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A CYTOLOGICAL STUDY OF

THIOPLOCA INGRICA WISLOUCH

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Siegfried Maier, B.S., M.Sc.

The Ohio State University

1963

Approved by

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INTRODUCTION

The intriguing question of the relationship of the filamentous sulfur bacteria to other microorganisms has been complicated by the discovery of Thioploca. Although this microorganism occurs in mud below the maximum penetration of light through the overlaying water, it possesses a faint, blue-green pigmentation. Many investigators described Thioploca as just an association of trichomes of Beggiatoa which occurred under unusual circumstances. In its gross morphology Thioploca has been compared to the blue-green alga Microcoleus.

The discovery of Thioploca ingricta in the bottom mud of the Bass Island Area of Lake Erie (Randles, 1956) made this interesting, though little known, sulfur bacterium available for study. It was hoped that a cytological investigation of T. ingricta might clarify the relationship of Thioploca to Beggiatoa and to Microcoleus.

In cytological studies it is advisable to control the age of the organisms. Therefore it would be necessary to grow T. ingricta in the laboratory, preferably under defined conditions and in pure cultures. Understanding the conditions necessary for growth would also provide important information about the physiology of T. ingricta. However, if this aspect of the study was not successful, T. ingricta from natural sources could be used for the cytochemical investigations. Without the factor of age being controlled, a cytological comparison of T. ingricta, Beggiatoa alba, and Microcoleus vaginatus could be done.
to clarify the relationship of these three organisms. Since the order Beggiatoales is currently included in the class Schizomycetes, trichomes of Caryophanon latum of the order Caryophanales would also be included in the cytological investigation.
LITERATURE REVIEW

In 1907, Lauterborn reported the discovery of a new genus of filamentous sulfur bacteria in the calcareous mud of Lake Constance in Germany. He observed bluish tinted trichomes which resembled trichomes of *Beggiatoa*. The trichomes were 5 to 9 µm in diameter and contained numerous sulfur granules. Easily detectable crosswalls separated the individual cells, the length of the cells was 1.5 times greater than the width. The trichomes were enclosed in a wide, firm, colorless sheath which was sharply demarcated on the outside by mineral incrustations and varied in diameter from 50 to 160 µm, depending on the number of trichomes inside. Lauterborn observed from 1 to several dozens trichomes within the sheath which was at least 3 to 4 cm long and did not branch. Thick sheaths showed ringlike indentations. The trichomes, which were parallel or interwoven, showed an active, independent gliding motion. He considered that this organism was a close relative of *Beggiatoa*, and that it resembled in gross morphology the blue-green alga *Hydrocoleum*. This new sulfur bacterium was called *Thioploca schmidlei*.

A few years later another species of *Thioploca* was discovered by Wislouch (1912), who named the organism *Thioploca ingrica*. He had found it in the mud of the delta of the Neva River in Russia at a depth of water of 3 to 4 m. Up to 20 trichomes, with tapered ends, could be found in a sheath, the length of which was 1 cm and up to 80 µm in
diameter. The width of the trichomes varied from 2 to 4.5 μ, and the length of the individual cells from 1.5 to 8 μ. The gliding trichomes also contained numerous sulfur inclusions. Trichomes with few inclusions showed a greenish-blue color.

In the same year, Kolkwitz (1912) reported that he found *T. schmidlei* in two locations in the Rhine River and *T. ingrica* in calcareous mud in brackish water of the Baltic Sea. However, he was unable to observe any color in the trichomes which partially emerged from the open end of the sheath and then retreated again.

Koppe (1924) found *T. schmidlei* in the mud of several lakes of Northern Germany and noted also that the trichomes occasionally would leave the sheath.

*Thioploca ingrica* was observed in two more locations in Central Germany by Kolkwitz in 1955. Bergey's Manual (1957) states that *T. ingrica* was observed in various localities in Central Europe. In 1956, however, Randles had found *Thioploca* in the bottom mud near Rattlesnake and South Bass Islands in Lake Erie. Since the individual cells were 2.0 to 3.5 μ wide and 3.5 to 7.5 μ long, the organism was probably *T. ingrica*. Taylor (1957) recovered this species from mud samples taken from many locations in the Bass Island Area of Lake Erie.

Koppe (1924) noted that the individual blue-green trichomes of *T. schmidlei* corresponded in size to the trichomes of *Beggiatoa arachnoidea*. He felt that all species of *Thioploca* had similar counterparts among the *Beggiatoa*. According to the seventh edition of Bergey's Manual, *T. ingrica* would correspond to *Beggiatoa alba*. 
A third species, *Thioploca minima*, with trichomes 0.8 to 1.5 µ wide, would correspond to *Beggiatoa leptomitiformis*. *Thioploca mixta* appears to be an association of trichomes of *T. schmidlei* and *T. minima* enclosed in a common sheath. The close relationship of *Beggiatoa* and *Thioploca* was also noted by Koppe (1924), who reported that trichomes of *T. schmidlei* which had emerged from the sheath could not be distinguished from trichomes of *B. arachnoidea*. A distinction between free trichomes of *T. ingricta* and *B. alba* should, however, be possible since the latter possess rounded ends while the trichomes of *T. ingricta* have tapered ends. Taylor (1957) felt that this was not a constant characteristic, since he observed trichomes of *T. ingricta* with rounded ends.

*Beggiatoa* and *Thioploca* have segmented trichomes of similar dimensions which show gliding movements when in contact with a substrate and both contain sulfur globules. The distinctive morphological dissimilarity is the firm sheath around the several trichomes of *Thioploca*, while the trichomes of *Beggiatoa* are free. According to Buchanan (1955), the "existence of a sheath [for *Beggiatoa* has] not [been] definitely established," although Keil (1912) observed a thin mucilage sheath around individual trichomes of *Beggiatoa*.

Bergey's Manual (1957) lists two more genera of filamentous sulfur bacteria, *Thiospirillopsis* and *Thiothrix*. The trichomes of the genus *Thiospirillopsis* are spirally wound and exhibit a creeping and rotational motility. In distinction from the other genera of the family *Beggiatoaceae*, the segmented trichomes of *Thiothrix* are non-motile and sessile and are individually surrounded by a delicate sheath.
Although Buchanan (1955) and Wille (1902) reported that the reproductive conidia of Thiothrix have a creeping motility, Keil (1912) and Pringsheim (1949) did not observe motility of the conidia of the species of Thiothrix which they studied.

The correct taxonomic position of the filamentous sulfur bacteria has presented a problem. Migula (1894) wanted to combine the Beggiatoaceae with the Oscillatoriaceae among the Schizophyceae. He emphasized also in 1895, "in view of their internal structure the species of Beggiatoa are so similar to those in the genus Oscillatoria that they can hardly be separated generically." The same opinion was expressed by Pringsheim (1949) when he called the Beggiatoa colorless Myxophyceae rather than sulfur bacteria. This suggestion was based on the fact that Beggiatoa and Oscillatoria possess the same organization of trichomes, the same type of gliding motility and excretion of a thin mucilagenous "sheath." Furthermore, the great variation in the diameter of the trichomes of species of Beggiatoa is paralleled in some species of Oscillatoria.

Lauterborn (1907), in describing Thioploca, called attention to the morphological counterpart among the Cyanophyceae Hydrocoleum. Wislouch (1912) also compared Thioploca to the blue-green genera Hydrocoleum and Microcoleus. Yet, Lauterborn and Wislouch described the firm sheath of Thioploca, while Hydrocoleum and Microcoleus have wide, diffluent sheaths according to Smith (1950). The kinship of the filamentous sulfur bacteria with the Oscillatoriaceae is thus based on morphological criteria and the peculiar gliding motility.

On a broader basis, Robinow (1956) called attention to the relationship
between bacteria and blue-green algae. They possess the same range of basic shapes, do not form gametes or other visible structures for sexual reproduction, and diaminopimelic acid seems to be confined to these groups. Stanier and Van Niel (1962) added to this list of similarities the presence of muramic acid in the cell walls of bacteria and blue-green algae.

Winogradsky showed in 1887 that hydrogen sulfide is absolutely necessary for the growth of Beggiatoa. Keil (1912) reported autotrophic growth of Beggiatoa and Thiothrix in pure cultures when hydrogen sulfide was the source of energy. Bavendamm (1924) also grew Beggiatoa and Thiothrix according to Keil's method, although he did not obtain pure cultures. Ruhland and Hoffmann (1926) failed to grow Beggiatoa mirabilis by Keil's method, but succeeded using slide cultures. Bahr and Schwartz (1957) obtained growth in slide cultures of B. alba and Thiothrix nivea. In all of these experiments hydrogen sulfide was necessary for growth. Therefore, the filamentous sulfur bacteria were generally considered to be obligate chemoautotrophs.

In 1956, Bahr and Schwartz produced evidence that there were ecologic strains within species which required different tensions of the various gases and different electrode potentials. Previously, Johnson and Baker (1947) had grown B. alba in biologically clarified sewage, and in 1961, Faust and Wolfe grew pure cultures of B. alba on organic media without hydrogen sulfide. Scotten and Stokes (1962) isolated pure cultures of five heterotrophic strains of Beggiatoa. However, isolation of gliding trichomes was only successful after enrichment with hay of a medium which especially favored heterotrophic
variants. Trichomes of these variants were devoid of sulfur inclusions. When these heterotrophic isolates were grown in the presence of hydrogen sulfide, the cells contained refractive granules. In certain organic media the strains isolated by Scotten and Stokes (1962) required hydrogen sulfide for growth. Thus, they suggested that the organic matter was the source of carbon, while energy was obtained from the oxidation of hydrogen sulfide.

Lauterborn (1907) observed a light bluish color in trichomes of *T. schmidleii* and Wislouch (1912) concluded that the blue-green color observed in *T. ingrìca* was due to pigments similar to those found in blue-green algae. However, considering the habitat of *Thioploca*, Wislouch remarked that the pigments had no function. Yet, living blue-green algae had also been recovered from considerable depth in soil, where only heterotrophic nutrition could occur. Russell (1950) stated that although many soil algae can grow in the dark in media containing sugars, the majority of those in lower layers of soil had either been washed down or carried down by tillage. According to Gainey and Lord (1952), many Cyanophyta are capable of heterotrophic existence and may be abundant in polluted waters. Pringsheim (1949) discounted the reports of color in *Thioploca* and suggested that the color might be due to some optical effect. Apparently, Pringsheim saw neither *Thioploca* nor the paper of Wislouch which he cited as reference from a secondary source. The cited report of Wislouch was not concerned with *Thioploca*, and Wislouch (1912), in reporting *T. ingrìca*, did not say the color was observed "only when the sulfur content was high." Furthermore, Koppe (1924) observed the color best in starved trichomes without
sulfur inclusions. Kolkwitz (1912, 1955), however, was unable to detect any color. The existence of functional photosynthetic pigments in some strains of *Thioploca* has not been definitely established.

It may be that the common concept of chemoautotrophic *Beggiatoa* and photoautotrophic *Oscillatoria* is not necessarily correct. Between these two extremes may be found various intermediates: colored chemoautotrophs, forms which require hydrogen sulfide for energy but use an organic source of carbon, colorless heterotrophs capable of oxidizing sulfide, colorless heterotrophs not capable of oxidizing sulfide, and heterotrophs with functional photosynthetic pigments.

With morphological and physiological evidence pointing to a close relationship between the family *Beggiatoaceae* of the order *Beggiatoales* and the *Oscillatoriaceae* of the order *Oscillariales*, a similar correspondence should be expected from cytological investigations. According to Stanier and Van Niel (1962), this is true not only for *Beggiatoa* and *Oscillatoria* but for bacteria and blue-green algae in general. They point out that both of these large groups are uniquely characterized by the procaryotic state: the nuclear material has no membrane and divides amitotically. Higher organisms and all other protists—other algae, protozoans, fungi—are eucaryotic, they possess distinct limiting membranes around nuclei and cell organelles.

Such fundamental similarities, of course, do not clarify the relationship between the subgroups, specifically between the family *Beggiatoaceae* of the order *Beggiatoales* and the family *Oscillatoriaceae* of the order *Oscillariales*. Relatively little effort has been extended to show this relationship and the few reports in the literature are
highly conflicting. Variations in cytological techniques may, in part, be responsible for the variability in findings. Furthermore, reports are available only for Beggiatoa and Thiothrix.

Hinze (1901) used hematoxylin to stain sections of B. mirabilis and found a double wall surrounding a protoplasmic cylinder. He also observed an interior vacuole which was dissected by protoplasmic lamellae. Sulfur inclusions were irregularly distributed in the finely granular protoplasmic components. Instead of a definite nucleus he found numerous small chromatinic bodies. He also saw larger, round to oval bodies in the protoplasm which were removed by amylase. Delaporte (1939) used both, hematoxylin and the Feulgen technique, to stain chromatinic elements in B. mirabilis. She found a large number of Feulgen-positive granules irregularly distributed in the cytoplasm. She did not, however, confirm Hinze's observation of vacuoles. Delaporte obtained identical results in studies of Beggiatoa gigantea. With smaller species, such as B. leptomitiformis, Delaporte (1939) found chromatinic granules either irregularly distributed or in the form of an axial filament. This was especially true for cells with few sulfur inclusions. The axial filament was also deformed by the presence of lipid granules.

Guilliermond (1926) studied B. alba and found sulfur inclusions, lipid droplets, and metachromatic granules in the cytoplasm. Guilliermond stained trichomes with hematoxylin after fixation with formalin and observed neither a nucleus nor a central body but saw numerous siderophilic granules. Bahr and Schwartz (1956) observed a yellow-green fluorescent axial filament in unfixed B. alba, using
acridine orange. They used trichomes, fixed for 2-3 seconds in osmium tetroxide vapors, hydrolyzed at 45°C in 1 N HCl and stained by the Feulgen reagent, Giemsa, and basic fuchsin, and observed a stained axial filament. In trichomes stained by methyl green-pyronin or hematoxylin, they also saw axial filaments which were deformed or dislocated by sulfur inclusions. Drawert and Metzner-Küster (1958) observed a green-fluorescing central body in B. alba stained with acridine orange. However, they were never able to observe any Feulgen-positive structures or metachromatic granules in the cells. The intensity of staining of the lipids in the cells was correlated with the number of sulfur inclusions. Faust and Wolfe (1961) also observed lipid granules in cells of heterotrophic isolates of B. alba. Scotten and Stokes in 1962 observed one or two discrete ovoid or spherical bodies in cells of trichomes of heterotrophic strains of Beggiatoa. They used trichomes fixed in Helly's solution and stained with Giemsa. Other cytochemical tests for chromatinic material were not employed. Lipoidal and volutin granules also were regularly present. Petter (1935) obtained only a weak and diffuse Feulgen reaction with several species of Beggiatoa.

Cytochemical studies of Thiothrix have given divergent results. Delaporte (1939), who observed Feulgen-positive axial filaments in spirilla and bacilli, reported similar filaments in Thiothrix nivea. She felt that supravital staining and staining with hematoxylin corroborated the existence of the axial filament. Bringmann (1952b) demonstrated metachromatic bodies in old cells of T. nivea. The net-like appearance of old cells was ascribed to the depletion of sulfur inclusions which left empty spaces in the cytoplasm. Bahr and Schwartz
in 1957, using the same techniques which they used for *B. alba*, observed an axial filament of chromatinic material in *Thiothrix*. Drawert and Metzner-Küster (1958) studied *T. nivea* obtained neither specific fluorescence of chromatinic material nor Feulgen-positive structures. Fourment (1926), investigating *Thiothrix tenuis* and *Thiothrix tenuissima*, was unable to demonstrate metachromatic granules, nucleus, or central body. Only scattered siderophilic granules in the cytoplasm in direct contact with the sulfur inclusions were observed after cells were stained with hematoxylin.

Unlike the findings in the family *Beggiatoaceae* of the order *Beggiatoales*, there is a wide accord as to the configuration of the chromatinic material in the family *Oscillatoriaceae* of the order *Oscillatoriales* and *Cyanophyta* in general. However, a few divergent results have been reported. Bringmann (1950, 1952a) used phase contrast microscopy and observed in each cell of *Lyngbia* one, two, or more "empty" bodies which gave a positive Feulgen reaction without hydrolysis. In order to obtain a positive Feulgen reaction following hydrolysis, prior precipitation with lanthanum acetate was necessary. The method for treatment with lanthanum acetate was not described. The granules also stained with methyl green. The presence of ribonucleic acid in the same bodies was demonstrated with pyronin and of metaphosphate by metachromasy with toluidine blue. In the electron microscope these basophilic structures were electron-dense. Bringmann called these granules "Karyoide" which means nuclear equivalents. But Winkler (1953) contended that the "Karyoide" described by Bringmann were metachromatic granules and not nuclear equivalents at all. She
cited evidence that pyronin and methyl green are not reliable indicators of the type of nucleic acids of lower forms. Bahr and Schwartz (1957) used the techniques which they used for Beggiatoa and Thiothrix and stained chromatinic material in Oscillatoria and Phormidium in the form of an axial filament or central strand.

Delaporte (1940) who had observed axial filaments in members of the family Beggiatoaceae, stained nuclear material in Oscillatoria, Phormidium, and Schizothrix as a central body. Zastrow (1953) examined 13 species of Oscillatoria, Lyngbia, and Phormidium and found in all of these forms a central body of varying configuration, which stained with methylene blue and pyronin, fluoresced with acridine orange, and gave a positive Feulgen reaction. Drawert and Metzner (1956) also obtained selective fluorescence of the central body in Microcoleus and Oscillatoria. A thorough study of the central body in certain Myxophyceae was made by Cassel and Hutchinson (1954). They investigated Oscillatoria, Phormidium, and Microcoleus vaginatus along with other blue-green algae. Robinow's (1944) acid-Giemsa technique and the Feulgen reaction were checked with perchloric acid extraction. The appearance of stained cells was compared to the appearance of living cells in the phase contrast microscopes. In all organisms examined, the chromatinic material was located in the central body showing three general types of arrangement: "1. a loose, sometimes granular, net-like organization; 2. rodlike elements oriented parallel to the longitudinal axis of the cell groups; and 3. a very much condensed organization of various shapes." It is particularly inter-
esting to note that all three types could be encountered in the same species. Therefore, the configuration of the chromatinic material in the central body does seem to vary considerably within each species.

The nature of the characteristic inclusions of *Beggiatoaceae* has been interpreted in various ways. Thus, Wille (1902) contended that the inclusions in *Thiothrix* were gas vacuoles. Winogradsky (1887) had shown earlier that the highly refractive bodies in *Beggiatoa* were plastic, elemental sulfur. After comparing the behavior of the inclusions of *Beggiatoaceae*, flowers of sulfur, and plastic sulfur, treated with physical and chemical agents, Corsini (1905) agreed with Winogradsky. Wislouch (1912), in reporting the new species *T. ingrica*, observed also the change from cell inclusions to characteristic sulfur crystals. Indirect evidence by other investigators supported these findings. Thus, Kolkwitz (1912) reported that the sulfur granules of *T. ingrica* disappeared during storage of the organisms. Bahr and Schwartz (1957) controlled the amount of sulfur inclusions in *Beggiatoa* and *Thiothrix* by changing the amount of hydrogen sulfide in the culture fluid. Winogradsky (1887) also showed that the sulfur content is a function of cultural conditions and not a constant morphological characteristic.

Because of the flexible walls, members of the family *Beggiatoaceae* of the order *Beggiatoales* clearly resemble members of the family *Oscillatoriaceae* of the order *Oscillatoriales*. Some studies on the nature of wall and septa of these organisms have been made. Hinze (1901) observed that the wall of *B. mirabilis* stained with ruthenium red and methylene blue. This was indicative of the presence
of pectins. The cellulose reaction was negative. Johansen (1940) stated that ruthenium red was the classical indicator for pectic substances, while the violet color obtained with methylene blue is less specific. Ruhland and Hoffmann (1926) confirmed the violet staining of the membrane of *B. mirabilis* with methylene blue and the absence of a reaction for cellulose. They reported that hypertonic solutions did not separate the membrane and the protoplasm, but the membrane could be split into two parts with 10 per cent potassium nitrate, concentrated mineral acids, or dilute bases. Since the inner, thinner part, still adhering to the protoplasm, could be digested with trypsin, it was proteinaceous. Ruhland and Hoffman postulated that in the living condition the protein portion of the membrane was elastically stretched and firmly bound to the outer, pectin layer, which was elastically contracted. In electron-micrographs of *B. alba* and *T. nivea*, Drawert and Metzner-Küster (1958) did not find evidence of the double nature of the wall. Faust and Wolfe (1961) concluded that the external wall of *B. alba* must be quite strong, since it kept the trichome connected even though a cell had ruptured and lost its contents during acute bending of the trichome. After mordanting with tannic acid, Scotten and Stokes (1962) stained membranes and septa of *Beggiatoa* with crystal violet. A wall distinct from the membrane was not observed.

In 1926, Ullrich reported that the membrane of *Oscillatoria jenensis* would dissolve in a solution of potassium hypochlorite, and therefore was neither cellulose nor hemicellulose. He obtained similar results (1929) with *Oscillatoria sancta* and observed that the septa also dissolved. Pectinase from cherry gum digested the membrane,
but not the septa. The latter swelled in alkaline media and were resistant to pepsin. The iodine reaction for cellulose was negative for membrane and septa. However, under the polarizing microscope the septa were birefringent in the diagonal position only. Ullrich felt this was evidence for the presence of cellulose embedded in a matrix which dissolved in potassium hypochlorite. Geitler (1932) believed that the membranes of blue-green algae were composed primarily of pectins, while the sheath contained cellulose, hemicelluloses, and pectins. The membranes of Cyanophyta contain some cellulose, according to Smith (1950), while the sheath is composed of pectic substances. In the blue-green genus Calothrix, which does not belong to the family Oscillatoriaceae, Kylin (1944) obtained a weakly violet coloration of the wall with the iodine-zinc chloride reagent for cellulose. Ruthenium red also colored the wall, but after extraction of pectins no color was obtained with this dye. He concluded that the sheath of this genus was a polymer of galactose, since the hydrolysis product was oxidized by nitric acid to mucic acid. It appears therefore, that pectins are structural components of the membranes of Beggiatoaceae and Oscillatoriaceae.

Although the morphology of members of the order Beggiatoales and members of the order Oscillatoriales is similar, there are other filamentous forms which are commonly included in the class Schizomycetes. However, none of these exhibit the peculiar gliding motility found in Beggiatoales and Oscillatoriales. In Chlamydo bacteriales and Caryophanales, motility is produced by flagella, and members of these orders possess rigid cell walls. Caryophanon latum was studied
extensively by Kelley (1952), who reported that the trichomes of this species are rigid and motile, owing to peritrichous flagellation. Walls and crosswalls did not stain with thionine, but stained with many basic dyes after mordanting with tannic acid. Each of the discoid cells in a trichome contained a discoid chromatinic body. Volutin bodies were present in almost all cells, but lipid inclusions could not be demonstrated.
MATERIALS AND METHODS

Organisms

*Thioploca ingrica* was found in mud which was obtained with an
Ekman dredge in approximately 30 feet of water in the Bass Island
Area of Western Lake Erie. The mud samples were placed into large,
brown ointment jars and overlaid with water from Lake Erie. These
jars were stored at 20°C in the laboratory. The organism remained
viable under these conditions for at least 3 years. When filaments
were needed for investigations, 50 ml of mud and 50 ml of water were
placed into a beaker. The mixture was thoroughly agitated, and the
flocs of filaments were fished from the mixture with an inoculating
needle. With the aid of another needle the flocs were brushed off
in water in a petri dish.

*Beggiatoa* was grown in a medium which contained 0.8 per cent
extracted hay (Faust and Wolfe, 1961). The autoclaved media were
inoculated with mud, decaying leaves, or both from natural waters
in the area of Columbus, Ohio. After incubation for 6 to 10 days at
28°C, characteristic tufts of *Beggiatoa* appeared on the walls of the
Erlenmeyer flasks, 3 to 5 mm below the surface of the liquid. A
strong odor of hydrogen sulfide was apparent. The growth was removed
with capillary pipettes and washed for use in cytochemical studies.
Microcoleus vaginatus M-7-1-1 was received from Dr. M. B. Allen, Kaiser Foundation Research Institute, Richmond, California. The organism was grown on a synthetic agar medium (Allen, 1961) under continuous illumination from a 15 watt daylight fluorescent lamp, 7 inch above the slants, at room temperature. In the preparation of the medium, equal volumes of autoclaved solutions A and B were mixed aseptically. The mixture was dispensed aseptically into clean, sterile culture tubes. Solution A contained: potassium nitrate (0.02 M), magnesium sulfate (0.001 M), sodium chloride (0.004 M), calcium chloride (0.0005 M), and 2 ml of solution A7 for every liter of solution A. Solution B contained: dibasic potassium phosphate (0.002 M) and 2 per cent agar (Difco). One liter of solution A7 contained: 4.0 mg ferrous iron (as sodium sequestrene), 0.5 mg boron (as boric acid), 0.5 mg manganese (as manganese sulfate), 0.05 mg zinc (as zinc sulfate), 0.02 mg copper (as copper sulfate), 0.01 mg molybdenum (as molybdenum oxide), and 0.01 mg vanadium (as ammonium vanadate). All solutions were prepared with water which was distilled in pyrex equipment. For each experiment a mass of trichomes was taken directly from the slant with an inoculating loop.

Caryophanon latum, OSU 688, was maintained on slants of trypticase soy agar (BBL) to which had been added 0.5 to 1.0 g of fresh cow dung for every 100 ml of medium. Plates of the same medium, incubated at 28°C, were used to grow the organism for each experiment.
The Cultivation of *Thioploca ingrica*

The Hay-Enrichment Method

The hay-enrichment method of Faust and Wolfe (1961) was modified in an attempt to grow *T. ingrica*. Water from Lake Erie, 60 ml, was placed into Erlenmeyer flasks of 125 ml capacity together with 1 to 2 ml mud from Lake Erie. The concentration of extracted hay was varied from 0.05 to 0.8 per cent. The flasks were inoculated with active flocs of *T. ingrica* immediately after mixing the ingredients and incubated at 28°C. The contents of the flasks were examined microscopically for growth every week and re-inoculated if no living trichomes could be recovered.

Pieces of hay with flocs of the organism attached and flocs picked directly from mud were washed in sterile tap water and placed on agar plates (1 per cent) containing 0.2 per cent yeast extract (Difco). After 2 hours, blocks of agar showing isolated trichomes were cut out and transferred to tubes containing 5.0 ml of 0.2 per cent yeast extract and 0.2 per cent agar. Some of the media were overlaid with either 2.0 ml or 5.0 ml water (tap water diluted 1:10 in distilled water) and incubated at 28°C in air; in an atmosphere of 0.2 mm Hg hydrogen sulfide in air; in an atmosphere of 0.1 mm Hg hydrogen sulfide, 40 mm Hg air, and 25 mm Hg carbon dioxide; and in an atmosphere of nitrogen. These atmospheres were replaced daily.
In order to determine the effect of various gases on the growth and survival of *T. ingrica* an apparatus (Fig. 1) was developed which allowed for a permanent connection of a mercury manometer (m) with:

1. a cylinder of commercial carbon dioxide (20 lbs, 98.96 per cent purity, Liquid Carbonic Div., General Dynamics),
2. a cylinder of nitrogen (112 cu ft, high purity dry, Liquid Carbonics Div.),
3. a flask in which hydrogen sulfide was mixed with nitrogen ($H_2S$-flask),
4. a 2-way glass stopcock connected to a vacuum pump,
5. an outlet to connect to the culture vessel.

The $H_2S$-flask (3) was prepared by fusing a 2-way glass stopcock to the mouth of a 6 liter Florence flask. The flow of gas from the $H_2S$-flask was controlled by a screw clamp on the connection made of pressure tubing. When hydrogen sulfide
was to be mixed with nitrogen in the H\textsubscript{2}S-flask, a cylinder of hydrogen sulfide (8 oz Lecture Bottle, Matheson Co.) was connected to the apparatus at outlet (5).

In the early experiments, 1000 ml separatory funnels were used as H\textsubscript{2}S-flasks. To ensure that air was removed, a funnel was evacuated and refilled with nitrogen. This procedure was repeated twice. The funnel was evacuated again, and the required amount of hydrogen sulfide was introduced. The funnel was then equilibrated with nitrogen to atmospheric pressure. By this method, eight separatory funnels were filled with hydrogen sulfide and nitrogen in proportions so that 25 mm Hg of mixed gases contained 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0 mm Hg of hydrogen sulfide respectively. When H\textsubscript{2}S-flasks were needed which contained less than 0.1 mm Hg of hydrogen sulfide in 25 mm Hg of the mixture, the contents of a H\textsubscript{2}S-flask (0.2 mm Hg H\textsubscript{2}S in 25 mm Hg mixture) which was prepared the previous day were diluted. For this purpose, the flask was partially evacuated and then equilibrated with nitrogen to atmospheric pressure. For the permanent H\textsubscript{2}S-flask (3) the proportions of hydrogen sulfide and nitrogen were selected so that 25 mm Hg of mixed gases contained 0.1 mm Hg of hydrogen sulfide. From this mixture, a relatively wide range of hydrogen sulfide tensions could be obtained readily in different culture vessels.

The amount of oxygen in the system was controlled by providing various residual pressures of air in comparison to atmospheric pressure by partial evacuation. Air was considered to contain 20 per cent oxygen.
Two kinds of culture vessels were used to maintain proper concentrations of gases. Separatory funnels of 500 ml capacity containing 10 ml of liquid medium were used. The stem of the funnel was inserted into the outlet (5) of the apparatus shown in Fig. 1. By closing the stopper of the funnel the system was also closed. Vacuum desiccators of 9 liter capacity were used for the incubation of tubes and petri dishes.

To establish the controlled atmosphere, fresh air was blown through the vessel with the aid of a portable air pump. The vessel was then connected to the apparatus, the entire system was closed and evacuated to the desired residual air pressure. Closing of the stopcock (4) disconnected the system from the vacuum pump. Next, the desired amount of hydrogen sulfide was introduced into the vessel from the H₂S-flask (3), then the desired amount of carbon dioxide. The amounts of gases were measured on the manometer (m) in mm Hg of pressure. The system was then equilibrated with nitrogen to atmospheric pressure, and the vessel was closed and disconnected. The following variations of gas pressures (mm Hg) were used:

1) hydrogen sulfide, 0 to 1.0 mm,
2) oxygen, 0 to 80 mm (400 mm air),
3) carbon dioxide, 0 to 60 mm.

The atmospheres in the culture vessels were replaced either daily or every other day, depending upon the experiment.
The Preparation of Liquid Media

Natural sulfur water from a spring on the campus of the Ohio Wesleyan University was filtered through Whatman #1 filter paper and then through a 02 Selas filter to remove dissolved gases. Water and an extract of mud, both from Lake Erie, were filtered in the same way. Ten ml amounts of these liquid media were placed into 500 ml separatory funnels and inoculated with washed flocs of *T. ingricta*. These culture vessels were then filled with the various atmospheres and incubated at 4°C, 20°C, 28°C, and 37°C, in light and in darkness. Flocs were checked microscopically every 2 days. Cessation of the gliding motion was taken as the sign of death.

The Preparation of Solid Media

Agar (Difco) in concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 per cent was added to the liquid media. Plates of silica gel were prepared according to the method of Hanks and Weintraub (1936). Sodium silicate (40-42°Be, Mallinckrodt) was used to give final concentrations of approximately 1.5, 1.8, 2.0, and 2.2 per cent silicate. The gels were dialyzed at 40°C against water from Lake Erie (LE-water) to which had been added 0.05 per cent ammonium sulfate. The pH of the LE-water was adjusted to 7 with hydrochloric acid. Solid media received surface and sub-surface inoculations of washed flocs of *T. ingricta*. The plates were incubated in the optimum atmospheres of hydrogen
sulfide, oxygen, and carbon dioxide. The atmospheres were replaced every 2 days. Trichomes were checked in situ for motility, using the 10X objective of the microscope.

Organic Compounds Added to Media

The following compounds were added to the liquid and solid media singly in final concentrations of 0.02 and 0.1 per cent: ammonium succinate, sodium lactate, sodium formate, potassium pyruvate, sodium acetate, yeast extract, beef extract, glucose, maltose, galactose, and cellobiose. In addition, nutrient broth, brain heart infusion broth, and heart infusion broth (all Difco) in serial doubling dilutions up to 1:2048 in LE-water were used.

In other experiments fresh mud from Lake Erie (LE-mud) was added in various concentrations to LE-water, or to the agar media cooled to 45°C. Sometimes a layer of LE-mud in a petri dish was overlaid with 2 layers of sterile agar in LE-water. Duplicates of all inoculated media were incubated in the optimum atmospheres of hydrogen sulfide, oxygen, and carbon dioxide at 28°C. One duplicate was incubated in the light, the other in darkness. Media containing LE-mud as the nutrient were also incubated in air or in candle jars at 28°C, either in light or in the dark.

Adjustment of the Oxidation-Reduction Potential

The oxidation-reduction (O/R) potential of LE-water, containing 0.5 per cent of agar, was adjusted to the range of the O/R potential of
fresh LE-mud. To accomplish this, cysteine hydrochloride and sodium thioglycollate were used in concentrations ranging from 0.1 to 0.0005 per cent. The tubes of media in the series were prepared with or without addition of sodium acetate. The inoculated media were incubated in controlled atmospheres at 28°C in the dark. The atmospheres were replaced every second day.

In an effort to create a suitable physical, inert environment for _T. ingricta_, which occurs naturally in physical contact with solid particles, washed Fuller's Earth was added to LE-water. In this manner an artificial "mud" sediment was created. Washed flocs were placed into this sediment and incubated at 28°C in an atmosphere of 0.1 mm Hg hydrogen sulfide, 8 mm Hg oxygen, 25 mm Hg carbon dioxide, and nitrogen. The atmosphere was changed daily. Flocs were checked every 10 days.

**Sulfur Compounds Added to Media**

Solid and liquid media prepared with LE-water were used to determine whether or not hydrogen sulfide could be replaced by either sodium sulfide or ferrous sulfide. Media were saturated with ferrous sulfide and serial doubling dilutions up to 1:1024 were prepared. The percentage of sodium sulfide was varied from 0.4 to 0.00005. These media were incubated at 28°C in air; in an atmosphere of air, carbon dioxide, and nitrogen; and in a candle jar, and the flocs were checked daily.
Cytochemical Investigations

Living Organisms

The appearances of the four organisms were compared by examining wet mounts of living trichomes by bright field microscopy under oil immersion. For observations with the phase contrast microscope, living trichomes were placed into drops of cooled agar and covered with a coverslip, which was gently pressed down. Immediately after the trichomes ceased gliding, photographs were taken.

Methods of Fixation

Liquid fixatives, such as Bouin's, Chabaud's, Formalin, Helly's, Schaudinn's and alcohols caused an immediate clumping of T. ingrica. Attempts to fix through agar in the above liquids were not successful. Because of the presence of the sheath, the flocs would not adhere to the coverslips. Fixation in vapors of osmium tetroxide could not be used for the same reason.

The following method of fixation of T. ingrica was used. Flocs of the organism, fished from the mud or from hay-enrichment media, were washed in LE-water and transferred with the aid of a capillary pipette to a Wassermann tube which contained 4 ml of LE-water. The tube was vigorously shaken, and the contents were emptied into a small petri dish. The floc was separated into smaller pieces and allowed to remain in the fluid for about 15 minutes. By microscopic examination at this time, it was shown that many trichomes had emerged
from the ends and points of breakage of the sheath. Most of the water was then removed and about 10 volumes of a 1 per cent solution of osmium tetroxide in distilled water was added to the petri dish containing *T. ingrica*. After fixation for 5 minutes the flocs were transferred through 3 changes of distilled water with the aid of a dissecting needle. The flocs were then placed on chemically clean coverslips with a capillary pipette, and the excess moisture was withdrawn with bibulous paper. After the floc had dried to the coverslip, the preparation was placed into a columbia dish containing either water or 70 per cent ethanol.

*Microcoleus vaginatus* was transferred from a slant to 1 per cent osmium tetroxide with an inoculating loop. The fixation and washing procedures were the same as for *T. ingrica*.

Liquid osmium fixation was also used for *Beggiatoa* and *Caryophanon*. However, the results were similar to those obtained with fixation in osmium vapors. Washed tufts of *Beggiatoa* were spotted on plates of 1 per cent agar in tap water. The trichomes were allowed to spread out from the points of inoculation. Plates of *Beggiatoa* and plate cultures of *Caryophanon* were fixed for 4 minutes in the vapor of a 1 per cent solution of osmium tetroxide in distilled water. Agar blocks were cut from the plates, and impression smears were prepared on clean coverslips. The impression smears were allowed to dry in air for 15 seconds and were placed either into distilled water or 70 per cent ethanol.

The preparations on coverslips stored in distilled water were used for simple stains and staining procedures for volutin, lipids,
cellulose, pectins, and wall. Preparations stored in 70 per cent etha­
nol were transferred to 95 per cent ethanol for 10 minutes and then
to absolute ethanol, in which they remained for 15 hours. Occasion­
ally they were then placed into acetone and ether, each for 24 hours.
Then the preparations were transferred through a series of solutions
of ethanol (95, 70, 50, 30, and 10 per cent) for 5 minutes each to
distilled water. This procedure effectively removed all traces of
sulfur inclusions of T. ingrica and Beggiatoa. Preparations of the
other organisms were treated in the same way. Preparations on cover­
slips treated in alcohol were used in the staining procedures for
chromatinic material.

Mounting of Preparations for Observation

Stained and washed preparations on coverslips were mounted in
a drop of distilled water on chemically clean microscope slides.
Excess moisture was absorbed with bibulous paper. The edges of the
coverslips were sealed with melted paraffin. Preparations were
examined with the oil immersion objective of the microscope.

Methods for the Demonstration of Volutin Inclusions

Fixed preparations on coverslips were stained for one minute
in a solution consisting of 1.0 ml of a saturated alcoholic solution
of methylene blue diluted with 10 ml distilled water. After washing,
the trichomes were differentially decolorized by instantaneous rinses
in 1.0 per cent sulfuric acid. Decolorization was stopped by washing in tap water (Meyer, 1912).

Metachromatic properties of intracellular granules were demonstrated by staining with 0.25 per cent thionine in distilled water for 0.5 to 60 minutes. Preparations were then washed, mounted, and observed in the usual manner.

Methods for the Demonstration of Lipid Inclusions

Fixed preparations on coverslips were mounted in freshly filtered 70 per cent ethanol saturated with sudan black B (Hartman, 1940). Photomicrographs were taken within an hour of mounting. Preparations were also stained in the same solution for 30 minutes, rinsed in 70 per cent ethanol, washed in water, and mounted in distilled water. For T. ingrica, an unstained water mount was photographed. The preparation was then rinsed with 70 per cent ethanol and mounted in the solution of sudan black B, and the same field was photographed again.

Method for the Demonstration of Cellulose

The zinc chloride-iodine reagent was prepared according to Frey (1927) and filtered before use. Preparations were stained in the solution for 2 to 10 hours. The preparations were rinsed in water, mounted, and observed in the usual manner.
Methods for the Demonstration of Pectins

The test for pectins by successive treatments in boiling water, in hydrochloric acid, and in ammonium hydroxide was performed on living flocs of *T. ingricta* (Johansen, 1940). At the beginning and after each step in the procedure, a floc was stained in methylene blue, 1:10,000, for 10 minutes, washed, and mounted. Pectins stain violet with methylene blue. Fixed preparations on coverslips of all organisms were stained in the same way.

Unfixed trichomes and fixed preparations were stained for 30 minutes in solutions of ruthenium red (Johansen, 1940). The washed preparations were mounted in the usual manner and observed.

Method for the Demonstration of Mucopolysaccharides

According to Widra (1956), alcian blue is specific for acid mucopolysaccharides. Preparations on coverslips were stained for 5 minutes in a solution of 1 per cent alcian blue and 0.25 per cent basic fuchsin in water. The preparations were then washed and mounted in the usual manner.

Methods for the Demonstration of the Nature of Cell Surfaces

Fixed preparations on coverslips were mordanted in a 1 per cent aqueous solution of phosphomolybdic acid for 3 to 11 minutes. After thorough washing in tap water, the preparations were stained in 1 per cent solutions of methyl green in distilled water for 1 to 10 minutes. Preparations were washed and mounted in the usual manner.
In order to separate the wall from the cytoplasm of Thioploca, Baggiatoa, and Microcoleus, living trichomes of these organisms were mounted in 10 per cent solutions of potassium nitrate and chloral hydrate (Ruhland and Hoffmann, 1926) and examined at intervals. After 15 hours, methylene blue was allowed to diffuse into the mounts, which were then again examined. Living trichomes were also mounted in the lactophenol reagent (Johansen, 1940) containing 0.05 per cent cotton blue and observed microscopically at intervals.

Methods for the Demonstration of Chromatinic Material

Three methods were used to stain chromatinic material after hydrolysis of fixed preparations with normal hydrochloric acid. Preparations were hydrolyzed at room temperature for varying intervals up to 3 hours, at 45°C for varying intervals up to 2 hours, and at 60°C for varying intervals up to 30 minutes. To remove deoxyribonucleic acid, fixed preparations were extracted at 60°C with a 10 per cent solution of perchloric acid before dehydration in ethanol, as outlined before. After re-hydration, preparations were hydrolyzed in hydrochloric acid at 60°C.

The acid-Giemsa procedure

Four tenths ml of an aged solution of Giemsa was diluted with distilled water to 10 ml (Robinow, 1944). Hydrolyzed preparations and controls of T. ingrlica and B. alba were stained for 90 minutes or more at room temperature. The other organisms were stained for
either 30 or 60 minutes. The preparations were then washed in distilled water or differentiated in 10 per cent aqueous tannic acid, washed, and mounted.

**The thionine-thionyl chloride procedure**

The staining mixture was prepared just before use by adding 1 drop of thionyl chloride to 10 ml of a 0.25 per cent aqueous solution of thionine (DeLamater, 1931). Hydrolyzed preparations and controls were stained for 2 to 48 hours, washed for 2 minutes each in 3 changes of distilled water to which had been added thionyl chloride (3 drops in 100 ml of distilled water), then washed in water, and mounted.

**The Feulgen reaction**

Solutions of leuco basic fuchsin were prepared according to Lillie (1951) and Rafalko (1946). Hydrolyzed preparations and controls were stained for 3 to 48 hours in the reagents at room temperature in sealed staining jars. Preparations were then washed for 2 minutes each in 3 changes of solutions of sulfur dioxide in distilled water (Ludford, 1928). After thorough washing in distilled water, preparations were mounted in the usual manner.

**The Gram Reaction**

Preparations on microscope slides were fixed by heat. The slides were flooded with a 1 per cent solution of crystal violet in tap water for 1 minute, washed, and flooded with Gram's iodine solution for 1 minute. The slides were washed, blotted dry, and
decolorized with a solution containing 30 ml of acetone and 70 ml of 95 per cent ethanol. Washed slides were counterstained for 15 seconds in a solution of 2.5 per cent safranin in distilled water. The washed and dried preparations were observed directly in oil. Cultures of Staphylococcus aureus and Escherichia coli served as controls.

Hydrolysis by Lysozyme

A 0.1 per cent solution of crystalline lysozyme (Armour) was prepared in LE-water. Part of this solution was heated at 90°C for 1 hour. All four organisms were exposed to lysozyme, heated lysozyme, and LE-water without lysozyme. Living organisms were placed into a drop of each of the 3 liquids on separate slides and covered with coverslips. The preparations were kept on moist filter paper in a petri dish and were observed microscopically at intervals up to 2 hours.

Flocs of T. ingricta and trichomes of M. vaginatus were suspended in the above 3 solutions contained in Wassermann tubes and incubated at room temperature. After incubation for 24 hours, each preparation was washed and examined microscopically in wet mounts.

Hydrolysis by Pectic Enzymes

One per cent solutions of pectinesterase (GBI) and pectinase (NB Co.) in pH 4.1 acetate buffer were used at 40°C in an attempt to hydrolyze the sheaths of T. ingricta and M. vaginatus. Living flocs and fixed preparations of T. ingricta and M. vaginatus were stained with
ruthenium red as outlined above. Other living flocs and preparations on coverslips were subjected to the following solutions in columbia dishes: (1) 144 hours in acetate buffer, (2) 72 hours in the solution of pectinesterase, (3) 72 hours in the solution of pectinase, (4) 72 hours in the solution of pectinesterase and 72 hours in the solution of pectinase, (5) 72 hours in the solution of pectinase and 72 hours in the solution of pectinesterase, (6) 24 hours in a 0.1 percent solution of lysozyme and 48 hours in the solution of pectinesterase and 48 hours in the solution of pectinase. Following enzymatic treatment, the preparations were washed in water, stained in ruthenium red, washed, mounted, and observed microscopically.

Sterile coverslips, broken in half, were placed on freshly inoculated slants of *M. vaginatus*. After 18 days, the coverslips with filaments adhering were removed and either stained at once or dried and subjected to the same treatment with pectic enzymes as outlined above. The preparations were then stained with alcian blue-basic fuchsin, as outlined before, dried, and examined directly in oil.

Microscopy and Photography

For phase contrast observation a Model BS microscope (Leitz) with phase contrast equipment was employed. The microscope was equipped with a Heine condenser, an apochromatic oil immersion objective *Pv* 90X NA 1.10, and a 6X periplan eyepiece. Immersion oil was placed between the slides and the condenser.

Bright field observations were made through a Microstar trinocular microscope (American Optical Co.) equipped with an Abbe
condenser NA 1.25; the following achromatic objectives: 97X NA 1.25 oil immersion, 43X NA 0.66 high dry, 10X NA 0.25, and 3.5X NA 0.09 low power; and 10X wide field Huygenian eyepieces.

Illumination was provided by a focusing research lamp, 12 volts, 100 watt. Neutral density filters and an interference filter (all Bausch and Lomb) with a peak transmission at 500 μ were used.

Photomicrographs were taken with a Model H Camera (Bausch and Lomb) using 34 x 44 Panatomic X sheet film (Kodak) at an initial magnification of 1340 diameters with the oil immersion objective, 136 diameters with the 10X objective, and 47 diameters with the 3.5X objective. A Simon Omega D2 enlarger was employed for the production of enlarged prints on Kodabromide F5 or F3 photographic paper. The magnification of the prints is 2680 diameters, unless otherwise indicated.
RESULTS AND DISCUSSION

The Cultivation of Thioploca ingrica

Growth and Survival of Thioploca ingrica in Hay-Enrichment Media

Scotten and Stokes (1962), in using the hay-enrichment method for the isolation of Beggiaota, observed a strong odor of hydrogen sulfide in the flasks about 10 days after the inoculation, at which time appreciable growth was observed in the medium. On the other hand, Bavendamm (1924) reported that the mud containing Thioploca ingrica must have a very low content of hydrogen sulfide, since there was no odor apparent. Consequently, it was not surprising that during the first few weeks T. ingrica failed to grow, and seldom survived, in the hay-media. About 3 weeks after the hay-media were prepared, the production of hydrogen sulfide declined. After the fourth to fifth week no odor was apparent. Re-inoculation after the fourth or fifth week usually resulted in growth, if the concentration of hay was below 4 per cent. In the flasks with higher concentrations of hay, T. ingrica seldom grew.

When T. ingrica grew in the hay-medium, numerous gliding and unsheathed trichomes were observed by microscopic examination of material from the dark-gray, fluffy coating of debris which surrounded...
the pieces of hay. A week later active filaments were found along with free trichomes in the debris around the pieces of hay. Kolkwitz (1955) observed that trichomes of *T. ingrica* left the sheath under adverse conditions, moved to a better habitat, and synthesized a new sheath. In the flask containing 0.05 per cent hay, no viable trichomes were observed after 10 weeks. In the media containing 1 to 4 per cent hay, *T. ingrica* grew and remained active for 16 weeks. Based on these results, media were then prepared with 3 per cent hay and inoculated after standing at 28°C for 4 and 5 weeks. As may be expected under such undefined conditions, growth of *T. ingrica* was not obtained in every flask.

Scotten and Stokes (1962) obtained growth of *Beggiatoa* in a surface film or just below (2 to 5 mm) the surface of the liquid on the walls of the flasks containing the hay-medium. However in these experiments, the growth of *T. ingrica* always was limited to the layer of mud around pieces of hay in the bottom of the flask. This finding corresponded to previous observations of differing oxygen requirements of the two organisms made by Kolkwitz (1912) and Koppe (1924).

Unfortunately, attempts to grow *T. ingrica* on 0.2 per cent yeast extract, which Faust and Wolfe (1961) had used for *B. alba*, were unsuccessful. Inocula derived directly from the mud generally yielded isolated trichomes on plates containing 0.2 per cent yeast extract and 1 per cent agar in distilled water. After 2 hours on the solid, organic medium, no more gliding motion was apparent. Isolated trichomes on the plates could be transferred on agar blocks, free from
contaminants, to tubes containing 0.2 per cent yeast extract and agar. However, trichomes from the hay-enrichment cultures did not move far enough from the inoculum to be isolated on agar blocks. Apparently, organisms from the cultures in mud were physiologically more active than organisms from the hay-media. This seems to indicate that the hay-medium does not provide optimal conditions for the growth of *T. ingrica*. Growth of *T. ingrica* was never obtained in the tubes containing 0.2, 0.14, or 0.1 per cent yeast extract to which trichomes were transferred. Variations of the tensions of hydrogen sulfide and oxygen in the atmosphere in which the tubes were incubated had no effect on the survival of *T. ingrica*.

Growth and Survival of *Thioploca ingrica* in Controlled Atmospheres

In spite of the fact that Bavendamm (1924) did not detect any odor of hydrogen sulfide from mud containing *T. ingrica*, it was observed that the organism remained active for several days in tubes of fresh water from a sulfur spring on the campus of the Ohio Wesleyan University. One hundred ml of this water contained 0.083 mg of hydrogen sulfide and gave a distinct odor of this gas. At 20°C in an atmosphere of 760 mm Hg of moist hydrogen sulfide, 380 mg of this gas will dissolve in 100 g of distilled water (Lange, 1956). The amount in the natural sulfur water would dissolve from an atmosphere with a partial pressure of hydrogen sulfide of 0.16 mm Hg. The difference in solubility between distilled water and the various media used was disregarded. It was recognized that in 500 ml of atmosphere
the final equilibrium conditions with 10 ml liquid would not exactly correspond to the expected values. The difference in solubility at different temperatures was also disregarded. In addition, it was assumed that oxygen and hydrogen sulfide, which are generally regarded as antagonistic, would dissolve independently of each other.

Optimal pressures of oxygen

Keil (1912), in culturing *Beggiatoa* and *Thiothrix* under defined atmospheric conditions, found that the pressure of carbon dioxide was not critical. He routinely used 25 mm Hg of carbon dioxide. In the first experiments with controlled atmospheres, fixed pressures of hydrogen sulfide (0.2 mm Hg) and carbon dioxide (25 mm Hg) were used. The partial pressure of oxygen was varied from 0 to slightly above one half atmospheric oxygen pressure, since the requirement of *T. ingrica* for this gas is apparently low. Trichomes remained motile for the longest time when the partial pressures of oxygen were between 14 and 17 mm Hg. In the absence of oxygen, however, a few trichomes with sulfur inclusions remained active for up to 30 days. Kolkwitz (1955) also reported the temporary survival of *Beggiatoa* and *Thioploca* in the absence of oxygen.

Optimal pressures of hydrogen sulfide

In trying to determine optimal pressures of hydrogen sulfide, the partial pressure of oxygen was fixed at 14 mm Hg and the partial pressure of carbon dioxide at 25 mm Hg. In the absence of hydrogen sulfide trichomes soon ceased moving. The best time of survival was
obtained with partial pressures of 0.1 and 0.2 mm Hg of hydrogen sulfide. The higher the pressure above 0.2 mm Hg or the lower below 0.1 mm Hg the sooner the trichomes of T. ingrica died. The trichomes in higher concentrations of hydrogen sulfide seemed completely filled with sulfur inclusions. At lower pressures, many trichomes were devoid of sulfur.

**Optimal pressures of carbon dioxide**

When the effect of carbon dioxide was investigated, partial pressures of 0.1 mm Hg of hydrogen sulfide and 8 mm Hg of oxygen were used. Without the addition of either calcium carbonate or carbon dioxide, T. ingrica died considerably sooner in sulfur water than in LE-water or mud extract. It was thought that this might be due to the different amounts of dissolved carbonates in these liquids. The Bass Island Area of Lake Erie consists of lime stone, while the sulfur spring at the Ohio Wesleyan University comes from a shale formation (White, 1947). As long as the liquids were saturated with calcium carbonate it made little difference how much carbon dioxide was added. Survival was greatly diminished without the addition of carbonate, and this was attributed to the effect of the pH. Without carbonate the pH of the LE-water, sulfur water, and mud extract fell below 6, while with carbonate and carbon dioxide a pH between 7.1 and 7.6 was maintained. As a result, media saturated with calcium carbonate and a partial pressure of 25 mm Hg of carbon dioxide in the atmosphere were used routinely.
Thioploca was first discovered in calcareous mud by Lauterborn (1907) and Bavendamm (1924) reported similar observations. Kolkwitz (1912, 1955) detected T. ingrica in four locations in calcareous mud with a neutral or slightly alkaline pH. The calcareous mud from Lake Erie, from which T. ingrica was obtained, had a neutral reaction also. Assuming that Thioploca is a chemoautotroph, it is possible that the bacteria in the mud supplied the hydrogen sulfide and carbon dioxide, and the carbonate in the mud would have a buffering effect. Atmospheric oxygen dissolved in the water would diffuse into the mud layer. Since the relatively shallow water of the Bass Island Area was kept in continuous circulation by wind and currents throughout most of the year, the content of dissolved oxygen was high (Chandler, 1940). Britt (1955) reported that the chemical composition and water temperature from top to bottom in the same area were usually uniform. Consequently it may be assumed that in the area from which T. ingrica was obtained the water above the mud is nearly saturated with oxygen. However, the concentration of oxygen in the mud would be quite different, and the content of oxygen in the water would give little information about the percentage of oxygen to be used in a defined atmosphere.

Relationship between the concentrations of oxygen and hydrogen sulfide

In order to investigate further the effect of relative concentrations of oxygen and hydrogen sulfide, the solubility of oxygen in water was taken into consideration. At 20°C in an atmosphere of moist oxygen of 760 mm Hg pressure, 4.3 mg of oxygen dissolve in
100 g of water (Lange, 1956). At a partial pressure of 0.2 mm Hg of hydrogen sulfide, 0.10 mg of hydrogen sulfide would dissolve in 100 g of water. To satisfy the equation, \(2 \text{H}_2\text{S} + \text{O}_2 \rightarrow 2 \text{H}_2\text{O} + 2 \text{S}\), 0.047 mg of oxygen would have to dissolve. This amount dissolves at a pressure of oxygen of 8.3 mm Hg, which was rounded to 8 mm. However, for the complete oxidation of hydrogen sulfide, 4 times more oxygen would be required: \(2 \text{H}_2\text{S} + 4 \text{O}_2 \rightarrow 2 \text{H}_2\text{SO}_4\). This amount would dissolve at a 4 times higher pressure of oxygen, 32 mm Hg. Fractions or multiples of the pressure of hydrogen sulfide used would have to be applied also to pressures of oxygen. It was understood that these considerations of solubility were of a purely theoretical nature and did not reflect the rate at which the dissolved gases became available to \text{T. ingrlica}. However, they provided a convenient basis for comparison.

For each partial pressure of hydrogen sulfide, 4 partial pressures of oxygen were employed: (1) The pressure to dissolve enough oxygen for the oxidation of hydrogen sulfide to sulfur; (2) twice this pressure; (3) four times pressure (1), or enough for complete oxidation to sulfate; (4) five times pressure (1). It was found that for all pressures of hydrogen sulfide tested in the range of 0.06 to 0.3 mm Hg the intermediate partial pressure of oxygen (2) was best. Although no growth of \text{T. ingrlica} was detected, a maximum survival for 80 to 100 days was obtained with partial pressures of oxygen of 8 mm Hg and of hydrogen sulfide of 0.1 mm Hg, or of 16 mm Hg of oxygen and 0.2 mm Hg of hydrogen sulfide. These results were independent of the temperature over a range of 4°C to 28°C.
A temperature of 37°C killed *T. ingricta* within 8 to 12 hours. Light or darkness had no effect on the survival of the trichomes. Results with the 3 liquid media (LE-water, LE-mud extract, sulfur water) were comparable when all were saturated with calcium carbonate.

The amount of intracellular sulfur correlated well with the relative partial pressures of oxygen and hydrogen sulfide. Generally, the higher the partial pressure of hydrogen sulfide the more sulfur accumulated in the cells. Yet, for each pressure of hydrogen sulfide, different amounts of sulfur were present in the trichomes with each of the different partial tensions of oxygen that were tested. The smallest amount of sulfur occurred in the trichomes when the highest partial pressure of oxygen was used. The quantity of sulfur inclusions regularly increased as the partial pressure of oxygen was decreased. Winogradsky (1887) also observed that the amount of sulfur inclusions in *Beggiatoa* was a function of cultural conditions and not a constant characteristic. The effect of the relative concentrations of oxygen and hydrogen sulfide in the atmosphere on the amount of intracellular sulfur was an important indication that *T. ingricta* oxidized hydrogen sulfide as well as intracellular sulfur. Consequently, oxygen and hydrogen sulfide must be present in the environment in which *T. ingricta* is grown, even though the concentrations needed are low.

The effect of solid and organic media

When a floc of *T. ingricta* was incubated in liquid media under defined atmospheric conditions, it soon contracted, but the trichomes remained motile. Although contraction of flocs was not observed in
solid or semisolid media, the trichomes did not survive long under such conditions. Surface inocula generally were dead in a few days, while occasional trichomes of subsurface inocula remained active for up to 30 days. Survival of organisms on silica plates was essentially the same as on media containing agar. When organic compounds were added to liquid or solid media, trichomes of *T. ingrica* showed no signs of motion after 1 to 2 days, and the growth of contaminating organisms complicated these experiments. However, even in experiments in which uncontaminated, isolated trichomes were inoculated into media containing various organic compounds, growth never occurred. Since mud from Lake Erie also contains organic substances, it was assumed that this organic material was not used by *T. ingrica*. Possibly organisms in the mud produce growth factors which *T. ingrica* required. Yet, the addition of mud to many different media did not stimulate growth of *T. ingrica*. Since fresh mud evolved small quantities of hydrogen sulfide, even when incorporated into agar media, incubation of cultures containing mud without controlling the atmosphere was also tried. However, no growth of *T. ingrica* occurred.

The failure of cultures to grow in media containing mud seemed curious, since the organism grew in the mud samples derived from Lake Erie which were stored in the laboratory. It was observed that disturbance of the mud samples, brought about by harvesting *T. ingrica*, soon caused the trichomes to die. If the jars then remained undisturbed for several weeks, active flocs of *T. ingrica* were recovered again. The detrimental effect of disturbing the micro-environment might be due either to a change in the oxidation-reduction potential or
to physical effects. Either of the two changes could explain why additions of mud to media had no beneficial effect, even if it supplied growth factors.

The effect of the oxidation-reduction potential

Attempts to stimulate growth of *T. ingrica* by manipulation of the O/R potential were not successful. Cysteine hydrochloride and sodium thioglycollate, which were used to lower the O/R potential, are organic compounds. As such, they had the same effect as other organic compounds added to the medium, within a few days the organism was dead. The uniformly detrimental effect of organic compounds and the failure to isolate heterotrophic variants from hay-enrichment cultures strongly suggested an obligate chemoautotrophic nutrition for *T. ingrica*. It must be remembered that Winogradsky (1887) reported the detrimental effect of organic compounds on *B. alba*, but using the correct concentration of nutrients in media, Faust and Wolfe (1961), Morita and Maharadjah (1961), and Scotten and Stokes (1962) have grown *Beggiatoa* heterotrophically.

To control certain physical factors, a suspension of Fuller's Earth in LE-water was used. In this environment, the flocs in physical contact with the solid particles did not contract. However, growth of *T. ingrica* did not occur, and survival was limited to 30 days. Since the equilibration of the gases between atmosphere and medium would be different in liquid media and media containing either Fuller's Earth or agar, results obtained with this medium cannot be compared with the results of other experiments.
The effect of various sources of sulfur

In order to determine whether sulfides other than hydrogen sulfide could be utilized by *Thioptera ingrica*, sodium and ferrous sulfide were employed. If these compounds could be oxidized by *T. ingrica*, conditions of incubation would be simplified. Unfortunately, concentrations of sodium sulfide above 0.01 per cent stopped the motility of *T. ingrica* within 20 minutes, and concentrations as low as 0.00005 per cent killed the trichomes within 24 hours. Taylor (1957) had made similar observations on *T. ingrica*. When ferrous sulfide was added to the medium, trichomes survived only for a maximum of 3 days. Consequently, neither sodium nor ferrous sulfide can replace hydrogen sulfide under the conditions tested in this study. Although one would not expect to find *T. ingrica* in mud containing sodium ions in appreciable quantities, Kolkwitz (1912) detected *T. ingrica* in mud from brackish water of the Baltic Sea. It must be assumed that the organism described by this author represented a particular ecologic strain that was adapted to saline conditions.

Cytochemical Investigations

Living Organisms

The filaments of *Thioploca ingrica*, taken directly from the mud, appeared to the unaided eye as fine, tangled, white threads mixed with debris. The characteristic milky white appearance was due to the numerous inclusions of plastic sulfur in the trichomes, as Corsini
has shown for \textit{Beggiatoa} in 1905. Although occasionally straight filamentsof \textit{T. ingrica} were recovered, flocs of tangled filaments were seenmore often. A typical field of a floc of \textit{T. ingrica} is shown in
Fig. 2. An enlarged section of Fig. 2 is shown in Fig. 3. The transparentsheath, which surrounded from 1 to 20 parallel or interwoven,gliding trichomes, was demarcated on the outside by mineral incrustations (Fig. 3). Empty sheaths are plainly visible in the lower righthand corner of Fig. 3. Within the large loop of a filament in Fig. 3,trichomes may be seen which have emerged from the open end of a sheath.The diameter of trichomes of \textit{T. ingrica} varied from 2.2 to 3.5 u. The lengths of the individual cells were between 3.3 and 7 u, thoughoccasionally longer cells (9.3 u) were encountered. The foregoingdescription of the \textit{Thioploca}, as determined by this investigation,corresponds closely to the original description of \textit{T. ingrica} byWislouch (1912) and reports by Kolkwitz (1912, 1955).

The strains of \textit{Beggiatoa} which were observed in this study couldnot be placed into a single species, as was possible for \textit{T. ingrica}.According to Bergey’s Manual (1957), the species of \textit{Beggiatoa} arequite arbitrarily differentiated on the basis of the diameters of the trichomes. Organisms with trichomes from 1.0 to 2.5 u in diameter areclassified as \textit{B. leptomitiformis}, and organisms with trichomes from2.5 to 5.0 u in diameter as \textit{B. alba}. Faust and Wolfe (1961) calledtheir isolated heterotrophic strains \textit{B. alba}, although the trichomeshad a diameter of 2.2 u. The strains of \textit{Beggiatoa} isolated byScotten and Stokes (1962) had trichomes 2.0 to 2.2 u wide. Accordingto Bergey’s Manual (1957), the strains of Faust and Wolfe and of
Fig. 2. *T. ingrica*, portion of a floc, unstained water mount (47X)

Fig. 3. *T. ingrica*, enlarged section of Fig. 2. (136X)
Scotten and Stokes should be called *B. leptomitiformis*. The strains of *Beggiatoa* used in this study varied in diameter from 2.0 to 2.7 μ. Apparently there is no sharp break in diameters of the trichomes that can be used to separate *B. leptomitiformis* from *B. alba*. The variation in the diameter of the trichomes of *Beggiatoa* might have been obviated by the isolation of heterotrophic strains from single trichomes. Because of the large size of the trichomes, *Beggiatoa* in this study was called *B. alba*.

When living trichomes of *T. ingrica* were compared with trichomes of *B. alba*, their similarity was at once apparent. The gliding motility seemed identical and both contained sulfur inclusions which appeared as dark, refractive rings. Trichomes of *T. ingrica* possessed a faint, blue-green tint, which was especially noticeable in trichomes with either a few sulfur inclusions or without sulfur inclusions. Since it was not possible to grow *T. ingrica* in pure cultures, the extraction and characterizations of the pigments were not feasible. *Beggiatoa alba* was hyaline, and septa were not seen. In *T. ingrica*, septa could be observed occasionally in trichomes with few sulfur inclusions. The sulfur inclusions of *B. alba* seemed to be located in the periphery of the cells. In *T. ingrica*, on the other hand, the sulfur seemed to be located in a central, irregular zig-zag fashion. An indication of this zig-zag arrangement can be seen in the free trichome extending to the left margin of Fig. 3 and in Figs. 4 and 30. The rounded tip of trichomes of *B. alba* is apparent in Fig. 5.
Fig. 4. *T. ingricta* (left)  
*M. vaginatus* (right)  

Fig. 5. *B. alba*

Fig. 6. *C. latum*

Figs. 4–6. Unstained water mounts, osmium fixed
Living, unstained trichomes of Microcoleus vaginatus glide in the same way as trichomes of *T. ingrica* and *B. alba*. In comparison to *T. ingrica*, *M. vaginatus* had a pronounced blue-green color. In the area of the central body in each cell, the color seemed much lighter. The position of the septa was detected with relative ease at various levels of focus. These features of *M. vaginatus* are illustrated in Fig. 4.

Trichomes of Caryophanon latum were much harder to see in the living state. Fig. 6 was over-exposed to obtain contrast. The bands which completely divided the trichomes of *C. latum* crosswise have been shown to be crosswalls by Kelley (1952). The dark bands are the protoplasmic units (cells) of the trichomes. The darker outer edges of the cells appeared to be discontinuous at the crosswalls. The outer boundary of the trichomes of *T. ingrica*, *B. alba*, and *M. vaginatus* (Figs. 4 and 5) was continuous, and septa were not marked as clear, crosswise bands. *Caryophanon latum* also differed from the other three organisms in this study, since it possesses peritrichous flagella (Bergey's Manual, 1957).

Three different terminal segments have been observed in trichomes of *T. ingrica*. The rounded tip (Fig. 7), according to Taylor (1957), resulted when a trichome divided into two parts by the dissolution of an intercalary cell. The conically tapered tip (Fig. 8) seemed to be an intermediary form between the rounded and the greatly extended tip (Fig. 9). The extreme tip of the extended segment usually was hooked and consequently is out of focus. In *B. alba*, only rounded terminal segments have been observed.
Figs. 7-9. Different terminal segments of trichomes of *T. ingriza*

Fig. 10. *T. ingriza*  
Fig. 11. *B. alba*

Figs. 10 and 11. Living, unstained trichomes, phase contrast (2000X)
Fixed and unfixed trichomes of *T. ingrica*, devoid of sulfur inclusions, occasionally were vacuolated. It was therefore of interest to observe living trichomes with the phase contrast microscope. The amount of intracellular sulfur could be reduced if flocs were incubated for 24 hours in a beaker of water from Lake Erie. Fully motile trichomes of *T. ingrica*, treated as described above, contained numerous, rounded bodies (Fig. 10) which were not sulfur inclusions. The number and size of these rounded bodies varied considerably from trichome to trichome. The rounded bodies could be stained with simple basic stains. Hydrolysis with normal hydrochloric acid destroyed the basophilic properties. These rounded bodies were therefore considered to be protoplasmic units rather than vacuoles. When trichomes contained sulfur inclusions, the diffraction halos caused by the sulfur completely obliterated the visualization of the rounded protoplasmic units.

The arrangement of the rounded protoplasmic units in *T. ingrica* could explain the observed distribution of the sulfur droplets, since the sulfur seemed to be located in the interstices between the spherical protoplasmic units. The irregular zig-zag arrangement of the sulfur inclusions would then necessarily follow.

The beaded internal appearance of *T. ingrica* was not seen in *B. alba*. Most trichomes of *B. alba* contained so many sulfur inclusions that details were obscured, and incubation to reduce the amount of sulfur was not successful. However, occasional cells could be found which were devoid of sulfur. These cells also contained rounded bodies in a cloudy matrix (Fig. 11). However, in later experiments it
became evident that the rounded bodies of *B. alba* were lipid inclusions and not the rounded protoplasmic units seen in *T. ingrica*. The oblique, gray bands in Fig. 11 were caused by two gliding trichomes of *B. alba*.

Obviously there appeared to be a fundamental difference between the cellular organizations of *B. alba* and *T. ingrica*. The cells of *T. ingrica* were filled with spherical protoplasmic units between which the sulfur inclusions were located. The cells of *B. alba* contained sulfur inclusions in the peripheral area of a relatively uniform cytoplasm, which also contained conspicuous lipid inclusions.

The appearance of *M. vaginatus* in phase contrast was comparable to the excellent photomicrographs of *M. vaginatus* published by Cassel and Hutchinson (1954). The light central body, containing numerous dark granules, corresponded to the location of chromatinic structures in preparations stained for chromatinic material. In the observation of *C. latum* by phase contrast only alternating dark and light bands along the trichomes and dark granules were seen. In *T. ingrica*, *B. alba*, and *C. latum*, structures which corresponded to the chromatinic material in stained preparations were not observed by phase microscopy.

**Volutin Inclusions**

When Meyer's stain for volutin was used to stain fixed trichomes of *T. ingrica* (Fig. 12), highly basophilic, blue granules were not observed. In *T. ingrica*, the walls, the septa, and the surfaces of the protoplasmic units stained a faint blue. For *B. alba*, the staining time with methylene blue had to be increased to 30 minutes in order to obtain a blue color (Fig. 13). The cytoplasm which was faintly blue
Fig. 12. *T. ingricta*

Fig. 13. *B. alba*

Fig. 14. *M. vaginatus*

Fig. 15. *C. latum*

Figs. 12-15. Meyer's volutin stain
appeared vacuolated. Although the walls of *B. alba* were slightly
darker than the cytoplasm, the septa did not stain darker than the
cytoplasm. All the dark granules in Figs. 12 and 13 were sulfur
inclusions. Only occasional trichomes of *Beggiatoa* with a diameter
of 1.3 μm possessed dark blue granules among the sulfur inclusions.
None of the trichomes of *B. alba* with diameters of 2.0 to 2.7 μm
contained volutin. However, all 5 of the heterotrophic strains of
*Beggiatoa* isolated by Scotten and Stokes (1962) contained many dark
blue "amorphous" granules of various sizes when stained with 1.0 per
cent methylene blue. It would be interesting to determine whether
the intracellular deposition of volutin is associated primarily with
heterotrophic metabolism. However, Guilliermond (1926) sometimes
observed metachromatic granules among the sulfur inclusions of *B. alba*.

Using the phase contrast microscope, Cassel and Hutchinson (1954)
observed dark granules within the light central body of *M. vaginatus*.
When trichomes of *M. vaginatus* were stained for volutin (Fig. 14),
dark blue granules were seen within the weakly stained central body.
The granules of volutin of various sizes appeared with different
frequencies. Furthermore, volutin was observed in *M. vaginatus* at
any age, and the location of volutin corresponded to the dark granules
which were seen in the phase contrast microscope. In *C. latum*,
volutin can be stained regularly (Fig. 15). Marked variation in
size and number of granules of volutin in trichomes of *C. latum* of
various ages has been demonstrated by Kelley (1952). The location of
volutin granules corresponded to the location of dark granules
observed by phase contrast microscopy.
In order to show the metachromatic properties of intracellular granules, fixed trichomes were stained with thionine. Since neither *T. ingricta* nor *B. alba* contained volutin, metachromatic granules were not observed in the cells of these trichomes. However, an interesting characteristic of *T. ingricta* became apparent. Walls and septa always stained distinctly purple and darker than the rest of the cells (Figs. 16 and 17). But the septa appeared more reddish than the walls, which had a bluish tint. Two different appearances of the interior of cells of *T. ingricta* were observed. Sometimes the spherical protoplasmic units stained purple, at other times the surfaces of the spheres stained darker than the interior of the spheres, giving a net-like appearance to the cytoplasm (Fig. 17). Unfortunately, the short depth of focus makes the visualization of the stained, overlapping spheres in Fig. 16 difficult. Irrespective of the method of staining with thionine, both staining characteristics occurred at random.

In order to obtain even a faint color, trichomes of *B. alba* had to be stained for 1 hour in thionine (0.25 per cent). Although the walls appeared slightly darker than the faintly purple cytoplasm, the septa of *B. alba* did not stain (Fig. 18). Unstained lipid inclusions and the sulfur inclusions can be distinguished within the cells.

Fixed trichomes of *M. vaginatus* stained readily with thionine (Fig. 19). Blue walls and faintly blue septa divided the trichomes into segments. The purple central bodies contained darker purple granules, which corresponded in location to the granules of volutin. As is obvious from Fig. 19, there was poor contrast between the blue-
Figs. 16-19. Stained with 0.25 per cent thionine
green cytoplasm and the purple central body and granules. Fewer granules were seen in cells stained with thionine than in cells stained for volutin by Meyer's method (Fig. 14).

Metachromatic granules in *C. latum* could not be demonstrated with thionine. The cells in the trichomes always stained uniformly purple. The appearance was not changed when the preparations were stained for shorter periods, although the color was less intense. Unstained areas in the cells, which would correspond to the location of chromatinic material, were not observed.

**Lipid Inclusions**

In the unstained trichomes of *T. ingrica* in Fig. 20 few sulfur inclusions are apparent. The spherical protoplasmic units and some of the septa are clearly discernible in the trichome on the right. The same two trichomes mounted in a solution of sudan black B are shown in Fig. 21. Walls and septa were stained darkly, and the surfaces of the protoplasmic units were clearly apparent. When the sections of the trichomes which are devoid of sulfur (Fig. 20) are compared to the same sections stained for lipid (Fig. 21), it is obvious that the granules of lipid are located outside of the protoplasmic spheres. The location of lipids corresponded therefore to the general area in which sulfur inclusions were usually located. This is apparent by comparison of Fig. 22 with Fig. 30. Approximately one half of the trichomes of *T. ingrica* which were stained with sudan black did not contain lipid inclusions. The other half of the trichomes of *T. ingrica* had the same appearance as trichomes in
Fig. 20. *T. ingrica*
unstained

Fig. 21. *T. ingrica*
same field as Fig. 20.

Fig. 22. *T. ingrica*

Fig. 23. *B. alba*

Fig. 24. *M. vaginatus*

Figs. 21-24. Stained for lipids with sudan black B
Figs. 21 and 22. However in Fig. 21, the lipid inclusions are less conspicuous than in Fig. 22, since the trichomes in Fig. 21 were not stained as long as the trichomes of Fig. 22. In trichomes of *T. ingrica* examined with phase contrast (Fig. 10) no granules were observed which corresponded to the lipid inclusions.

*Beggiatoa alba* regularly contained inclusions of lipids which appeared as solid bodies with the phase contrast microscope (Fig. 11), while the sulfur inclusions appeared as bright, refractive rings. The rounded lipid bodies appeared blue-black when stained with sudan black B and could be distinguished readily from the refractive sulfur globules. In Fig. 23, the black dots and rings are the sulfur globules, while the lipid inclusions appear gray with a fuzzy edge. Septa and walls of *B. alba* (Fig. 23) did not stain with sudan black. Although massive, intracellular lipoidal structures were characteristic of heterotrophic strains of *Beggiatoa* (Faust and Wolfe, 1961; Scotten and Stokes, 1962), numerous lipid inclusions have been observed in autotrophic strains of *B. alba* (Guilliermond, 1926).

When trichomes of *M. vaginatus* were mounted in sudan black B (Fig. 24), the entire chromoplasm stained gray. Blue-black granules of lipoidal material were not apparent. It was assumed therefore that lipid substances were present in the chromoplasm in a diffuse form and not in discrete granules. The darkly stained septa are apparent in Fig. 24, especially when cells had separated slightly. The walls also stained darker, but this is not seen in the photomicrograph because of the plane of focus. Intracellular lipoidal
inclusions were not observed in *C. latum* under the conditions used in this investigation. Kelley (1952) also failed to demonstrate lipid inclusions in *C. latum*.

**Cellulose**

A reagent described by Frey (1927) imparts to cellulose a deep purple color. In trichomes of *M. vaginatus* only a faint, purple color was apparent in the wall after preparations were stained with Frey's reagent. The purple color might indicate the presence of small amounts of cellulose in the wall. According to Smith (1950), a certain amount of cellulose is found in the walls of blue-green algae. There was no indication of the presence of cellulose in the walls of either *B. alba* or *T. ingrica*. Cellulose has never been definitely demonstrated in the walls of the true bacteria (Lamanna and Mallette, 1953). The absence of cellulose in the walls of *T. ingrica* and *B. alba* seems to indicate a relationship to the order *Eubacteriales*.

**Pectins**

The sheath of *T. ingrica* was not hydrolyzed in the solubility test for pectins (Johansen, 1940). Furthermore, hydrolysis did not affect the color reaction of sheath, walls, and septa. Before hydrolysis, after each step, and at the end of the procedure the sheath of *T. ingrica* stained a faint purple to blue with methylene blue, while the walls were a purplish hue, and the septa were blue. According to Johansen, methylene blue stains pectins violet.
Since the color of filaments of *T. ingrica* stained with methylene blue was not affected by the solubility test, unhydrolyzed trichomes of *T. ingrica*, *B. alba*, *M. vaginatus*, and *C. latum* were stained with methylene blue. In *T. ingrica*, the purplish walls and blue septa were clearly apparent (Fig. 25). The surfaces of the protoplastic units of *T. ingrica* stained a deeper blue than the interior of the units. Although the trichome of *B. alba* in Fig. 26 appears darker than the trichome of *T. ingrica* in Fig. 25, the color of *B. alba* observed in the microscope was fainter than the color of *T. ingrica*. The septa of *B. alba* did not stain, and the slight contrast between wall and cytoplasm could not be increased by prolonged staining. The walls and septa of *M. vaginatus* were easily discernible (Fig. 27). The walls of *M. vaginatus* were a purplish hue, while the septa were blue. A regular sequence in the intensity of the stain was observed in successive septa. A similar pattern was not observed in the septa of *T. ingrica*. The granular, diffusent sheath of *M. vaginatus* (not shown in Fig. 27) also appeared faint purple. The central body was slightly deeper blue than the chromoplasm. Intensely stained granules (voluin) appeared in the area of the central body. For Fig. 28, trichomes of *C. latum* devoid of voluin were selected. The narrow protoplasmic bands stained intensely blue, while walls and crosswalls remained unstained. Wide sheaths of *T. ingrica* were often observed to be longitudinally lamellated. When methylene blue was allowed to diffuse into the preparation from one edge of the coverslip, the lamellation was readily apparent in the partially stained sheath (Fig. 29).
Fig. 25. *T. ingricta*  
Fig. 26. *B. alba*  
Fig. 27. *M. vaginatus*

Fig. 28. *C. latum*  
Fig. 29. Sheath of *T. ingricta*, partially stained

Figs. 25-29. Stained with 1:10,000 methylene blue
Ruthenium red, a classical indicator, imparts a red color to pectic substances (Johansen, 1940). When *T. ingraca* was stained with this dye, no part of the trichomes was red. Consequently, pectins were either absent or were not present in sufficient quantities to give a red color in the trichomes. The sheath of *T. ingraca* did not stain uniformly with ruthenium red. The color was a faint red, a faint purple, or a faint blue-gray. Sometimes a reddish central core was surrounded by a faint, bluish, outer layer. The presence of a red color in some sheaths of *T. ingraca* stained with ruthenium red indicated the presence of pectic substances.

Since the diffluent sheath of *M. vaginatus* stained a faint red, purple, or blue-gray with ruthenium red, it probably contained some pectic substances. A reddish hue of the walls stained with ruthenium red was not observed with certainty. No color was observed when preparations of *Beggiatoa* were stained with ruthenium red.

**Mucopolysaccharides**

The variable coloration of sheaths of *T. ingraca* and *M. vaginatus* stained with ruthenium red was also observed when preparations were stained with alcian blue-basic fuchsin. The firm sheath of *T. ingraca* stained blue (polysaccharide), red, or purple. Sometimes a red central core was surrounded by a blue layer. Similarly, the sheath of *M. vaginatus* stained blue, pink, or purple. A definite layering of the diffluent sheath in two colors was not observed.

The trichomes of *T. ingraca* stained with alcian blue-basic fuchsin appeared light purple with red septa and bluish walls. The
trichomes of *M. vaginatus* appeared pink with slightly darker central bodies. The septa of *M. vaginatus* stained red, the walls bluish. The differential staining of wall and septa of *T. ingrissa* and *M. vaginatus* does not indicate necessarily that wall and septa have a different composition, since Widra (1956) observed that alcian blue did not readily penetrate cell walls. *Beggiatoa alba* was stained for 10 minutes in alcian blue-basic fuchsin. The trichomes were a light pink and had a vacuolated appearance, while septa were a slightly darker pink. A bluish tint in the wall could not be discerned. There was no evidence of a sheath.

**Cell Surfaces**

Relatively prolonged times for mordanting and staining were necessary for the successful staining of cell walls by the phosphomolybdic acid (PMA) - methyl green (MG) procedure. Usually when cell walls are stained, the cells are fixed in Bouin's fixative. The use of osmium tetroxide for fixation in this study might explain the requirement for prolonged treatment. *Thioploca ingrissa* and *B. alba* required the longest periods for mordanting and staining, *C. latum* the shortest.

The appearance of *T. ingrissa* stained by the PMA-MG procedure is illustrated in Fig. 30. *Beggiatoa alba* is shown in Fig. 31 and *M. vaginatus* in Fig. 32. For all 3 organisms, the wall appeared darker than the septa. It will be recalled that wall and septa of *T. ingrissa* and *M. vaginatus* also stained darker with simple stains without previous mordanting. This darker staining with simple stains
was also true for the walls of B. alba. The septa of B. alba, however, appeared darker only when stained with alciar blue-basic fuchsin, otherwise the septa were not apparent with simple stains. The walls and crosswalls of C. latum, on the other hand, did not stain with simple stains and required mordanting with PMA. The appearance of trichomes of C. latum stained by the PMA-MG procedure is shown in Fig. 33. On the basis of the reaction of the walls with different stains, it appears that the walls of T. ingrica, B. alba, and M. vaginatus may be similar, but they are different from the walls of C. latum and true bacteria.

Ruhland and Hoffmann (1926) were able to split the walls of Beggiatoa mirabilis with either 10 per cent potassium nitrate or chloral hydrate. Neither potassium nitrate nor chloral hydrate had any observable effects on the walls of T. ingrica, B. alba, and M. vaginatus. Beggiatoa mirabilis possesses trichomes 15 to 21.5 μ in diameter (Bergey's Manual, 1957), and the effects of these reagents might be easily observed in such large cells, while comparable effects in the smaller species used in this study would not be resolved.

When living trichomes of T. ingrica were mounted in lactophenol cotton blue (Fig. 34), separation of the wall and the cytoplasm was observed in many trichomes. The cells were a faintly cloudy, light blue, and the cytoplasmic membrane stained dark blue. The barely visible septa extended to the wall (see arrows in Fig. 34). A similar effect was obtained in B. alba (Fig. 35), but the cytoplasmic membrane did not stain darker than the distinctly vacuolated cytoplasm. The vacuolated appearance was due to the dissolution of the sulfur
Figs. 34-36. Living trichomes mounted in lactophenol cotton blue
granules and to the regular lipid inclusions. In T. ingrica (Fig. 34), the sulfur inclusions were still present. It was observed repeatedly that the sulfur inclusions of B. alba dissolved faster than the sulfur inclusions of T. ingrica. The shrinkage of the trichomes of B. alba in the lactophenol cotton blue reagent caused the cells to separate at the septa. Separation of the cells at the septa was not observed in T. ingrica (Fig. 34) and in M. vaginatus (Fig. 36).

In the left trichome of B. alba in Fig. 35, the wall can be seen to extend far beyond the cells. By phase contrast microscopy, remnants of dead cells and septa were seen in the extension of the wall. A similar effect was seen in M. vaginatus (Fig. 36), where the wall and the septa of a dead portion of a trichome extended beyond the tip of the intact trichome. However, in M. vaginatus the cytoplasm did not separate from the wall. Unlike the septa of T. ingrica and B. alba, the septa of M. vaginatus, stained distinctly with the lactophenol cotton blue reagent and were also observed in the dead portion of the trichome by light microscopy (Fig. 36).

Chromatinic Material

Although an optimum time of hydrolysis for staining of chromatinic material could not be determined, hydrolysis at 60°C was best. In most preparations hydrolyzed for 6 to 12 minutes, trichomes with well differentiated chromatinic material and trichomes which were either solidly stained or completely colorless could be observed. The variability in differentiation was less in M. vaginatus and C. latum, since preparations of uniform age were used.
Chromatinic material stained by the acid-Giemsa procedure

The chromatinic material of *T. ingrica*, stained by the acid-Giemsa procedure (Robinow, 1944), appeared as numerous, dark, purple granules which were distributed between the spherical protoplasmic units which did not stain after hydrolysis (Figs. 37 to 41). The particular arrangement in which the purple granules were found varied considerably with individual trichomes. Irregular zig-zag patterns (Figs. 37, 38, and 41) were apparent, as well as circular and semi-circular arrangements (Fig. 39). Sometimes the chromatinic granules were condensed into large accumulations in which individual particles could be resolved (Figs. 38 and 40). Sometimes the individual granules could not be resolved (Fig. 37). The appearance of the chromatinic material, however, changed considerably with the focal level. With an organism of such a large diameter it is to be expected that by focusing up and down details can be resolved which photomicrographs of a single plane cannot illustrate. In Figs. 38 and 40, chromatinic granules can be observed in the extended tips. Chromatinic material was often observed to accumulate around the septa (See arrows in Figs. 37 and 40). Septa and walls also stained purple. From the appearance of trichomes of *T. ingrica* stained by the acid-Giemsa procedure it can be concluded that the chromatinic granules were located in the spaces outside of the spherical protoplasmic units.

Chromatinic material in *T. ingrica* could not be stained after extraction of cells with 10 per cent perchloric acid at 60°C which removes deoxyribonucleic acid (DNA) from cells (Cassel, 1950).
Figs. 37-40. *T. ingrica*, acid-Giemsa procedure for chromatinic material
Similarly, after extraction with perchloric acid, the chromatinic material of B. alba, M. vaginatus, and C. latum did not stain.

In trichomes of B. alba stained by the acid-Giemsa procedure (Fig. 42), chromatinic bodies of irregular configuration were observed. Single granules, short granular rods, and V-shaped bodies were encountered. The arrangement of chromatinic material in B. alba was quite different from the arrangement in T. ingrica (Figs. 41 and 42). Likewise, the appearance of chromatinic material in B. alba differed considerably from that described by Scotten and Stokes (1962) for heterotrophic strains, in which one or two discrete, ovoid chromatinic bodies were seen in the cells. Since lipid inclusions were numerous in heterotrophic and autotrophic strains, crowding by sulfur inclusions in autotrophic strains of B. alba might explain the configuration of the chromatinic material.

The appearance of trichomes of C. latum stained by the acid-Giemsa technique (Fig. 43) corresponded closely to the photomicrographs of Kelley (1952). The discrete chromatinic bodies of this organism stained readily with Giemsa stain and were quite distinct from the granular chromatinic material in T. ingrica and the configuration observed in B. alba. In M. vaginatus (Figs. 44 to 46), the chromatinic material in the central bodies appeared also granular. Although the chromatinic granules were located regularly in the central areas of the cells, considerable variation in the arrangement could be observed. Cassel and Hutchinson (1954) reported similar variations of the appearance of the chromatinic material in M. vaginatus and other blue-green algae.
Fig. 41. *T. ingrica*  
Fig. 42. *B. alba*  
Fig. 43. *C. latum*  

Fig. 44. *M. vaginatus*  
Fig. 45. *M. vaginatus*  
Fig. 46. *M. vaginatus*  

Figs. 41-46. Acid-Giemsa procedure for chromatinic material
The observation of Bahr and Schwartz (1957) that the location of the chromatinic material in *Beggiatoaceae* and *Oscillatoriaceae* was confined to an axial filament has not been confirmed. In this study, the chromatinic structures were distinct for each of the organisms (*T. ingrica*, *B. alba*, *M. vaginatus*, and *C. latum*), though within each species considerable variations occurred. Robinow (1956) observed that the "usual" time of osmium fixation left chromatinic bodies in a labile form subject to condensation or fragmentation. The unusually short time of fixation (2 to 3 seconds) employed by Bahr and Schwartz might have resulted in a labile state and subsequent condensation of the chromatinic material.

**Chromatinic material stained by the thionine-thionyl chloride procedure**

The thionine-thionyl chloride method of DeLamater (1951) failed to stain chromatinic structures of fixed and hydrolyzed preparations of *T. ingrica* and *B. alba*, even when the preparations were stained for 48 hours. With *M. vaginatus* (Figs. 47 and 48) and *C. latum* (Fig. 49) the method was successful, although the chromatinic material did not stain as darkly as with the acid-Giemsa procedure. Due to the short depth of focus, it was difficult to show the fine chromatinic structures on the photomicrographs. However, the general appearance of the chromatinic material corresponded well to the appearance of preparations stained by the acid-Giemsa procedure.
Figs. 47-49. Thionine-thionyl chloride for chromatinic material
Chromatinic material stained by the Feulgen reaction

As with DeLamater's method, the Feulgen procedure failed to stain chromatinic material in *T. ingricta* and *B. alba* in spite of numerous variations in the procedure. The failure to reveal DNA by the Feulgen technique in *T. ingricta* and *B. alba* might cast doubt on the results obtained by the acid-Giemsa procedure. However, nuclei in the leaf epidermis of begonias also failed to stain by the Feulgen procedure (Petter, 1933). Robinow (1956) contended that the weak or negative Feulgen reaction in microorganisms is due to technique. It is hoped that a technique will be developed that shows the DNA-nature of chromatinic structures in *B. alba* and *T. ingricta*. Until then, the evidence derived from the acid-Giemsa procedure must suffice.

The chromatinic material in *M. vaginatus* (Fig. 50) and in *C. latum* (Fig. 51) was Feulgen-positive when the reagent was prepared according to Lillie (1951). The Feulgen reagent of Rafalko (1946), which was supposedly superior for coloring small, diffuse chromatinic elements, failed to stain chromatinic material in all four organisms. The Feulgen reaction in *M. vaginatus* was relatively weak. In *C. latum*, the chromatinic bodies stained intensely. The general configuration of the Feulgen-positive chromatinic apparatus of *M. vaginatus* and *C. latum* corresponded closely to the configurations which were observed in preparations stained by either DeLamater's or the acid-Giemsa procedures. The sheath of *T. ingricta* and *M. vaginatus* in preparations stained by the Feulgen technique was positive even in unhydrolyzed preparations. A similar, nonspecific reaction was reported by Delaporte (1940) for the sheaths of many blue-green algae.
Figs. 50 and 51. Feulgen technique for DNA
The Gram Reaction

When fixed preparations of the four different trichomes were stained by the gram procedure, it was apparent that a simple classification as either a gram-positive or gram-negative organism was not possible. *Thioploca ingrica* contained fine gram-positive granules in a gram-negative trichome. The number of the dark purple granules showed marked variation. Sometimes the gram-positive granules were so numerous that the trichomes appeared almost completely purple with only gram-negative septa. The sheath was definitely gram-negative. *Beggiatoa alba* was gram-negative, except in thick portions of the preparations where some of the trichomes contained gram-positive granules. The trichomes of *M. vaginatus* appeared similar to the trichomes of *T. ingrica*, but the septa were always gram-positive. The mycelia of various fungi, like *T. ingrica* and *M. vaginatus*, have been shown to contain gram-positive granules (Lamanna and Mallette, 1953). The sheath of *M. vaginatus* was gram-negative. A 6 hour culture of *C. latum* was definitely gram-positive, which was also reported by Provost and Doetsch (1962).

Hydrolysis by Lysozyme

The effect of a 0.1 per cent solution of lysozyme on trichomes of *T. ingrica*, *B. alba*, *M. vaginatus*, and *C. latum* was determined using wet mounts and suspensions of trichomes in tubes. The results from both tests were identical. Lysozyme had no effect on *C. latum* and *M. vaginatus* under the conditions of the tests. *Thioploca ingrica*
and *B. alba* were not affected by heated lysozyme in which they remained motile. However in the solutions of fresh lysozyme, the trichomes ceased to glide, the walls bulged, sometimes the cytoplasm formed conspicuous protuberances through the walls, and the septa bulged. Although the cellular contents were lost, the displaced sulfur inclusions remained within the remnants of the walls and septa of the trichomes.

One of the products of lysozyme action is 3-O-carboxyethyl-D-glucosamine, muramic acid (Salton, 1960). According to Stanier and Van Niel (1962), muramic acid is uniquely and exclusively found in the walls of procaryotic protists, bacteria and blue-green algae. Although the presence of muramic acid in cell walls is necessary, the amount of muramic acid does not determine susceptibility of a wall to the action of lysozyme. There is a great variability in the susceptibility to lysozyme even among the species of a genus (Salton, 1957). According to Salton (1960), the proposed cell wall structure contains a backbone of alternating residues of acetyl-muramic acid and acetyl-glucosamine with alternating 1-4 and 1-6 beta glycosidic bonds. Short peptide chains, which probably cross-linked the backbone-chains, are attached to the carboxyl groups of muramic acid. The combination of glucosamine, muramic acid, and peptide residues is referred to as mucopepptide (Salton, 1961). It is the 1-4 bond that is hydrolyzed by lysozyme, and consequently different linkages between the amino sugars would confer resistance to lysozyme (Salton, 1960). Even if 1-4 beta glycosidic bonds were present, the configuration of adjacent molecules could make the sensitive bonds inaccessible to lysozyme (Perkins, 1963).
Numerous bacteria, both gram-positive and gram-negative, are susceptible to the action of lysozyme (Salton, 1957). Since no reports are known of the lysis of blue-green algae, the susceptibility to lysozyme of *T. ingrica* and *B. alba* might indicate a relationship to the *Eubacteriales*. However, future investigations might show that under suitable conditions a similar spectrum of sensitivity to lysozyme exists in blue-green algae. Furthermore, circumstantial evidence indicates that the substrate of lysozyme is present in the walls of blue-green algae. Stanier and Van Niel (1962) and Perkins (1965) emphasized the parallel between penicillin sensitivity and the inhibition of mucoprotein synthesis. Galloway and Krauss (1959) showed that the blue-green alga *Anabaena variabilis* was 10,000 times more sensitive to penicillin G than green algae and gram-negative bacteria. The concentration of penicillin which inhibited *A. variabilis* was comparable to the concentration of penicillin which inhibits sensitive strains of *Staphylococcus*. Salton (1960) stated that the mucoprotein was found in walls of *Microcoleus vaginatus*.

Since it was not possible to grow *T. ingrica* in pure cultures, the effect of penicillin and the presence of muramic acid in the walls of *T. ingrica*, *B. alba*, and *M. vaginatus* were not ascertained. It is hoped that future work will correlate lysozyme and penicillin sensitivity, staining reactions, and the presence of muramic acid in the walls of these three species.
Hydrolysis by Pectic Enzymes

When *T. ingrica* and *M. vaginatus* were stained with ruthenium red, the red color observed in some of the sheaths indicated the presence of pectins. After treatment with the various combinations of pectic enzymes, the same staining reactions with ruthenium red were observed as in untreated preparations. It must be concluded therefore, that either the reddish color obtained with ruthenium red did not indicate pectic substances, or that the pectic compounds present were resistant to the action of the pectic enzymes under the conditions of the test.

It will be recalled, that the solubility test for pectins was also negative. The reaction of the wall and the sheath of *M. vaginatus* with alcian blue-basic fuchsin likewise was not altered by treatment with pectic enzymes. It is realized that these investigations contribute relatively little to the understanding of the nature of the sheath of *T. ingrica* and *M. vaginatus*. Chemical investigations of the nature of the sheath of *T. ingrica* will have to await the cultivation of the organism in pure cultures.

Taxonomic Significance

Koppe (1924) was unable to distinguish free trichomes of *Thioploca* from trichomes of *Beggiatoa*. Bavendamm (1924) asserted that *Thioploca* may be just an association of trichomes of *Beggiatoa* which secreted a common sheath under unknown conditions. Kolkwitz (1955) concurred in this opinion, and Buchanan (1955) described *Thioploca* as *Beggiatoa* within a common sheath. This cytological study revealed
that the trichomes of T. ingrica and B. alba possessed different cellular organizations and different terminal segments. Consequently, the generic status of Thioploca seems to be justified.

Thioploca ingrica and B. alba possessed certain characteristics in common with the blue-green alga M. vaginatus. In all three species, the wall could be stained with simple basic dyes. Eubacteriales and Caryophanales require mordanting to stain the cell wall with the simple dyes used in these studies. The gliding motility of the highly flexible trichomes is possessed by members of the order Beggiatoales and members of the order Oscillatoriales. However, the chemical nature of the walls of T. ingrica and B. alba must be different from the chemical nature of the wall of M. vaginatus, since the latter was not lysed by lysozyme and gave a weakly positive reaction for cellulose. It should be remembered, that among the Eubacteriales there is considerable variation in the susceptibility to lysozyme within the species of a genus. Even among strains of the indicator organism of lysozyme action, Micrococcus lysodeikticus, less sensitive strains have been discovered (Salton, 1957). The color obtained in stained sheaths of T. ingrica and M. vaginatus was identical, although the physical characteristics of the sheaths of the two species differed.

The arrangement of chromatinic material was distinct for each of the four species in this study. Characteristic bacterial chromatinic bodies were observed in C. latum. The granular chromatinic material of M. vaginatus was characteristically located in the central body. In T. ingrica, the configuration of chromatinic granules seemed to be determined by the size and number of spherical protoplasmic units in
the cells, since the granular chromatinic material was located outside of these units. To the contrary, the chromatinic bodies of *B. alba* did not seem to have a definite location in relation to other cell constituents. The appearance of the chromatinic material of *T. ingrica* and of *B. alba* was neither distinctly bacterial nor distinctly cyanophycean. The sulfur and lipid inclusions of *T. ingrica* were located in the same area in which the chromatinic granules were located. In *M. vaginatus*, volutin bodies and chromatinic material were located in the central body. A similar relation in the location of sulfur, lipid, and chromatinic elements was not apparent in *B. alba*. Volutin bodies were located outside of the chromatinic area of *C. latum*.

This comparative study was concerned only with *T. ingrica* and *B. alba*, two members of the family *Beggiatoaceae* which is presently placed in the order *Beggiatoales* of the class *Schizomycetes* (Bergey's Manual, 1957), and *M. vaginatus*, a member of the supposedly related family *Oscillatoriaceae* of the order *Oscillatoriales* which is an order of the class *Schizophyceae*. The other families of the orders *Beggiatoales* and *Oscillatoriales* have not been considered, although investigations are needed to settle disputed relationships.

The distinction between photoautotrophic *Oscillatoriaceae* and chemoautotrophic *Beggiatoaceae* has been invalidated by the discovery of *Beggiatoa* and *Cyanophyceae* capable of heterotrophic metabolism and of chemoautotrophic *Thioploca* with faint, blue-green pigmentation. The morphological similarity of the *Beggiatoaceae* and the *Oscillatoriaceae* seems to relate the *Beggiatoaceae* more closely to the *Oscillatoriaceae* than to the bacteria. Since the intracellular
organization of *T. ingrica* and *B. alba* was markedly different from the intracellular organization of the Oscillatoriaceae, as represented by *M. vaginatus*, the family Beggiatoaceae should be maintained.

Perhaps an arrangement as it is found in the order Pseudomonadales should be followed. In the Pseudomonadales, the photosynthetic members are placed in the suborder Rhodobacteriineae, while the non-photosynthetic members are placed in the suborder Pseudomonadineae. Within the Pseudomonadineae, autotrophic families contain also facultative members (Bergey's Manual, 1957). A similar arrangement would place the families Beggiatoaceae and Oscillatoriaceae into different suborders of the order Oscillorales of the class Schizophyceae. It is hoped that other studies will assign to the other families of the order Beggiatoales consistent positions in the proposed taxonomic scheme. Stanier and Van Niel (1962) have pointed out that the bacteria cannot be clearly separated from the blue-green algae in view of their common procaryotic cellular organization. The relationship of the two groups is expressed in the classification in Bergey's Manual (1957) in which the classes Schizophyceae and Schizomycetes are united in the division Protophyta.
SUMMARY

Growth of *T. ingrica* has been obtained in flasks containing water and mud from Lake Erie and 0.05 to 0.4 per cent thoroughly extracted hay. Four or 5 weeks after preparation the media were inoculated. Isolation of possible heterotrophic variants from the hay-media was not possible.

Autotrophic growth of the organism under defined atmospheric conditions was not obtained. Survival for up to 100 days was observed in an atmosphere with partial pressures of oxygen of 8 mm Hg and hydrogen sulfide of 0.1 mm Hg, or oxygen of 16 mm Hg and hydrogen sulfide of 0.2 mm Hg. Although carbon dioxide and saturation of natural sulfur water, water from Lake Erie, and mud extract with calcium carbonate was essential for survival, the concentration of carbon dioxide was not critical and a partial pressure of 25 mm Hg was used.

Addition of organic compounds to cultures shortened the time of survival considerably. Mud from the natural habitat of the organism, when added to the media, had no effect. Sodium sulfide or ferrous sulfide could not replace hydrogen sulfide.

Observations of living trichomes by phase contrast microscopy and of stained trichomes revealed distinct differences in the cellular organizations of *T. ingrica* and *B. alba*. Cells of *T. ingrica* contained
spherical protoplasmic units. Lipid and sulfur inclusions and chromatinic granules were located outside of the spherical units. Volutin was not observed in *T. ingrica*, and the tips of trichomes were rounded, tapered, or extended. According to the tests used in this study, cellulose and pectic substances were not present in sheaths and trichomes of *T. ingrica*. The trichomes of *T. ingrica* were susceptible to lysozyme. Reactions to various special stains indicated a complex composition for the lamellated sheath of *T. ingrica*. The walls and septa of the organism stained with simple dyes. Lactophenol cotton blue effected a separation of the wall and cytoplasm of *T. ingrica*. Chromatinic structures were stained by the acid-Giemsa procedure but failed to stain with Delamater's thionine-thionyl chloride and the Feulgen procedures. The trichomes of *T. ingrica* contained gram-positive granules.

The interior of cells of *B. alba* was not organized into spherical units. Consequently, a definite orientation of lipid and sulfur inclusions was not observed. Volutin and cellulose were not observed in *B. alba*. The walls of the organism stained weakly with simple dyes, the septa did not stain. Walls and septa could be stained distinctly by the phosphomolybdic acid-methyl green procedure. Lactophenol cotton blue caused the cells to separate and effected a separation of the wall and cytoplasm. The irregular chromatinic bodies were colored intensely following staining by the acid-Giemsa procedure but were not stained by the thionine-thionyl chloride and the Feulgen procedures. The trichomes of *B. alba* were gram-negative and were susceptible to the action of lysozyme.
In trichomes of *M. vaginatus*, the typical cellular organization into chromoplasm and central body was apparent in unstained preparations. Volutin was present regularly as conspicuous granules in the area of the central body. Lipid inclusions were not observed. There was a possible indication of the presence of cellulose in the walls of the trichomes. Pectic substances, if present in the sheath and trichomes of *M. vaginatus*, were not affected by pectic enzymes. Lysozyme had no visible effect on the walls of *M. vaginatus*. Reactions of the sheath to special stains indicated a heterogenous nature of this diffluent structure. The walls and septa of the organism stained with simple dyes. Lactophenol cotton blue had no visible effect on the integrity of the trichomes of *M. vaginatus*. Chromatinic granules in the central body were observed following staining by the acid-Giemsa procedure, the thionine-thionyl chloride procedure, and the Feulgen procedure. The trichomes of *M. vaginatus* contained gram-positive granules and septa.

The typical banded appearance of trichomes of *C. latum* was observed in unstained and stained preparations. The wall and cross-walls of the organism did not stain with simple dyes but stained after mordanting with phosphomolybdic acid. Although volutin was present regularly in the cells, lipids were never observed. Lysozyme had no visible effect on *C. latum*. Chromatinic bodies were easily stained by the acid-Giemsa, the thionine-thionyl chloride, and the Feulgen procedures; *C. latum* was gram-positive.
BIBLIOGRAPHY


I, Siegfried Maier, was born in Stuttgart, Germany, on April 22, 1930. I received my secondary-school education in Germany. My undergraduate training was completed at Capital University, Columbus, Ohio, which granted me the Bachelor of Science degree in 1958. While a senior at Capital University, I was awarded a Danforth Graduate Fellowship, which was annually renewed until the requirements for the degree Doctor of Philosophy were completed. I received the Master of Science degree in 1960 from the Ohio State University. During the final year of graduate study, I obtained a graduate assistantship in the Department of Microbiology. I held this position while completing the requirements for the Doctor of Philosophy degree.

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