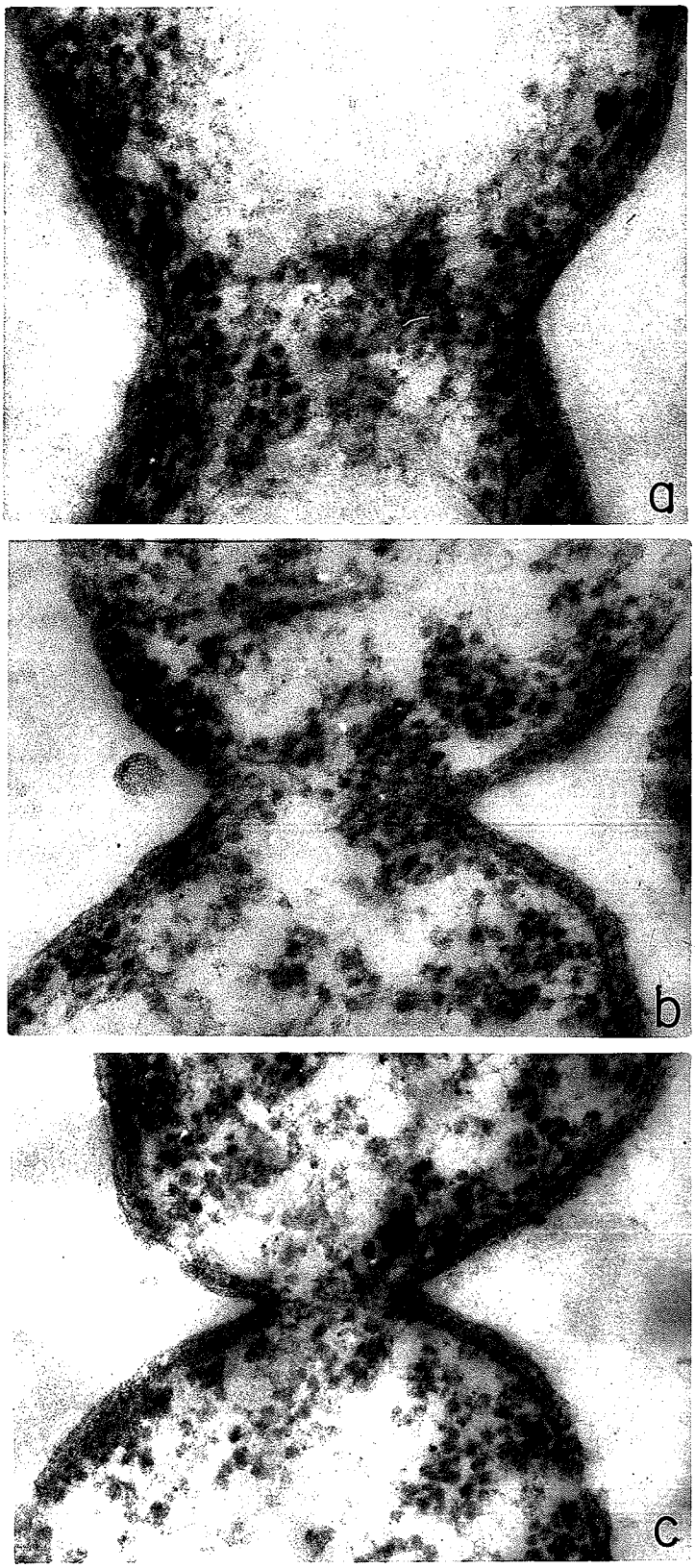

a
b
c

Fig. 23. Longitudinal section of N. agilis showing general structure, fibrillar whorl (F), nuclear material (N), internal membrane structure (IM), and individual layers of cell envelope. Cells were prepared as in Fig. 1. X 158,000.

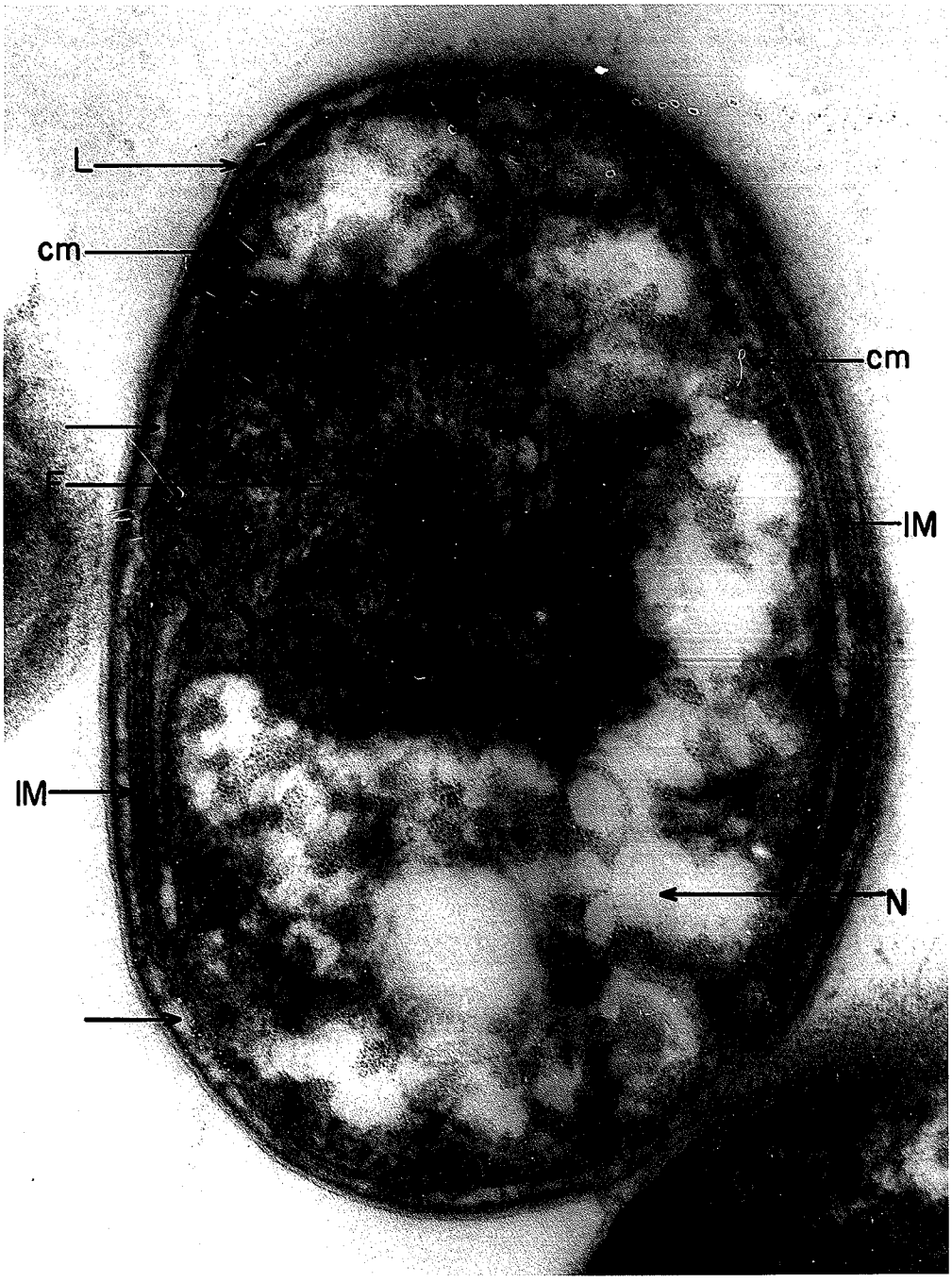

L
cm
cm
IM
IM
N

Fig. 24. Thin section of N. agilis. Cells were prepared as in Fig. 1.

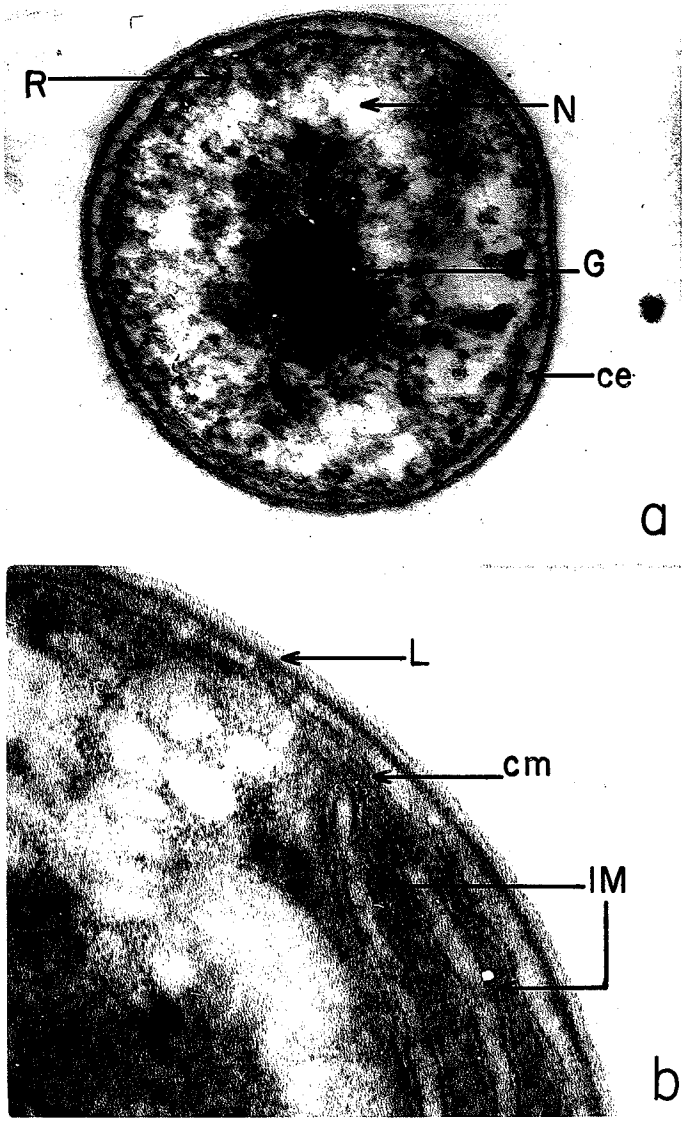
(a) Cross section of cell showing ribosome (R), nuclear material (N), cell envelope (ce) and granule (G). X 95,000.

(b) Section of cell wall showing outer layer (L), cytoplasmic membrane (cm) and internal membranes (IM). X 242,000.

Fig. 24. Thin section of N. agilis. Cells were prepared as in Fig. 1.

(a) Cross section of cell showing ribosome (R), nuclear material (N), cell envelope (ce) and granule (G). X 95,000.

(b) Section of cell wall showing outer layer (L), cytoplasmic membrane (cm) and internal membranes (IM). X 242,000.


R
N
G
ce
a
L
cm
IM
b

(Murray and Watson, 1965), it pinches off membranes which remain in close proximity to the cell envelope (Figs. 23 and 24b). The large majority of these membranes oriented themselves inside the cytoplasmic membrane but were not infrequently found between the cytoplasmic membrane and the remainder of the cell envelope (Fig. 23).

The cytoplasm of this organism was somewhat irregular (Fig. 23). A striking feature present in virtually all cells was a mass of fibril-like material thrown into a large whorl. It displaced the nuclear material from the centre of the cell and appeared to have an adverse effect on the membrane structures inside the cytoplasm. The nuclear material appeared stark white (Fig. 23) and was probably interspersed with globules of poly-β-hydroxybutyrate (Tobback and Laudelout, 1965). Also, in conjunction with the nuclear material were aggregations identical to the polyribosomal particles described in F. ferrooxidans (Lundgren et al, 1964). Occasionally an intensely stained inclusion (Fig. 24a) was observed. These were probably present in most cells but were small enough to be missed in sectioning.

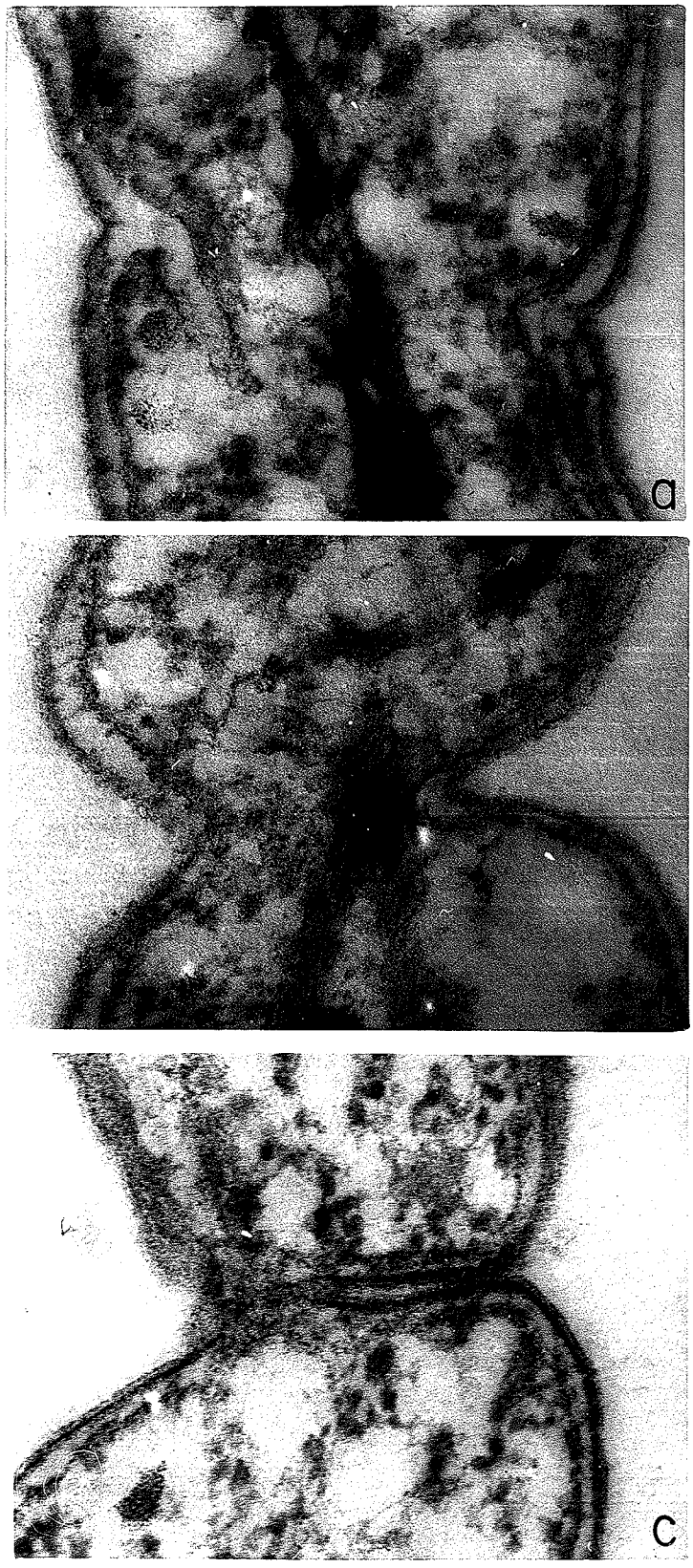
The cytokinetic properties of the organism (Figs. 25a, b and c) were ill-defined. At times a budding type of division could be seen, however, in the majority of cases a constrictive type of division as is shown was carried out. The division took place without involving the

Fig. 25. Thin sections of N. agilis. Cells were prepared as in Fig. 1.

(a) Cross section of cell in initial stages of division. X 186,000.

(b) Section showing intermediate stage of division. X 178,000.

(c) Section of cell in final stages of division. X 178,000.


a
c

internal membranes (Fig. 25a). The example shown appears atypical but this was probably caused by the extreme pliability of cells not containing an abundance of internal membranes.

8. N. europaea

This organism had a cell envelope typical of Gram-negative organisms. The L layer was 45 - 50 Å thick and formed blebs (Fig. 26a) when embedded in agar previous to fixation. The overall preservation of the cells mounted in agar cubes was superior to that of other techniques so that in the final analysis the organism was fixed and embedded in this way. The overall thickness of the cell envelope was 260 - 280 Å (Fig. 26b). The cytoplasmic membrane which was typically 55 - 65 Å thick formed a number of internal membranes by a pinching off procedure much the same as N. agilis. These membranes would align themselves concentrically around the entire cell or paritally around the cell depending on their stage of developement (Figs. 26 c and d).

The cytoplasm of the cell was quite fibrillar with nuclear material spread throughout. The presence of ribosomal material along the periphery of the cell was seen (Fig. 26a), but individual particles were masked by the intense staining in these areas.

The cell divided by a pinching off mechanism typical

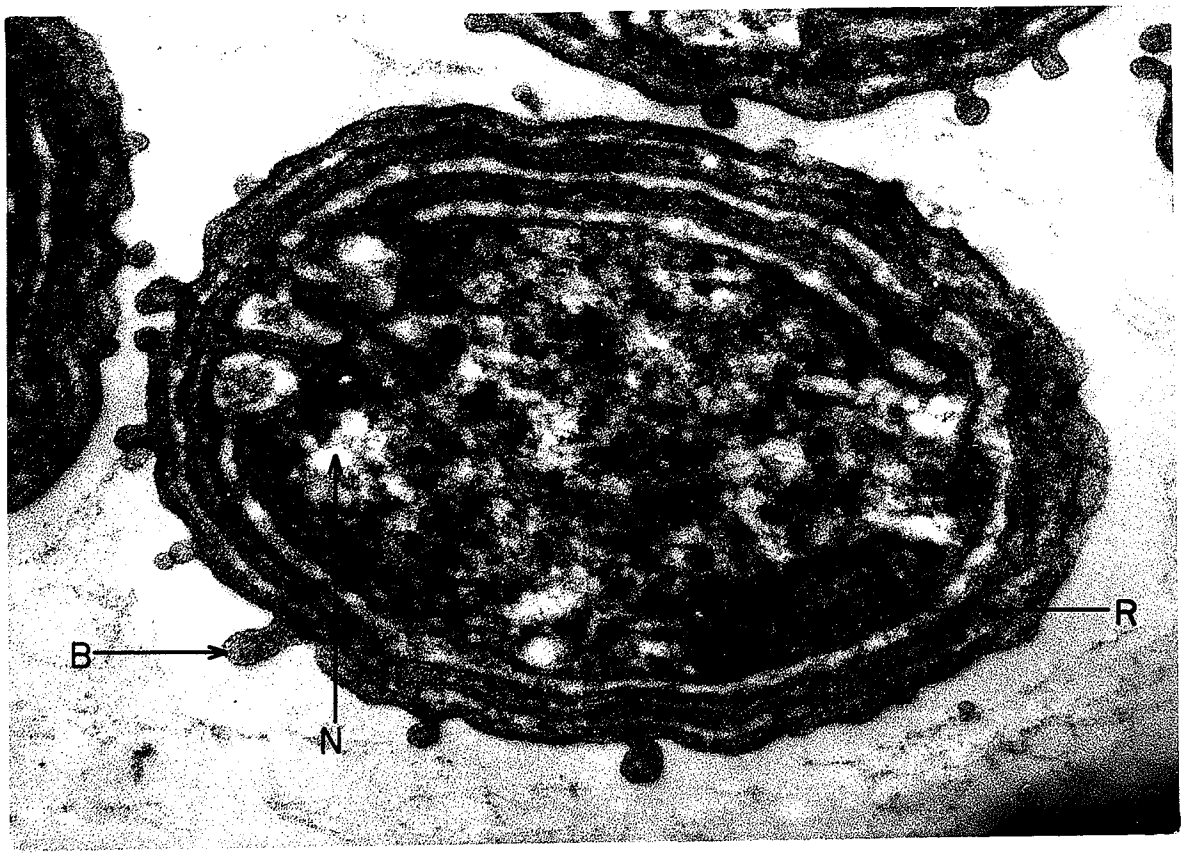
Fig. 26. Thin sections of N. europaea. Cells were prepared as in Fig. 1.

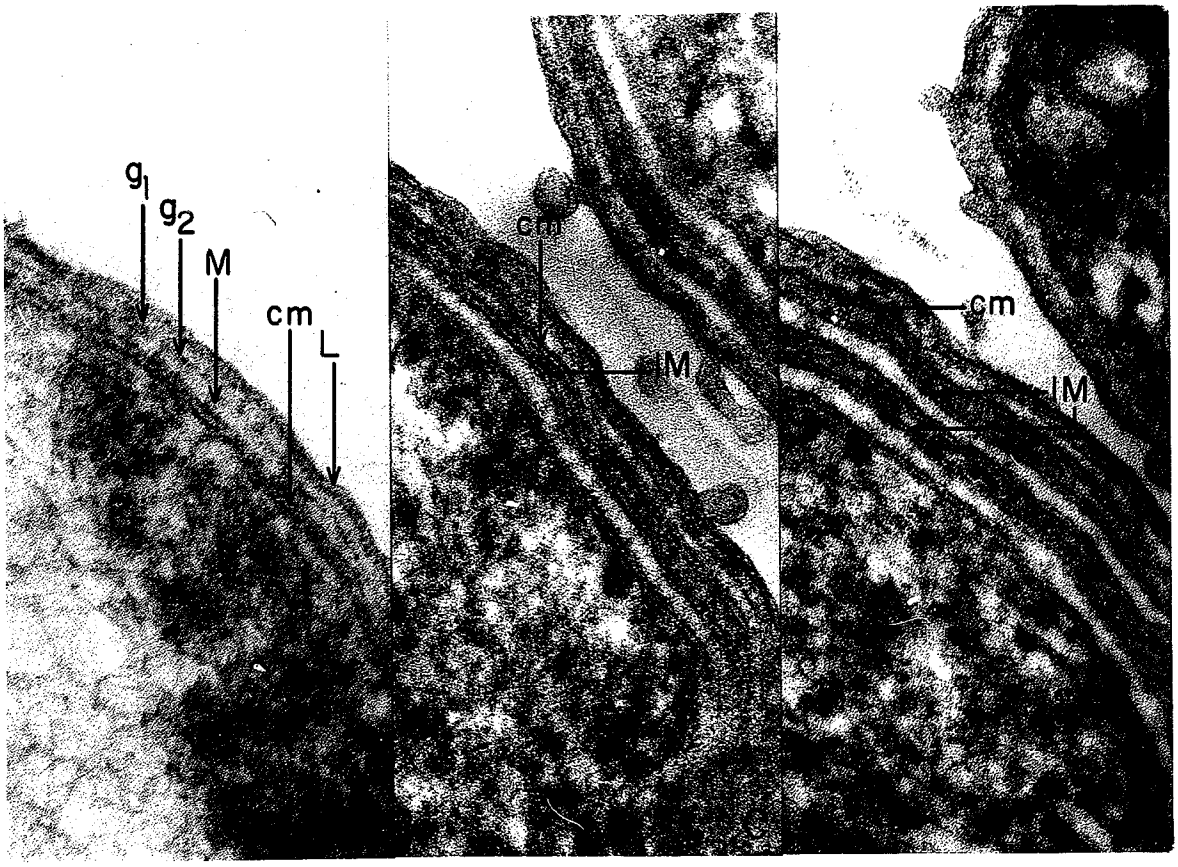
(a) Longitudinal section showing general structure, nuclear material (N) and concentration of ribosomal particles (R). Note presence of blebs (B) caused by embedding in agar previous to fixation. X 180,000.

(b) Section of cell envelope showing individual layers. X 302,000.

(c) Section of cell envelope with one set of internal membranes (IM). X 225,000.

(d) Section of cell envelope with two sets of internal membranes (IM). X 228,000.


B
N
R


g1
g2
M
cm
L
cm
M
cm
M

of Gram-negative bacteria (Figs. 27 a, b and c). The division often involved the internal membrane systems which appeared to be completed in the daughter cells (Fig. 27b) before the completion of the cell envelope. When the cell envelope had been completed in both daughter cells the two cells separated preventing the formation of chains of cells.

## Ammonia Oxidation Studies in *Nitrosomonas* Spheroplasts

*Nitrosomonas* spheroplasts, free from whole cells, were not able to oxidize ammonia (Fig. 28, Curve a) but regained the oxidizing ability in the presence of hydroxylamine (Curve c) or after preincubation with magnesium ion (Curve d) in agreement with Suzuki and Kwok (1969). There was some endogenous oxygen uptake by the control spheroplasts (Fig. 28, Curve a) which was not increased by the addition of ammonia. Hydroxylamine was rapidly oxidized (Curve b) but after the completion of its oxidation, the rate of oxygen uptake returned to the original level. In the presence of ammonia the addition of hydroxylamine resulted in an increased rate of oxygen uptake after the complete oxidation of hydroxylamine (Curve c) indicating a stable ammonia oxidizing system was present. Spheroplasts preincubated with magnesium ion before dilution in the reaction mixture also regained the ability to oxidize ammonia (Curve d). The oxidation of ammonia by magnesium treated as well as hydroxylamine

Fig. 27. Sections of N. europaea showing different stages of division. Cells were prepared as in Fig. 1.

(a) Section of cell showing initial stage of division. X 140,000.

(b) Section of cell showing intermediate stage of division. Note completion of internal membrane in daughter cell (arrow). X 122,000.

(c) Section of cell showing final stage of division. X 125,000.

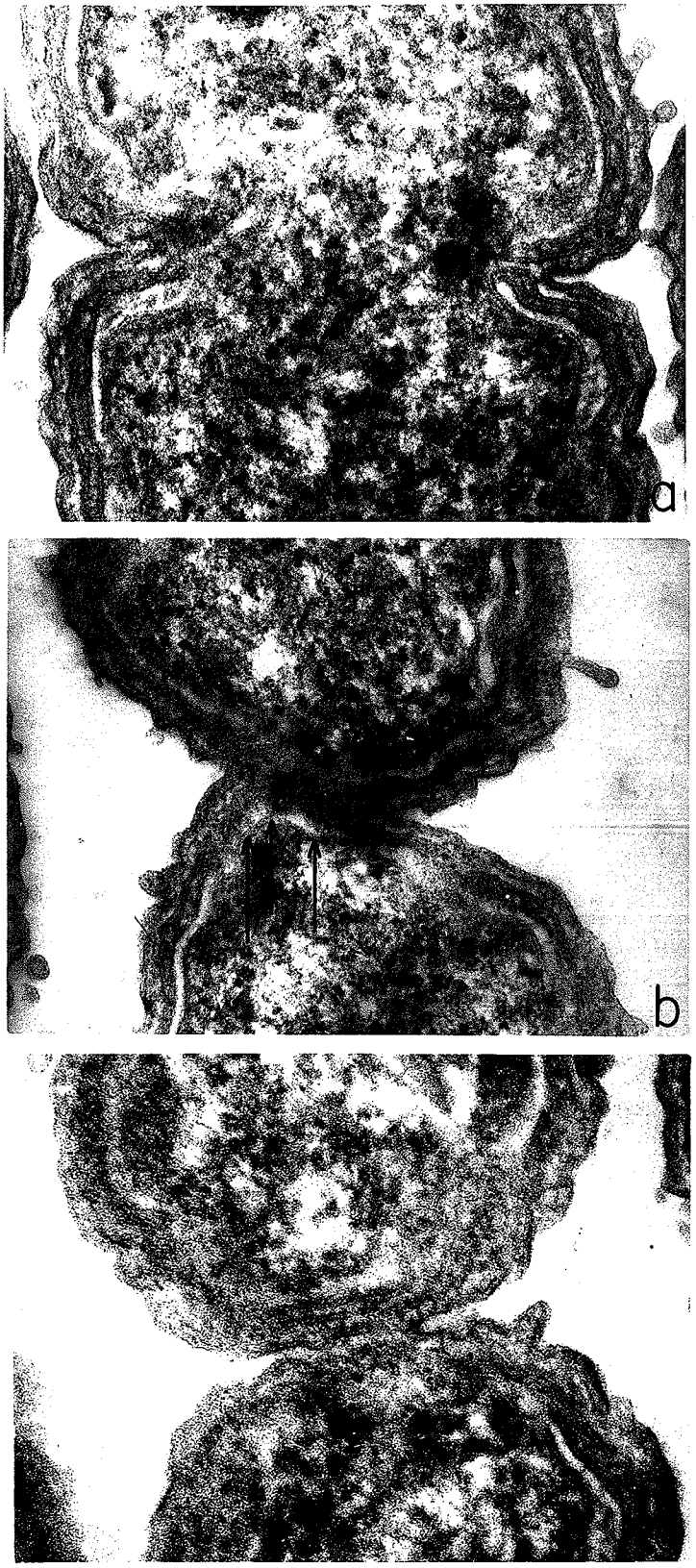
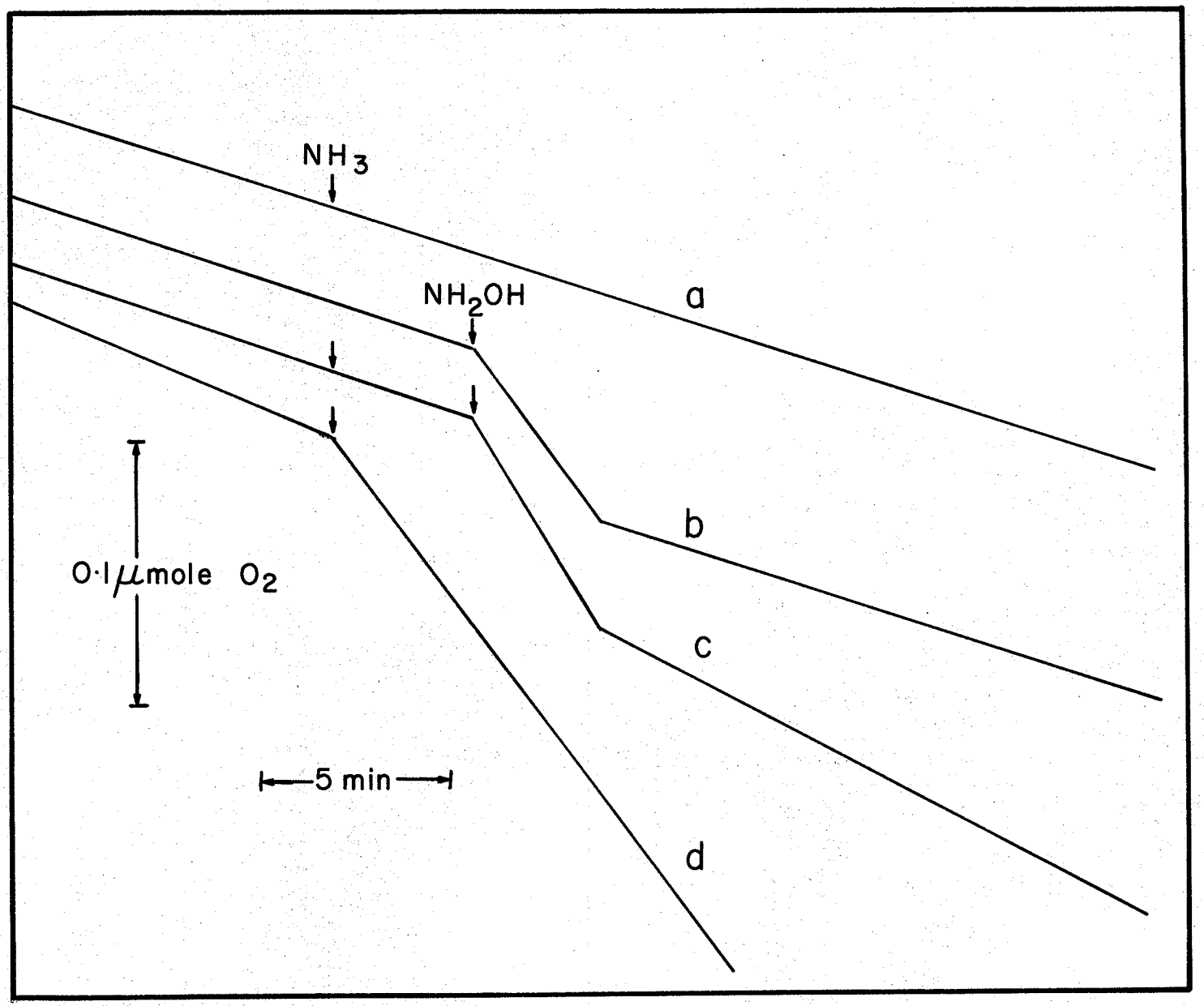

a
b

Fig. 28. Time course study of oxygen utilization by Nitrosomonas spheroplasts. At the times indicated by arrows, either 2.5 μ moles of $(NH_4)_2SO_4$ or 0.05 μ moles of $NH_2OH.HCl$ was added. The reaction mixture contained 0.1 ml of spheroplasts (derived from 10 mg of wet Nitrosomonas cells) and 1.375 ml of 0.25 M sucrose-0.1 M potassium phosphate buffer pH 7.5. Reactions were carried out at 20°. In experiment d, 0.1 ml of spheroplasts was pre-incubated with $MgSO_4$ (0.015 ml of 0.1 M) at 20° for 5 minutes before the initiation of the experiment.


NH3
NH2OH
a
b
c
d
0·1 μmole O2
5 min

activated spheroplasts was confirmed by nitrite determination.

Spheroplasts used in electron microscope studies were taken from a study with a Gilson Oxygraph. A representative field and single spheroplast (Figs. 29a and 30a) taken from a control mixture (Curve a) after ammonium sulfate addition showed typically smooth organisms with a minimum of disruption in the cytoplasmic membrane. Each spheroplast was bounded by an undisrupted triplet membrane which was probably the cytoplasmic membrane and a somewhat injured triplet membrane which was probably the L layer. The fibrilar nature of the cytoplasm was left intact indicating the absence of lysis.

A representative field and a single spheroplast (Figs. 29b and 30b) taken from a mixture to which ammonium sulfate and hydroxylamine had been added (Curve c, after hydroxylamine oxidation) showed spheroplasts which had evidently undergone an organizational change. Inside the organism a number of membrane bound inclusions were apparent. Inside the inclusions was a matrix which stained very intensely when treated by the same preparation procedure as the control sample. A triplet membrane remained intact around the entire spheroplast.

Ultrathin sections (Figs. 29c and 30c) from a sample containing 0.1 ml spheroplasts (derived from 10 mg wet weight Nitrosomonas cells) which had been preincubated

Fig. 29. (a) A typical field of sectioned spheroplasts of N. europaea taken from a reaction mixture containing ammonia. Spheroplasts were prepared as in Fig. 1. X 26,000.

(b) A typical field of sectioned N. europaea spheroplasts taken from a reaction mixture containing hydroxylamine and ammonia (after the complete oxidation of hydroxylamine). X 25,000.

(c) A typical field of sectioned N. europaea spheroplasts after pre-incubation with magnesium ion. X 26,000.

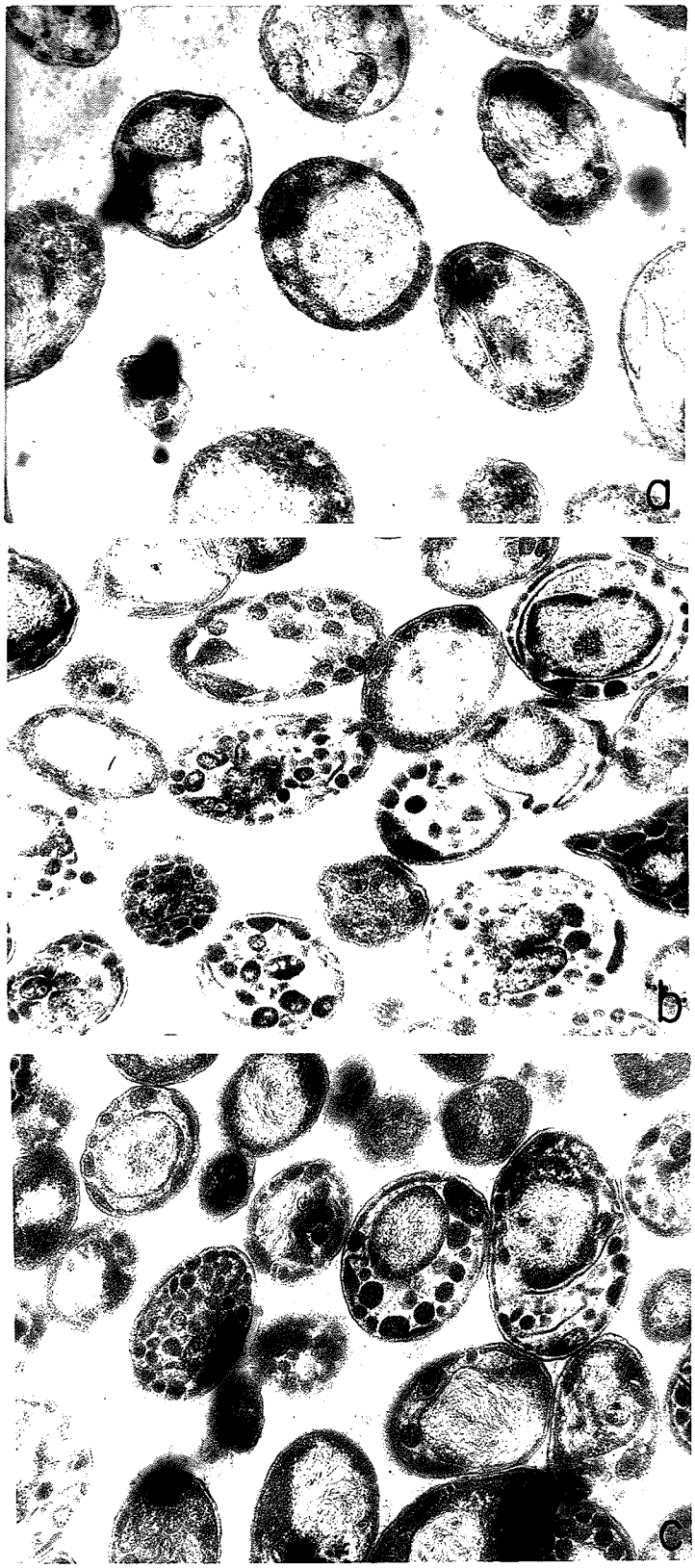
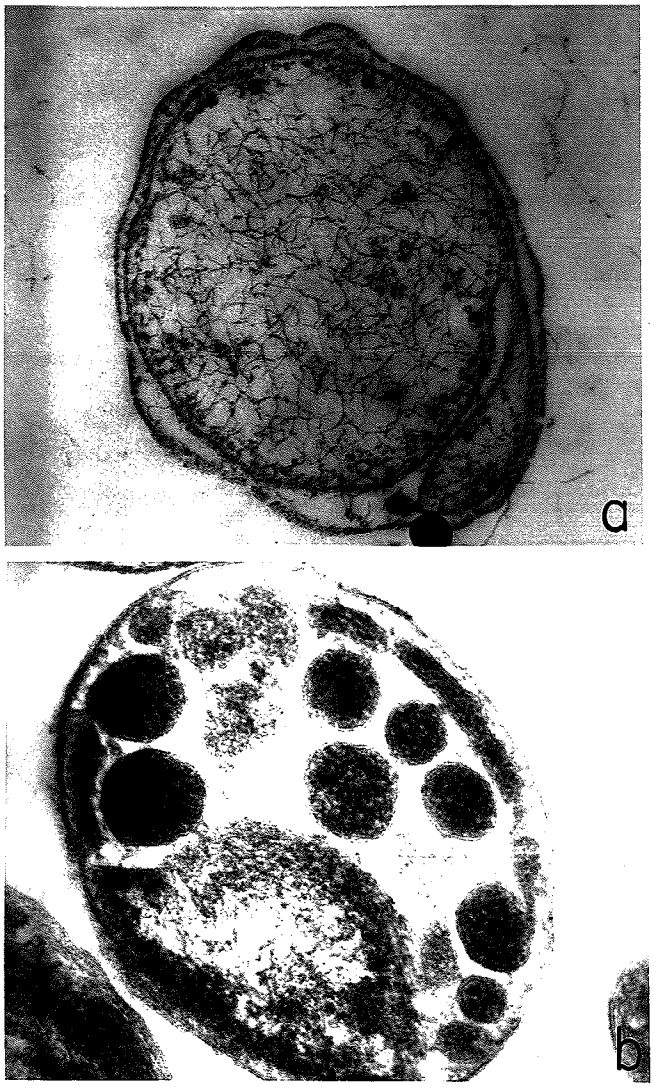

a
b
c

Fig. 30. (a) A typical sectioned N. europaea spheroplast taken from a reaction mixture containing ammonia. Spheroplasts were prepared as in Fig. 1. X 60,000.

(b) A typical sectioned N. europaea spheroplast taken from a reaction mixture containing hydroxylamine and ammonia (after the complete oxidation of hydroxylamine). X 66,000.

(c) A typical sectioned N. europaea spheroplast after pre-incubation with magnesium ion. X 66,000.


a
b
c

with 0.015 ml 0.1 M magnesium sulfate (Curve d, after ammonia addition) appeared to contain the same type of inclusions as spheroplasts treated with hydroxylamine. The staining properties of the matrix inside the bound inclusions was less intense, however the overall appearance of the spheroplasts indicated a similar structural change.

# DISCUSSION

## DISCUSSION

The thiobacilli, despite differences in energy sources and differences in preferred environments, appear to have similar cell envelopes. This result was not expected since some thiobacilli grow optimally under extreme conditions of pH and in the presence of toxic substrates while others are able to tolerate a pH only near neutrality. Properties of enzymes isolated from inside cells suggest that in general, the interior of cells remains near neutral pH, therefore it is expected that some component of the cell wall must form a barrier to the outside proton concentration. Some existing component of the cell wall must be altered to function as a barrier or a specific layer must be produced to function in this capacity. For example, T. thicoxidans not only exists but flourishes in a medium that is nothing more than a weakly buffered solution of 1 N sulfuric acid with a few normal essential inorganic elements and elemental sulfur. The only distinct difference in its cell profile with respect to heterotrophs is the presence of a dark $g_2$ layer. The supposition that the $g_2$ layer controls the hydrogen ion concentration inside the cell would not agree with existing knowledge of cell osmotic barriers. Marine pseudomonads which have a gradient of sodium chloride

across the cell membrane did not lose their ability to maintain this gradient nor did they lose the ability to transport amino acids into the cell after removal of the $g_2$ layer. Only the M layer and the cytoplasmic membrane from the cell envelope were left unaltered. They also retained the original shape of the organism (De Voe, Thompson, Costerton and MacLeod, 1970). It therefore seems likely that the proton barrier should also be localized in either the M layer or the cytoplasmic membrane. This was not evident in ultrastructural studies.

T. thiooxidans not only grows in a high hydrogen ion concentration but also uses a substrate which would be extremely toxic if left unbound in the cytoplasm. The organism uses sulfur as its sole energy source and probably reduces the sulfur to sulfide to transport it to the energy yielding site, oxidizes it and excretes it outside the cell where it forms sulfuric acid (Suzuki and Werkman, 1959). Sulfide is probably metabolized at or near the cytoplasmic membrane or in conjunction with a carrier protein to facilitate an easy entry and exit from the cell. It was thought that this arrangement would be evident in thin section studies but this did not prove to be so. In fact, the cytoplasmic membrane of T. thiooxidans appeared as delicate as that in any heterotrophic organism.

The remaining thiobacilli were grown with thiosulfate as the sole energy source. T. thioparus

metabolizes only the outer sulfur atom of thiosulfate ($S^*.SO_3$) (Skarzynski, Ostrowski and Krawczyk, 1957) leaving the other in the medium as sulfate. The outer sulfur atom is either used in protein structure or is deposited in the medium as elemental sulfur. Part of this elemental sulfur is then further oxidized to sulfate. This part of the energy yielding reaction is the same as that of T. thiooxidans and for that reason might be expected to produce a cell envelope similar to that of T. thiooxidans. In fact, T. thioparus had a cell envelope dissimilar to T. thiooxidans in the region of the $g_2$ layer. With the exception of T. novellus, all the thiobacilli examined maintained a cell envelope profile the same as normal Gram-negative bacteria grown in non-toxic, easily transportable substrates. It seems that the organisms in question are able to maintain a barrier to the potential toxicity of this substrate without drastic alteration of cell envelope parts.

T. novellus, the exception to the rule, did not display a typical cell envelope. This organism, although grown on thiosulfate, did not appear to have a $g_2$ layer in its cell envelope. This result was supported in a concurrent study of the thiobacilli (Shively et al, 1970). The $g_2$ layer was present in heterotrophically grown T. novellus which used glucose as its sole energy source. The fact that T. novellus which is able to grow hetero-

trophically or autotrophically does show appreciable structural difference between growth modes indicates that in some cases organisms do alter a major envelope structure to cope with environmental changes. It should be noted that the absence of the $g_2$ layer could be caused by a change in the layer on either side of it, therefore it is difficult to pinpoint the actual layer which has undergone change.

T. novellus, grown heterotrophically, contained large globules in its cytoplasm which proved to be made up of polysaccharide. Visual examination of these globules by standard staining techniques gave the impression that they were made up of poly-β-hydroxybutyrate as occurs in H. facilis (Schlegel et al, 1961). Chemical analysis of the cells disclosed an unusually high polysaccharide content in heterotrophic cells relative to autotrophic cells (11.2% difference). Heterotrophic cells contained only 4.6% by weight poly-β-hydroxybutyrate. This evidence in conjunction with results from a specific iodine staining technique devised for this study (Materials and Methods) leaves little doubt that the greater part of the globules are made up of polysaccharide. The relatively small percentage of poly-β-hydroxybutyrate could also be contained in these areas but this was not readily demonstrable. The depositing of polysaccharide probably reflects the fact that glucose is an excellent source of carbon and

energy while thiosulfate is a poor source of energy and is devoid of carbon.

T. denitrificans which is able to oxidize thiosulfate using either oxygen or nitrate as a terminal electron acceptor appeared the same under aerobic or anaerobic conditions. The difficulty that this organism undergoes in switching from aerobic to anaerobic metabolism (Vishniac et al, 1957) indicated that more than a simple induction of enzymes was necessary for the utilization of nitrate. If a structural requirement is necessary in this transformation it was not readily seen by ultrathin section study. Since both oxygen in aerobic growth and nitrate in anaerobic growth act as terminal electron acceptors in T. denitrificans respiration, the only difference expected when changing from anaerobic to aerobic growth would be in the cytochrome complex itself because neither substrate nor product is toxic. This level of structural change would not be detectable by present ultrastructural techniques.

The two species, T. ferrooxidans and F. ferrooxidans, appear the same in ultrastructural organization. The consistently present polyribosomal inclusions (Lundgren et al, 1964) which were not found in the other thiobacilli were arranged in the same patterns. The cell envelopes of the two species also behaved identically when subjected to the same fixation procedure. It therefore

appears from a purely ultrastructural standpoint that the two organisms are the same. This is in agreement with the findings of Margalith et al (1966) and Unz et al (1961) who found the species identical by chemical analysis and by nutritional studies.

H. facilis, considered by some (Davis et al, 1969) to be a pseudomonad, did not differ from the heterotrophic to the autotrophic growth mode. The physiology of this organism does not differ to a great extent from that of a pseudomonad. If the latter were supplied with the enzymes carboxydismutase, ribulosediphosphate kinase and a hydrogenase the two would be the same. It does not seem likely that a great deal of structural difference should be evident since the hydrogen molecule is not considered significantly removed from organic compounds such as formate which supports the growth of pseudomonads. Therefore, in essence, the organism is not changing modes of growth. The absence of bacteriophage particles specific to autotrophic organisms is somewhat baffling even considering difficulties in the isolation of such particles. The exception to this rule is H. facilis which is attacked by a phage from the soil (Pootjes et al, 1966). This, in a sense, supports the arguement that Hydrogenomonas is really a pseudomonad and not a typical autotroph.

The cytokinetic behavior of the thiobacilli

revealed some differences between species. T. thiooxidans displayed a distinct procedure which resembled the cytokinetic properties of some Gram-positive bacteria (Chapman et al, 1953). The completion of the cell envelope in both daughter cells in advance of the constrictive furrow is not characteristic of Gram-negative bacteria. The other species studied had cytokinetic properties which were very similar if not identical to heterotrophic Gram-negative bacteria. The defining of cytokinetic behavior of organisms has not been applied in a practical sense to date. The possibility of using this property as a key in taxonomic studies does not seem realistic because of the complexity of preparative procedures and the difficulty in keeping each parameter from affecting results.

The recognition of artifacts in ultrastructural studies has and will continue to be a major problem for cytologists. Rarely has a quantitative measure of artifacts been put forward for any specific case. Recently, a chemical evaluation of staining and fixing procedures has revealed that uranyl acetate plays a more important part in fixation than was previously thought (Silva, 1969). Silva found that when cells were dehydrated with ethanol or acetone after fixation in osmic acid or aldehydes the dehydrating agent extracted 18 - 22% of their lipid content. If the cells were fixed in the

same way but treated with uranyl acetate for a number of hours before dehydration only 1 - 2% of the lipid content was extracted. It is well known that autotrophic bacteria contain phospholipid in their cell walls (Korczynski et al, 1967). This finding may explain the peculiar undulated appearance of thiobacilli cell envelopes fixed by the Ryter-Kellenberger method (Shively et al, 1970). Those organisms containing less extractable lipid would appear smoother in profile than those with high lipid content.

The nitrifiers, N. europaea and N. agilis revealed somewhat more than was previously shown (Murray et al, 1965). The internal membrane structure of N. agilis (Schmidt strain) showed an unusual ability to displace the cytoplasmic membrane from the remainder of the cell wall and align itself between the two parts. This characteristic has not been reported to date. It is generally felt that these membranes contain the nitrite oxidizing systems of the cell yet it seems unusual that the organism would produce high energy intermediates and reduced pyridine nucleotides outside of the cytoplasmic membrane. This is not in keeping with the results of studies on heterotrophic organisms which carry on energy processes inside the cytoplasmic membrane. The cell chosen as representative of N. agilis in this study contains one of these unusual membrane structures but it would be unfair to suggest that all cells contained them. A fair estimate of the

incidence of these structures would be in the area of 30%. The possibility of this many artifacts seems unlikely.

The cell envelope profile of N. agilis is significantly different from heterotrophic Gram-negative bacteria. The inner aspect of the L layer was much more dense than that of other organisms studied. This property may be associated with the extreme toxicity of the energy substrate used by this organism. If nitrite, the energy substrate, were allowed to remain in solution in the cytoplasm of the cell there is little doubt it would destroy the organism because of the chemical reactivity of nitrous acid. The cell envelope profile of N. europaea, an organism which does not have a toxic energy substrate but does have a toxic energy metabolism product, appeared similar to normal Gram-negative bacteria.

The cytokinetic properties of the nitrifiers were more dramatic than those of the thiobacilli. It appeared that the internal membrane systems of N. agilis were not directly involved in the actual fission of the cell. In N. europaea, on the other hand, internal membrane systems were often split in two during cell fission. In some cases an unusual amount of these internal systems gathered at the fission site and obscured the mechanism involved. The behavior of the actual cell wall in both cases did not differ appreciably from that of Gram-negative organisms.

The property of Nitrosomonas that allows it to be stored for great lengths of time without losing its viability (Meikeljohn, 1954) combined with the fact that it loses the ability to oxidize ammonia faster than its ability to oxidize hydroxylamine (Lees, 1960) had led to the belief that it contained the ammonia oxidizing enzymes in its outer cell wall. The cell wall, when stripped away by lysozyme and EDTA treatment, did in fact cause a loss of ability to oxidize ammonia. Since EDTA chelates metal ions which may play a role in maintaining the integrity of the cell wall (Watson and Remsen, 1968), it was thought that the addition of a divalent ion such as magnesium to a suspension of spheroplasts might restore the lost oxidizing ability. This proved to be so but the dramatic effect of this ion on the internal membrane structure rather than the outer cell wall suggested that the ammonia oxidizing system lay within these internal structures rather than in the outer cell wall structure. Surprisingly, the addition of hydroxylamine which is not noted for its ability to alter structure caused the same effect. The internal structure again modified to an organized level and the lost ammonia oxidizing ability was restored. This suggested that the ammonia oxidizing system required a structural integrity to function. These findings eventually led to the isolation of a cell free system in both Nitrosocystis (Watson et al, 1970) and Nitrosomonas

(Suzuki and Kwok, 1970).

The alteration of cell structure mirroring a change in physiology has been shown in two cases. That of T. novellus as it changes from the autotrophic to the heterotrophic growth mode and in the case of N. europaea spheroplasts which regain lost ammonia oxidizing ability through structural change. It was not possible to show a trend through the species studied but interesting observations were made in most. A word of caution should be given to any wishing to use the electron microscope to observe structural difference as it appears that bacterial cells are able to cope with their environments with only delicate changes in their structure.

# REFERENCES

## REFERENCES


Adair, F. W. and Gunderson, K. (1968). Chemoautotrophic sulfur bacteria from the marine environment. II. Characterization of an obligately marine facultative autotroph. Can. J. Microbiol. 15:355-359.

Adair, F. W. and Gunderson, K. (1969). Chemoautotrophic sulfur bacteria in the marine environment. I. Isolation, cultivation and distribution. Can. J. Microbiol. 15:345-353.

Baalsrud, K. and Baalsrud, K. S. (1954). Studies on Thiobacillus denitrificans Arch. Mikrobiol. 20:34-62.

*Beijerinck, M. W. (1904). Über Bakterien welche sich im Dunkeln mit Kohlensäure als Kohlenstoffquelle ernähren können. Centr. Bacteriol. Parasitenk., Abt. II, 11:593-599.

Bratton, A. C. and Marshall, E. K. (1939). A new coupling component for sulfanilamide determination. J. Biol. Chem. 128:537-550.

Burge, W. D., Malavolta, E. and Delwiche, C. C. (1963). Phosphorylation by extracts of Nitrosomonas europaea. J. Bacteriol. 85(1):106-110.

Chapman, G. B. and Hillier, J. (1953). Electron microscopy of ultrathin sections of bacteria. I. Cellular division in Bacillus cereus. J. Bacteriol. 66:362-373.

Charles, A. M. and Suzuki, I. (1965). Sulfite oxidase of a facultative autotroph Thiobacillus novellus. Biochem. Biophys. Res. Commun. 19:686-690.

Colmer, A. R., Temple, K. L. and Hinkle, M. E. (1949). An iron-oxidizing bacterium from the acid drainage of some bituminous coal mines. J. Bacteriol. 59:317-328.

Conti, S. F. and Gettner, M. E. (1962). Electron microscopy of cellular division in Escherichia coli. J. Bacteriol. 83:544-550.

Davis, D. H., Doudoroff, M., Stanier, R. and Mandel, M. (1969). Proposal to reject the genus Hydrogenomonas. Taxonomic implications. Int. J. Syst. Bacteriol. 19:375-390.

DeCicco, B. T. and Stukus, P. E. (1968). Autotrophic and heterotrophic metabolism of Hydrogenomonas. J. Bacteriol. 95:1469-1475.

DeKruyff, C. D., Van der Walt, J. P., Schwartz, H. M. (1957). The utilization of thiocyanate and nitrate by thiobacilli. Antonie Van Leeuwenhoek J. Microbiol. Serol. 23:305-316.

Delwiche, C. C., Burge, W. D. and Malavolta, E. (1961). Reactions of cell free preparations of Nitrosomonas europaea. Fed. Proc. 20:350.

DePetris, S. (1965). Ultrastructure of the cell wall of Escherichia coli. J. Ultrastruct. Res. 12:247-262.

DePetris, S. (1967). Ultrastructure of the cell wall of Escherichia coli and chemical nature of its constituent layers. J. Ultrastruct. Res. 19: 45-83.

*de Saussure, T. (1839). Mem. Soc. Phys. Hist. Nat. Geneve 8:163.

De Voe, I. W., Thompson, J., Costerton, J. W. and MacLeod, R. A. (1970). Stability and comparative transport capacity of cells, mureinoplasts and true protoplasts of a Gram-negative bacterium. J. Bacteriol. 101:1014-1026.

Dugan, P. R. and Lundgren, D. G. (1965). Energy supply for the chemo-autotrophe Ferrobacillus ferrooxidans. J. Bacteriol. 89(3):825-834.

Fauré-Fremiet, E. and Rouiller, C. (1958). Etude au microscope électronique dúne Bactérie sulfureuse, Thiovulum majus Hinze Exptl. Cell Res. 14(1):29-46.

Frank, H. and Dekegel, D. (1965). Interpretation of cell wall structures in thin sectioned Gram-negative bacteria. Zentr. Bakteriol. Parasitenk Abt. I Orig. 198:81-87.

Fuhs, W., Iterson, W. van, Cole, R. M. and Shockman, G. P. (1965). Symposium on the fine structure and replication of bacteria and their parts. Bacteriol. Rev. 29:277-293.

Glauert, A. M., Brieger, E. M. and Allen, J. F. (1961). The fine structures of vegetative cells of Bacillus subtilis. Exptl. Cell Res. 22:73-85.

Godson, G. N., Hunter, G. D. and Butler, A. V. (1961). Cellular components of Bacillus megaterium and their role in protein biosynthesis. Biochem. J. 81:59-68.

Gould, G. W. (1959). Ph.D. thesis. University of Aberdeen.

Happold, F. C., Johnstone, K. I., Rogers, H. J. and Youatt, J. B. (1954). The isolation and characteristics of an organism oxidizing thiocyanate. J. Gen. Microbiol. 10:261-266.

Happold, F. C., Jones, G. L. and Pratt, D. B. (1958). Utilization of thiocyanate by Thiobacillus thioparus and Thiobacillus thiocyanoxidans. Nature 182:266-268.

*Harmsen, G. W. (1938). Biologische ijzeromsettingen in den bodem. Chem. Weekblad 35:495-500.

Hess, W. M. (1966). Fixation and staining of fungus hyphae and host plant root tissue for electron microscopy. Stain. Technol. 41:27-35.

Hofman, T. (1953). The biochemistry of nitrifying organisms. III. Composition of Nitrosomonas. Biochem. J. 54:293-295.

Hofman, T. and Lees, H. (1953). Composition and cytology of Nitrosomonas. Biochem. J. 53:v.

Hutchinson, M., Johnstone, K. I. and White, D. (1965). The taxonomy of certain thiobacilli. J. Gen. Microbiol. 41:357-364.

Iterson, W. van (1961). Some features of a remarkable organelle in Bacillus subtilis. J. Biophys. Biochem. Cytol. 9:183-192.

Jensen, H. L. (1950). Effect of organic compounds on Nitrosomonas. Nature 165:974.

Johnstone, K. I., Townshend, M. and White, D. (1961). Inter-species change in thiobacilli. J. Gen. Microbiol. 24:201-206.

*Kaserer, H. (1906). Centralbl. Bakt. Abt. II 16:681.

Kates, M., Yengoyan, L. S. and Sastry, P. S. (1965). A diether analog of phosphatidyl glycerophosphate in Halobacterium cutirubrum. Biochim. Biophys. Acta. 98:252-268.

Kay, D. H. (1965). Techniques for Electron Microscopy, The Alden Press, Oxford, England.

Kellenberger, E., Ryter, A. and Séchaud, J. (1958). Electron microscope study of DNA - containing plasma. II.Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. 4:671-678.

Kocur, M., Martinec, T. and Mazenec, K. (1968). Fine structure of Thiobacillus novellus. J. Gen. Microbiol. 52:343-346.

Korczynski, H. S., Agate, A. D. and Lundgren, D. G. (1967). Phospholipids from the chemoautotroph Ferrobacillus ferrooxidans. Biochem. Biophys. Res. Commun. 29(4):457-462.

Latta, H. and Hartmann, J. F. (1950). Use of a glass edge in thin sectioning for electron microscopy. Proc. Soc. Exptl. Biol. Med. 74:436-439.

*Lautrop, H., Reyn, A. and Birch-Anderson, A. (1964). Proc. 4th Scand. Congr. Pathol. Microbiol., Oslo, 1964, p. 157. Universitetsforlaget, Oslo.

Leathen, W. W. and Braley, S. A. (1954). A new iron oxidizing bacterium Ferrobacillus Ferrooxidans. Bacteriol. Proc. 44.

Lees, H. (1954). Autotrophic Micro-organisms. Symposium Soc. Gen. Microbiol., Cambridge Univ. Press, New York, New York.

Lees, H. (1960). Energy metabolism in chemolithotrophic bacteria. Ann. Rev. Microbiol. 14:83-98.

Lees, H. (1962). Some thoughts on the energetics of chemosynthesis. Bacteriol. Rev. 26:165-167.

Lewis, R. F. (1964). The biochemistry of the cell wall of Nitrosomonas europaea. Biochim. Biophys. Acta. 83:237-239.

Lockett, W. T. (1914). Oxidation of thiosulfate by certain bacteria in pure culture. Proc. Royal Soc. (London) B87:441-444.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin reagent. J. Biol. Chem. 193:265-275.

Lundgren, D. G., Anderson, K. J., Rensen, C. C. and Mahoney, R. P. (1964). Culture, structure and physiology of the chemoautotroph Ferrobacillus ferrooxidans. Develp. Ind. Microbiol. 6:250-259.

Mahoney, R. P. and Edwards, M. R. (1966). Fine structure of Thiobacillus thiooxidans. J. Bacteriol. 92:487-495.

Margalith, P., Silver, M. and Lundgren, D. G. (1966). Sulfur oxidation by the iron bacteria Ferrobacillus ferrooxidans. J. Bacteriol. 92: 1706-1709.

Marton, L. (1941). The electron microscope a new tool for bacteriological research. J. Bacteriol. 41:397-413.

Meiklejohn, J. (1954). Autotrophic micro-organisms. Symposium Soc. Gen. Microbiol., Cambridge Univ. Press, New York, New York. 68-83.

Mudd, S., Polvitsky, K., Anderson, T. F. and Chambers, L. A. (1941). Bacterial morphology as shown by the electron microscope. II. The bacterial cell wall in the genus Bacillus. J. Bacteriol. 42:251-264.

Mulder, E. G. (1964). Iron bacteria, particularly those of the Sphaerotilus Leptothrix Group, and industrial problems. J. App. Bacteriol. 27:151-173.

Murray, R. G. E. and Watson, S. W. (1963). An organelle confined within the cell wall of Nitrosocystis oceanus. Nature 197(4863):211-212.

Murray, R. G. E., Steed, P. and Elson, H. E. (1965). The location of the cell wall of Escherichia coli and other Gram-negative bacteria. Can. J. Microbiol. 11:547-560.

Murray, R. G. E. and Watson, S. W. (1965). Structure of Nitrocystis oceanus and comparison with Nitrosomonas and Nitrobacter. J. Bacteriol. 89:1594-1609.

*Nathansohn, A. (1902). Über eine neue Gruppe von Schwefel bakterien und ihren Stoffwechsel. Mitt. zool. Sta. Neapel 15:655-680.

Nelson, D. H. (1930). The isolation of Nitrosomonas and Nitrobacter by the single cell technique. Science 71:541-542.

*Niklewski, B. (1906). Extr. Bull. Acad. Sci. Cracovie 911.

O'brien, H. C. and McKinley, G. M. (1943). New microtome and sectioning method for electron microscopy. Science 98:455-456.

Parker, C. D. (1945). The isolation of a species of bacterium associated with the corrosion of concrete exposed to atmospheres containing hydrogen sulfide. Aust. J. Exptl. Biol. Med. Sci. 23:81-90.

Pate, J. L. and Ordal, E. J. (1965). The fine structure of two unusual stalked bacteria. J. Cell Biol. 27: 133-140.

Pease, D. C. (1964). Histological Techniques for Electron Microscopy. Academic Press, New York, London 136.

Peck, H. D. (1968). Energy-coupling mechanisms in chemolithotrophic bacteria. Ann. Rev. Microbiol. 22:489-518.

Pootjes, C. (1964). Isolation of a bacteriophage for Hydrogenomonas facilis. J. Bacteriol. 87:1259.

Pootjes, C. F., Mayhew, R. B. and Korant, B. D. (1966). Isolation and characterization of Hydrogenomonas facilis bacteriophages under heterotrophic growth conditions. J. Bacteriol. 92:1787-1791.

Rees, M. and Nason, A. (1965). A P-450 like cytochrome and a soluble terminal oxidase identified as cytochrome o from Nitrosomonas europaea. Biochem. Biophys. Res. Commun. 21:248-256.

Rees, M. K. (1968). Studies of the hydroxylamine metabolism of Nitrosomonas europaea. I. Purification of hydroxylamine oxidase. Biochem. 7:353-366.

Remsen, C. and Lundgren, D. G. (1966). Electron microscopy of the cell envelope of Ferrobacillus ferrooxidans prepared by freeze-etching and chemical fixation techniques. J. Bacteriol. 92:1765-1771.

Repaske, P. (1966). Characteristics of Hydrogen bacteria. Biotechnol. Bioeng. 8:217-235.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:208-212.

Schatz, A. and Bovell, C. (1952). Growth and Hydrogenase activity of a new bacterium Hydrogenomonas facilis. J. Bacteriol. 63:87-98.

Schlegel, H. G., Gottschalk, G. and von Bartha, R. (1961). Formation and utilization of poly-β-hydroxybutyrate acid by knallgus bacteria (Hydrogenomonas). Nature 191:463-465.

Seifter, S., Seymour, S., Novic, B. and Muntwyler, E. (1950). The estimation of glycogen with the anthrone reagent. Arch. Biochem. 25:191.

Shively, J. M., Decker, G. L. and Greenawalt, J. W. (1970). Comparative ultrastructure of the thiobacilli. J. Bacteriol. 101:618-627.

Silva, M. T. (1969). The fixation action of uranyl acetate in electron microscopy. Separatum Experientia 24:1074.

*Skarzynski, B., Ostrowski, W. and Krawczyk, A. (1957). Investigations on the metabolism of sulfur in Thiobacillus thioparus with radioactive $^{35}S$. Bull. Acad. Polon. Sci., Class II. 5:159-164.

Slepecky, R. A. and Law, J. H. (1960). A rapid spectrophotometric assay for beta-unsaturated acids and beta-hydroxy acids. Anal. Chem. 32:1967.

Starkey, R. L. (1925). The physiology of Thiobacillus thiooxidans, an autotrophic bacterium oxidizing sulfur under acid conditions. J. Bacteriol. 10: 135-163.

Starkey, R. L. (1934). Cultivation of organisms concerned in the oxidation of thiosulfate. J. Bacteriol. 28:365-386.

Starkey, R. L. (1935). Isolation of some bacteria which oxidize thiosulfate. Soil Science 39:197-215.

Suzuki, I. and Werkman, C. H. (1959). Glutathione and sulfur oxidation by Thiobacillus thiooxidans. Proc. Natl. Acad. Sci. U. S. 45:239-244.

Suzuki, I. and Kwok, S. C. (1969). Oxidation of ammonia by spheroplasts of Nitrosomonas europaea. J. Bacteriol. 99:897-898.

Suzuki, I. and Kwok, S. C. (1970). Cell-free ammonia oxidation by Nitrosomonas europaea extracts: effects of polyamines, $Mg^{++}$ and albumin. Biochem. Biophys. Res. Commun. 39:950-955.

Taylor, B. F. and Hoare, D. S. (1969). New facultative Thiobacillus and a reevaluation of the heterotrophic potential of Thiobacillus novellus. J. Bacteriol. 100(1):489-499.

Tilton, R. C., Stewart, T. J. and Jones, G. E. (1967). Marine thiobacilli. II. Culture and ultrastructure. Can. J. Microbiol. 13(11):1529-1534.

Tobback, P. and Laudelout, H. (1965). Poly-β-hydroxybutyrate acid in Nitrobacter. Biochim. Biophys. Acta. 97:589-590.

Tsien, H., Lambert, R. and Laudelout, H. (1968). Fine structure and the localization of the nitrite oxidizing system in Nitrobacter winogradsky. Antonie Van Leeuwenhoek J. Microbiol. Serol. 34:483-494.

Tsien, H. C. and Laudelout, H. (1968). Minimal size of Nitrobacter membrane fragments retaining nitrite oxidizing activity. Arch. Mikrobiol. 61(3):280-291.

Umbreit, W. W. and Anderson, T. F. (1942). A study of Thiobacillus thiooxidans with the electron microscope. J. Bacteriol. 44:317-320.

Unz, R. F. and Lundgren, D. G. (1961). A comparative nutritional study of three chemoautotrophic bacteria: Ferrobacillus ferrooxidans, Thiobacillus ferrooxidans and Thiobacillus thiooxidans. Soil Science 92:302-313.

Van Neil, C. B. (1953). Introductory remarks on the comparative biochemistry of micro-organisms. J. Cell Comp. Physiol. 41:11-38.

Vishniac, W. and Santer, M. (1957). The thiobacilli. Bact. Rev. 21:195-213.

von Ardenne, M. (1939). Z. wiss. Mikroskop. 56:8.

Waksman, S. A. and Joffe, J. S. (1922). Thiobacillus thiooxidans, a new sulfur-oxidizing organism isolated from the soil. J. Bacteriol. 7:239-256.

Wallace, W. and Nicholas, D. J. (1969). The biochemistry of nitrifying microorganisms. Biol. Rev. 44: 359-391.

Watson, S. W. (1962). Nitrosocytis oceanus sp. mov. Abst. Eighth Inter. Cong. Microbiol. Montreal.

Watson, S. W. and Remsen, C. C. (1969). Macromolecular sub-units in the walls of marine nitrifying bacteria. Science 163:685-686.

Watson, S. W.,Asbell, M. A. and Valois, F. W. (1970). Ammonia oxidation by cell-free extracts of Nitrosocystis oceanus. Biochem. Biophys. Res. Commun. 38(6):1113-1119.

Waygood, E. R. (1948). Adaptation of the method of Lowry and Lopez to the estimation of organic and inorganic phosphate in plant extracts. Can. J. Res. Sec. C, 26:461-478.

Williamson, D. H. and Wilkinson, J. F. (1958). The isolation and estimation of the poly-β-hydroxybutyrate inclusions of Bacillus species. J. Gen. Microbiol. 19:198-209.

*Winogradsky, S. (1888). Über Eisenbakterian. Botan. Ztg. 46:261.

*Winogradsky, S. (1891). Ann. Inst. Pateur 5:92.

Wischnitzer, S. (1962). Introduction to Electron Microscopy. Pergamon Press, New York, Oxford, London and Paris.

*Not read in the original.