STUDIES ON THE FILAMENTOUS SHEATHED IRON BACTERIUM SPHAEROTILUS NATANS

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The extensive literature on the iron bacteria and the present status of our knowledge of this interesting group of microorganisms recently have been reviewed critically and exhaustively by Pringsheim (1949*a*). It is clear from this review that every aspect of the iron bacteria—ecology, isolation, culture, morphology, nutrition, biochemistry, and taxonomy—requires extensive investigation if there is to be a sound knowledge of these microorganisms. The present investigations were begun as part of a systematic attempt to fill in some of the existing gaps.

The studies which are reported here are concerned with one of the commonest of the iron bacteria, *Sphaerotilus natans*. This organism was described and named first by Kützing (1833) who noted its presence in large numbers in polluted waters. It was rediscovered later by Cohn (1875) under similar conditions, but because the filaments appeared to be dichotomously branched, although falsely so, Cohn thought that he had found a new organism and accordingly named it *Cladothrix dichotoma*. Both names have appeared in the subsequent literature and were considered by some investigators (Linde, 1913) to apply to one and the same organism and by others (Zikes, 1915) to organisms that are different. But Pringsheim (1949*b*) has shown that *S. natans* exhibits dichotomous false branching whenever it is grown in media of low organic matter content. It seems best, therefore, to consider *C. dichotoma* as being the cladothrix form of *S. natans*. In this paper, therefore, we shall use the name *S. natans* exclusively.

Zopf (1882) claimed that *S. natans* is highly pleomorphic and described the occurrence of micrococci, vibrios, spirilla, and spirochetes along with the usual rod shaped cells. But this was based on observations of impure cultures and has not been supported by subsequent studies with pure strains. Büsgen (1894) was the first to obtain such pure cultures by streaking filaments of *S. natans*, originally obtained from flowing contaminated water, on meat extract-gelatin plates. Since then many other investigators (Höflich, 1901; Linde, 1913; Zikes, 1915; Cataldi, 1939; Lackey and Wattie, 1940; Pringsheim, 1949*b*) also have isolated pure cultures by employing the usual bacteriological techniques, and investigations made with these strains have supplied a great deal of information on the culture, morphology, nutrition, and general physiology of *S. natans*.

In the present investigation nine strains of *S. natans* were isolated from a variety of aqueous habitats. Their morphology, heterotrophic nutrition, and general physiology were studied. The results obtained confirm, modify, and extend those of previous investigators. No significant differences were observed among the nine strains although each one was isolated from a different source.

EXPERIMENTAL METHODS AND RESULTS

Isolation of cultures. Enrichment cultures were prepared in a medium which consisted simply of one per cent alfalfa straw in tap water. The straw had been cut previously into pieces about one inch in length and extracted three or four times by boiling with large volumes of water in order to remove most of the soluble organic matter. The presence of more than small amounts of organic matter in the medium permits the growth of a large number of varieties of microorganisms, and this makes the subsequent isolation of *Sphaerotilus* more difficult (Cataldi, 1939). The extracted hay medium was distributed in 50 ml quantities in 125 ml Erlenmeyer flasks, and each flask was inoculated with about 10 ml of water from streams, ponds, and ditches. The enrichment cultures were incubated at room temperature and examined microscopically at intervals for the presence of filaments of *Sphaerotilus*. These appeared in considerable numbers, usually

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after about a week, and were obtained with most of the water samples. Tufts of filaments were found attached to the pieces of straw and also to the sides of the flasks at or near the surface of the medium; single filaments in small numbers were distributed throughout the liquid portion of the medium.

Positive cultures were streaked on a solid medium composed of glucose, 0.1; peptone, 0.1; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2$, 0.005; $FeCl_3 \cdot 6H_2O$, 0.001; agar, 1.25 per cent in tap water and incubated at 28 C. Within two or three days, colonies of *Sphaerotilus* several millimeters in diameter could be seen and tentatively identified by their characteristically flat, dull, white, cottony appearance. The edges of the colonies are very irregular due to the presence of curled filaments of growth which extend from the center in all directions (figures 1–3). They resemble somewhat young, unsporulated mold colonies but are not as dry or coarse. Identification was confirmed microscopically by finding chains of rod shaped cells enclosed in sheaths. The latter fit closely about the cells and therefore are not easily seen except when the cells have moved out of a portion of the filament and have left a section of empty sheath. Even then they are not visible readily with the ordinary light microscope but can be seen more easily if the slide preparation is allowed to become almost completely dry or if a drop of one per cent aqueous crystal violet is added to the preparation. In the latter case the cells stain a deep purple and the sheaths lightly so. The sheaths, however, can be seen easily in unstained preparations with the phase microscope under dark contrast.

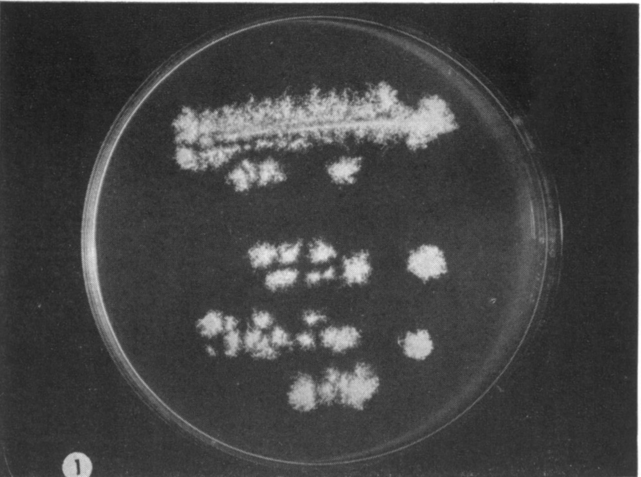
Pure cultures were obtained by restreaking fresh plates with material from isolated colonies. It is important to dry the surface of the sterile agar medium. Otherwise contaminating bacteria, attached to the gelatinous sheaths of the *Sphaerotilus* filaments, are carried along with the latter in the streaking process. Or if separation is obtained initially, the growths later become confluent because of the connecting surface water film. This difficulty was eliminated by storing the sterile plates at 37 C overnight to remove excess surface moisture. A total of nine pure cultures of *Sphaerotilus* was isolated and identified as strains of *S. natans* by their morphological and cultural properties (Pringsheim, 1949*b*). Also, the cultures were sent to Dr. Pringsheim, and he has confirmed our identifications.

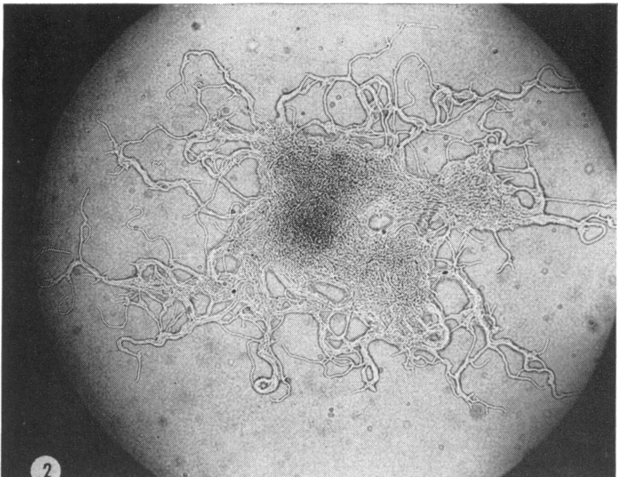
Morphology. Types of colonies. When isolated from natural habitats, *S. natans* is recognized most easily by its characteristically filamentous colonial form which is shown in figures 1–3. This form can be considered to be equivalent to the rough or R type which is found so commonly among bacteria. But on cultivation in the laboratory the R type dissociates and gives rise to a smooth or S type in which the colonies are strikingly different in that they are smooth, glistening, often hemispherical, and usually have a very regular edge. S type colonies are shown in figure 4. Intermediate forms in which the center of the colony is smooth but the edges are irregular and filamentous are common. The R type of colony is composed predominantly of filaments whereas the S type consists mainly of single cells. The R type is best for studying the relationship between cells and sheaths. The S type is most suitable for studying the morphology and motility of the individual cells. Also, because it can be made into uniform cell suspensions, the S type is very useful for manometric experiments.

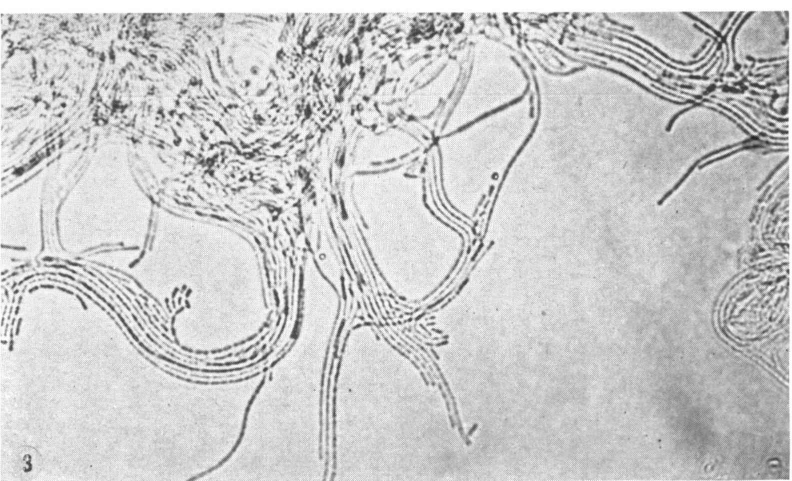
Höflich (1901) briefly mentions the occurrence of filamentous and smooth colonies in cultures of *S. natans*. And Pringsheim (1949*b*) has found R to S dissociation in the related species, *Sphaerotilus discophorus*. The factors which control the dissociation phenomenon are not known. However, we have found that a low content of organic matter in the medium seems to favor the formation of the R type whereas the S form appears more readily on rich media. The degree of dryness of the agar surface does not seem to influence dissociation.

It may be that the S type can also be isolated directly from enrichment cultures, but this is difficult to determine because the cells of this type look so much like other relatively short motile rods, i.e., pseudomonads, that it is impossible to identify them as cells of *S. natans*. Also, the frequent occurrence of the S type of growth in pure cultures makes it often difficult to decide whether one has a dissociated or a contaminated culture. Reisolations of the strains from single colonies were made whenever there was any suspicion of contamination and also as a routine preventative measure.

Structure of sheaths and cells. The most characteristic structures of *Sphaerotilus* are the filaments. These consist of chains of rod shaped cells with rounded ends which are enclosed in closely fitting envelopes or sheaths. This is







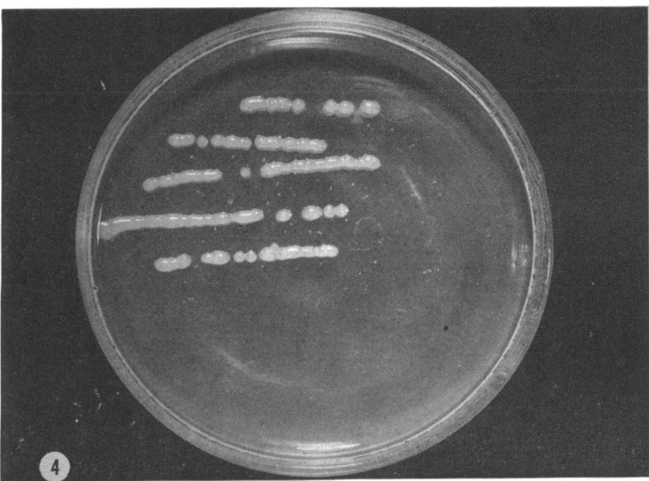
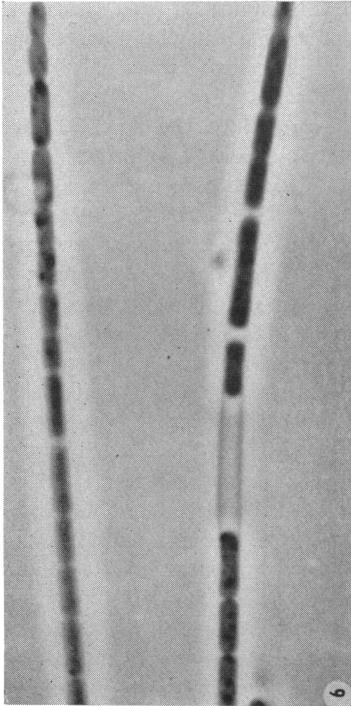
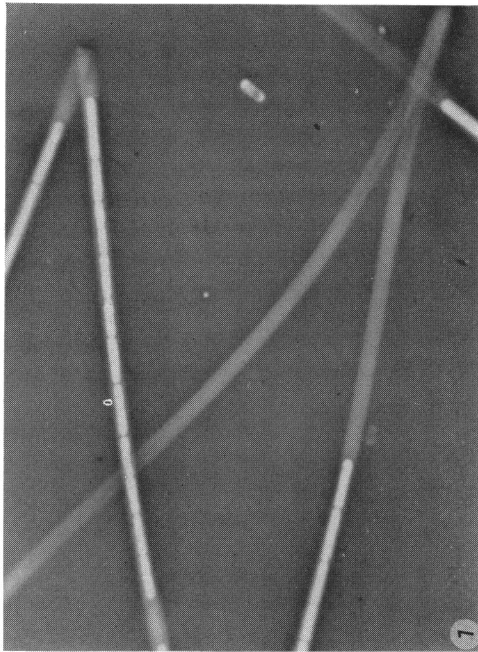


Figure 1. Rough colonies of strain 10; two days old; × 0.56.
Figure 2. Rough colony of strain 11; one day old; × 37.
Figure 3. Edge of rough colony of strain 6; two days old; magnified to show the rods which compose the filaments; × 480.
Figure 4. Smooth colonies of strain 11: three days old; × 0.56.





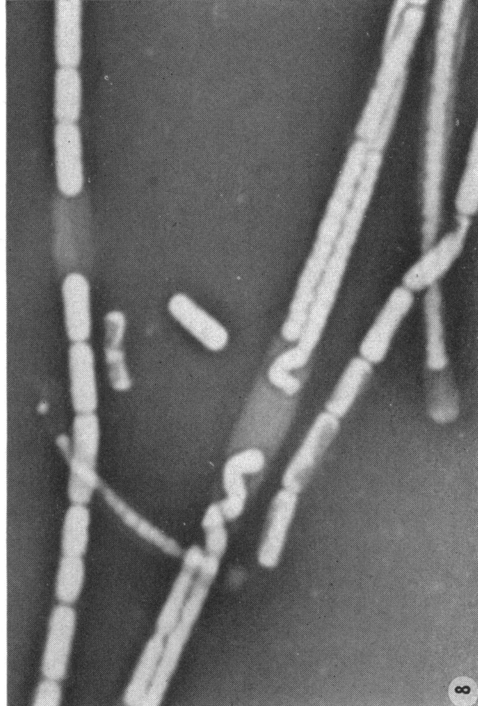


Figure 5. Sheath and cells of strain 8; stained with nigrosin; × 1,700.
Figure 6. Sheath and cells of strain 11; living material photographed with the phase microscope (dark contrast); × 2,300.
Figure 7. Nigrosin preparation of strain 13 which shows spaces between cells and sheath due to contraction of the cells on drying; × 1,250.
Figure 8. Nigrosin preparation of strain 12 which shows two strands of cells in a common sheath; × 2,500.

shown in figure 5. The sheaths are thin, transparent, and also coherent since they can be bent at very sharp angles without tearing. They are soft tubes and tend to collapse when they are not distended by the cells. The sheaths look very much like pieces of cellophane. Their chemical composition is not known. According to Linde (1913) the sheaths are soluble in 50 per cent H_2SO_4 but not in 5 per cent H_2SO_4, 60 per cent KOH, nor ammoniacal copper solutions. They may contain hemicellulose but give no reaction for chitin (Linde, 1913; Zikes, 1915).

There is a very close fit between cells and sheaths. This is shown clearly in figure 6 which is a photograph of living material taken with the phase microscope. Cell preparations that are dried and stained and also wet preparations stained with nigrosin and allowed to dry frequently show large spaces between cells and sheath. This condition, in a nigrosin preparation, is shown in figure 7. But it is an artifact due to the shrinkage of the cells on drying, and it was used erroneously by Fischer (1895) as evidence that there is sufficient space within the filaments for the cells to develop their flagella while still enclosed in the sheaths although Fischer's main conclusion appears to be correct (see below).

Occasionally two rows of cells are found within a common sheath as shown in figure 8. This phenomenon was first noted by Büsgen (1894) and later also by Linde (1913) who reported that as many as three rows of cells may be present in a single sheath.

The dimensions of unstained cells taken from three day old agar cultures ranged from 1.2 to 1.8 μ in width and from 2.5 to 16 μ in length although most cells were 3 to 8 μ long. These dimensions correspond fairly closely to those reported by other investigators. Since the sheath is very thin and clings to the cells, the width of the cells can be considered to be also the width of the sheaths and the filaments. Collapsed, empty sheaths, however, are broader by 50 per cent or more.

The cells contain prominent and numerous refractile bodies of various sizes, and some are quite large. They are present both in young and old cultures and appear as black, globular bodies when viewed in the phase microscope under dark contrast (figure 9). They look like fat globules and stain with Sudan Black B. Similar bodies have been noted in *S. natans* by most investigators, and apparently they misled Zopf (1882), Eidam (1879), and Büsgen (1894) into concluding that they are spores although no attempts were made to establish that they are resistant to heat.

In young cultures the filaments appear to consist of long strands of protoplasm without any subdivisions. As the cultures age, cross walls appear and the filaments become chains of rods connected by thin bridges of protoplasm. Eventually the rods separate completely, and gradually large spaces appear between individual cells and groups of cells as the rods begin to move out of the sheaths. Also, as the cultures age, the sheaths disintegrate and liberate cells. It is our impression that the latter process is the main one for the liberation of the cells, at least under our experimental conditions. The situation may be different in nature where one finds, in ochraceous deposits, enormous numbers of iron encrusted empty sheaths. The iron coating may prevent the sheath from disintegrating and thus force the cells to migrate out. Only traces of iron were present in our media. The disintegration of the sheaths into amorphous debris in our cultures was so extensive and complete as to suggest the possible participation of enzymes secreted by the cells.

Form and arrangement of the flagella. The individual cells liberated from the sheaths are frequently actively motile. Fischer (1895) has shown, by means of stained preparations, that these motile cells or swarmers possess a single bundle of flagella which is composed usually of 8 to 12 strands and that these arise from one locus on the side of the cell, generally near the rounded end. These observations have been amply confirmed by subsequent investigators, but Höflich and others have noted that the flagella may arise also from the ends although rarely at the apex. Also, the number of strands composing the fascicle appears to vary greatly. Pringsheim found only a single strand, but most investigators report the presence of 5 to 8 flagella. This variation may be due, at least in part, to the difficulty in obtaining a complete separation of the fascicle into individual strands since the flagella tend to stick together.

Our strains of *S. natans* are also flagellated lophotrichously as shown in figure 10. The flagella are intertwined to such an extent that they appear to be a single unit in most instances. The tuft arises from one locus which may be on the side or end of the cell or occasionally on the

apex. But one cannot be certain of the point of insertion of the tuft because the flagella may cling to the surface of the cell for some distance from the point of origin before they extend out from the cell. The fascicle is about 0.2 μ wide and may be as much as 80 to 90 μ long or four to five times the length of the cell. It is wavy, uniform in width, but tapers at the end. The fascicle is composed of strands as can be seen on the cell located at the bottom of figure 10; the tuft of this cell has separated into five strands.

Because some of the fascicles are wide enough to be within the resolving power of the microscope, they are visible on living, motile cells in the phase microscope under dark contrast. This permitted a study of the form and arrangement of the flagella without the danger of distortion inherent in making stained preparations. It turned out, however, that in general the stained preparations gave a reasonably accurate picture of the flagella. This is clear from a comparison of the stained cells in figure 10 with the living cells shown in figure 11. The flagella on living cells also are twisted into a single, wavy structure of uniform width but which tapers at the end. There is no sign of a separation of the tuft into strands, and therefore the occurrence of separation in the stained preparations must be considered an artifact. The dimensions of the unstained and stained tufts are essentially the same. The living material shows one aspect of the flagella which is not evident with the stained cells, and that is the spiral shape of the fascicle. This can be seen in figure 12 but perhaps is observed more clearly with living cells under dark field illumination as shown in figure 13. Also, the movement of the flagella as a single helicoidal unit can be seen very clearly with the dark field microscope. The flagella are always behind the moving cell and extend out from the apex.

Frequently we have seen motile cells, in the phase microscope, which have stopped moving, but their flagellar tufts continued to move. This apparently independent movement of the flagella and their sharply defined and uniform morphology suggest strongly that the flagella of *S. natans* are truly organs of locomotion rather than incidental, trailing, strands of inert slime as claimed by Pijper for the flagella of many bacteria (*cf* Knaysi, 1951).

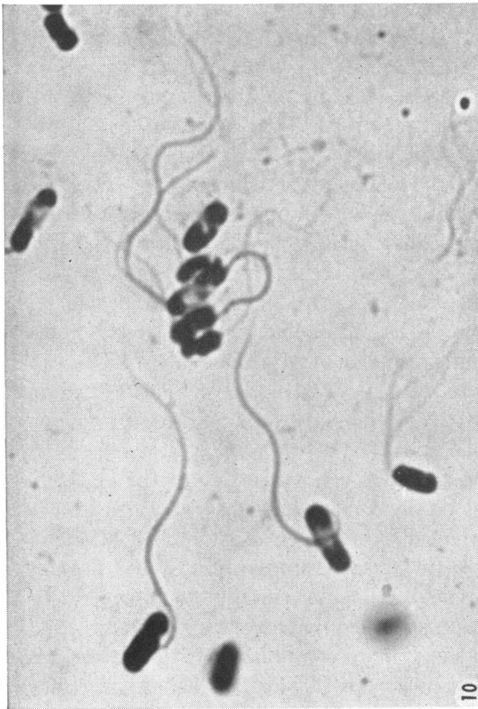
The detailed structure of the flagellar tuft is best seen under the high magnification of the electron microscope. The drying process and the high vacuum imposed seem to favor the unravelling of the fascicle into its individual strands or flagella. The electron photomicrograph of the cells in figure 14 reveals that the fascicle may be composed of more than 12 strands as reported by Fischer (1895). It is difficult to make an exact count of the number of strands because the unravelling is not complete. But it is estimated that there are about 20 strands in this particular tuft. One cannot be certain, of course, that the visible individual strands are not composed of a number of even finer fibers. The cell in figure 15 has 12 strands of flagella and is particularly interesting because the tuft arises from the apex.

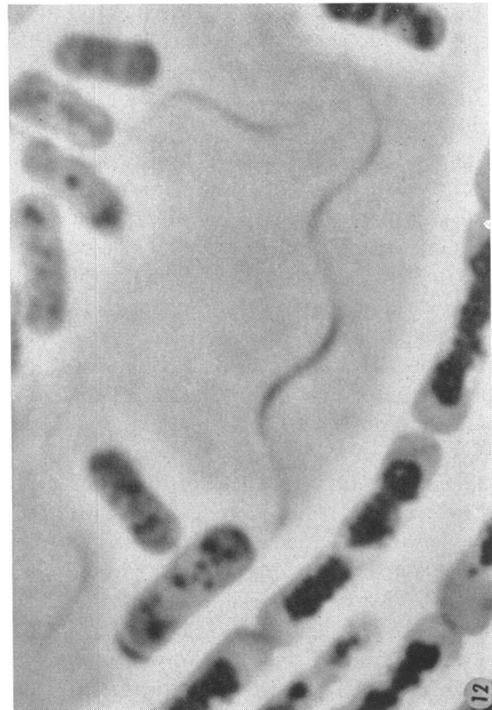
Since the cells can move out of the sheaths, it must be assumed that the flagella are formed while the cells are still encased. Indeed Fischer's drawings of stained preparations show flagella on sheathed cells. We could not, however, see flagella on sheathed living cells with either phase or dark field microscopy although the flagella on liberated cells were visible. This is not surprising because the tight fit between cells and sheaths could very easily obscure flagella pressed between the two. But we did obtain just one stained preparation, shown in figure 16, which contained a segment of sheath with a single cell inside with a sharply defined flagellar tuft.

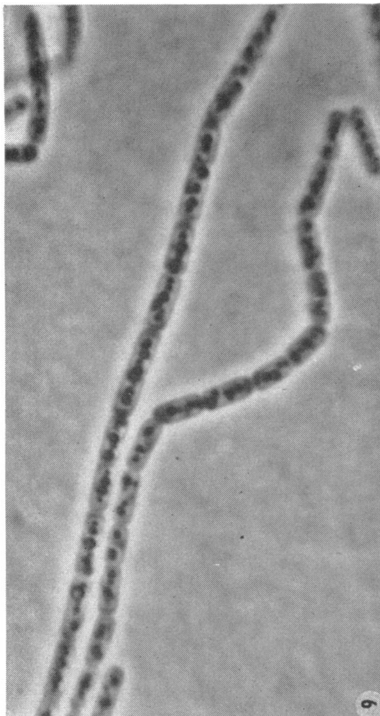
Nutrition. A survey was made of the carbon, nitrogen, and growth factor requirements of the *Sphaerotilus* strains.

Sources of carbon and energy. The basal medium contained 0.05 per cent casamino acids (casein hydrolyzate) as the source of nitrogen; the following minerals, in per cent: $MgSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.01; $CaCl_2$, 0.005; $FeCl_3 \cdot 6H_2O$, 0.001; M/100 K_2HPO_4-KH_2PO_4 buffer, pH 7.1; and distilled water. The medium was prepared without the phosphate buffer, adjusted to pH 7, distributed in 9.5 ml quantities into 50 ml Erlenmeyer flasks and autoclaved. To each flask was added 0.1 ml of sterile M/1 phosphate buffer and 0.5 ml of an autoclaved 5 per cent neutral solution of the carbon source to be tested. Pyruvate and alcohols, however, were sterilized by filtration. The concentration of the carbon source in the medium, therefore, was 0.25 per cent although in some instances lower and higher concentrations were used.

Phosphate was used in the form of buffer because it had been noted that cultures tended to







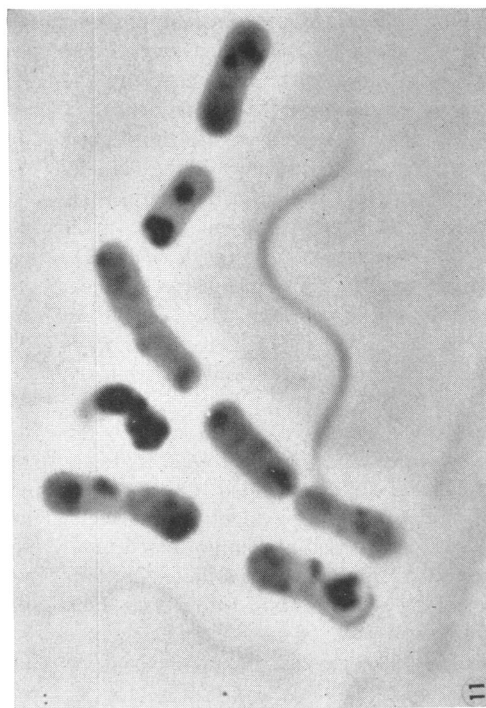
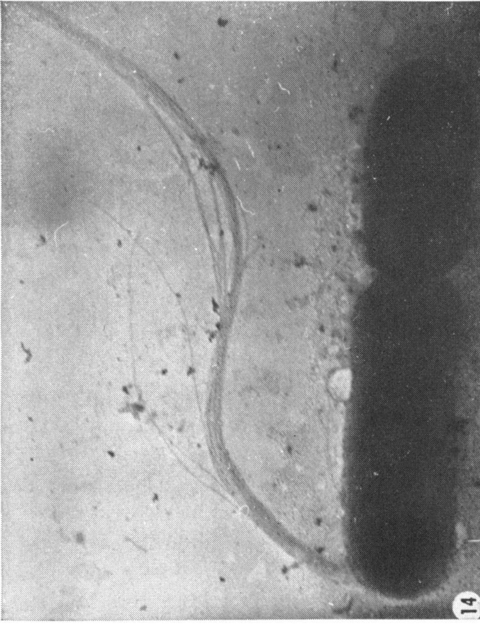
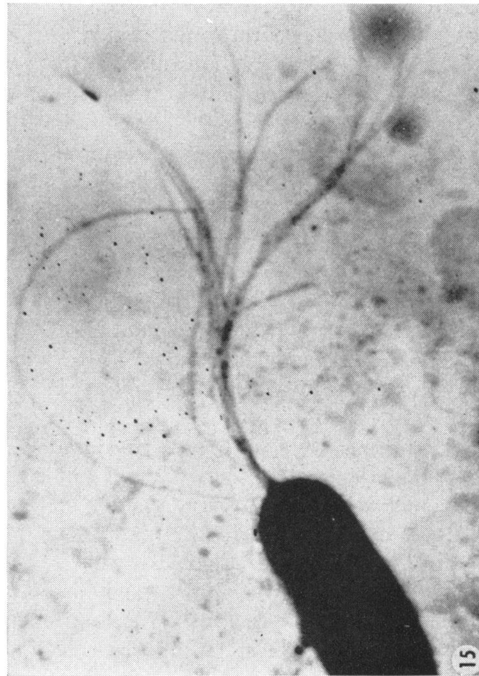


Figure 9. Fat globules in cells of strain 12; one day old live cells photographed with the phase microscope (dark contrast); × 1,900.
Figure 10. Flagellated cells of strain 11; stained with tannic acid and basic fuchsin; × 2,700.
Figure 11. Flagellar tufts on living, unstained cells of strain 4; photographed with the phase microscope (dark contrast); × 5,300.
Figure 12. Spiral shaped flagella on living, unstained cells of strain 4; photographed with the phase microscope (dark contrast); ×4,200.





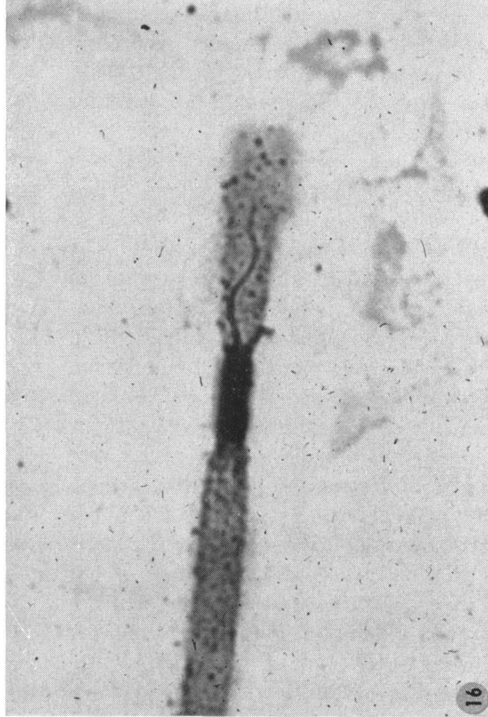


Figure 13. Spiral shaped flagella on a living, unstained cell of strain 11; photographed under dark field illumination; × 8,000.
Figure 14. Individual strands of flagella which compose the tuft of a cell of strain 11; electron photomicrograph; × 13,400.
Figure 15. Individual strands or flagella which compose the tuft of a cell of strain 11; electron photomicrograph; × 11,400.
Figure 16. Flagellar tuft on a sheathed cell of strain 11; prepared with Leifson's flagellar stain; × 3,200.

become somewhat acid, pH 5, during growth unless buffered. The buffer was added separately to the sterile medium because the medium would not support growth when autoclaved together with the phosphate.

The $MnSO_4$ is not essential for growth. It was introduced originally into the medium when attempts were being made to isolate strains of *S. discophorus* from enrichment cultures. The manganous ion is taken up by this organism, becomes oxidized, and therefore colors the organisms dark brown. This is of considerable help in recognizing *S. discophorus* in a mixed population. Only one strain of *S. discophorus* was isolated during the course of the present investigations, and therefore detailed studies of this species were deferred until more strains are available. Pringsheim (1949*b*) found that as little as 0.005 per cent $MnSO_4$ may inhibit the growth of *S. natans* although we failed to see any inhibition of our strains with as much as 0.01 per cent $MnSO_4$. The latter was eliminated eventually from the medium towards the end of the experimental work because it gives rise to a slight undesirable precipitate when the medium is autoclaved.

Organic nitrogen was used rather than inorganic salts, which would have been preferable in experiments of this type, because it had been noted previously that little or no growth occurred with either ammonium or nitrate nitrogen in a mineral salts-glucose medium. This led us to believe, initially, that *S. natans* cannot use inorganic nitrogen for growth. But this conclusion had to be modified later when carbon sources other than glucose were used (see below). In any event, the small amount of casamino acids in the medium supported very little growth in the absence of additional, available carbon compounds. Therefore one could determine readily whether a particular organic compound was utilized by observing whether it supported more growth of *Sphaerotilus* than could be obtained without it.

The stock cultures were kept on a medium consisting of 0.2 per cent peptone, 0.2 per cent glucose, 0.02 per cent $MgSO_4 \cdot 7H_2O$, 0.005 per cent $CaCl_2$, 0.001 per cent $FeCl_3 \cdot 6H_2O$, 1.25 per cent agar and tap water, and adjusted to pH 7. The phosphate buffer was not required and was not used because it gave rise to a marked precipitate in the agar medium. The stock cultures were transferred about once a month although they remained viable for at least two months when stored at room temperature. Growth is best if transfers are made on freshly slanted agar tubes which contain some surface moisture.

The flasks of complete medium and controls without added carbon were each inoculated with one drop of a water suspension of *S. natans*, prepared usually from two day old agar slant cultures. The inoculated media were incubated in a stationary state at 28 C, and the amount of growth formed was estimated by direct visual observation at intervals usually for a week. Most of the growth occurs as a coherent, rather slimy, surface mat or pellicle. Some of the growth clings to the sides and bottom of the flask in the form of a delicate film or as granules. The main body of the medium, however, remains clear although smooth type cultures, especially when shaken during growth, may produce a fairly uniform turbidity throughout the medium. Unless large inocula are used, growth is slight during the first day but is maximal usually within three days. And then the turbidity of the cultures begins to decrease gradually as the cells and sheaths disintegrate.

In the manner described above, it was found that *S. natans* can use a large variety of organic compounds as sources of carbon and energy. These include glucose, galactose, sucrose, maltose, mannitol, sorbitol, succinate, fumarate, butyrate, butanol, glycerol, sodium lactate, sodium pyruvate, sodium acetate, and ethanol; ethanol and butanol were tested in a concentration of 0.1 per cent. Acetate and butyrate appeared to be somewhat toxic since they failed to support growth when used in a concentration of 0.25 per cent but induced good development when the concentration was lowered to 0.1 per cent.

Little or no growth was obtained with 0.25 per cent lactose, xylose, arabinose, or 0.1 per cent sodium benzoate, sodium propionate, propanol, or methanol. There were no significant differences among the nine strains.

Our data, except for lactose, are in accord with the results of Linde (1913) who found that glucose, galactose, lactose, sucrose, maltose, mannitol, and glycerol are utilized by *S. natans*. And from Cataldi's investigations (1939) the list of available carbon compounds can be extended to include also citrate and asparagine. Linde reported, however, that polysaccharides—dextrin, gum arabic, starch, inulin, and cellulose—will not support growth of *S. natans*.

A few experiments were made with different concentrations of glucose to determine the effect of sugar concentration upon growth. More luxuriant growth was obtained by increasing the glucose concentration from 0.1 per cent to 0.2 per cent. Growth was delayed, however, by one per cent glucose and completely inhibited by two per cent glucose. To eliminate the possibility that inhibition at the higher sugar concentrations might be due to the formation of toxic substances in the medium during autoclaving, this type of experiment was repeated with separately autoclaved glucose. Under these conditions, luxuriant growth was obtained even with two per cent sugar although the total amount of growth did not seem to be greater than that obtainable with 0.2 per cent glucose. The organisms can therefore tolerate reasonably high concentrations of organic matter.

Sources of nitrogen. The general methodology used in the survey of the carbon requirements of *S. natans* was employed also for the determination of the availability of a variety of nitrogen compounds, mainly amino acids, as sources of nitrogen for growth. The basal medium was modified by the elimination of the casamino acids and the introduction of 0.1 per cent glucose. The nitrogen compounds were used in a concentration of 0.1 per cent. The control flasks included basal medium without added nitrogen and basal medium with casamino acids.

The following compounds supported good growth of *S. natans*: L-glutamic acid, L-aspartic acid, L-asparagine, DL-alanine, L-leucine, L-cystine, DL-tryptophan, L-arginine, and L-proline. Growth with the individual amino acids was usually not as rapid or extensive as with the mixture of amino acids in the form of casamino acids. Leucine, proline, and tyrosine were the best among the amino acids in supporting growth. Qualitatively, the results were uniform with all strains although some strains grew better with a particular amino acid than did others.

Little or no growth was obtained with urea, acetamide, glycine, DL-serine, L-lysine, DL-methionine, DL-isoleucine, DL-threonine, DL-valine, L-histidine, and DL-phenylalanine.

The only other detailed investigation of the nitrogen requirements of *S. natans* with chemically defined media is that of Lackey and Wattie (1940). They reported good growth with DL-alanine and L-asparagine; fair growth with urea; and poor growth with L-tyrosine, L-cystine, L-leucine, and L-glutamic acid. There are obviously some differences between our results and those of Lackey and Wattie, and these undoubtedly are due to differences in the strains used.

Many attempts to obtain growth of our strains with inorganic nitrogen in the form of ammonium or nitrate nitrogen were almost uniformly unsuccessful; occasionally a small amount of growth or even good growth was obtained with some of the strains. This situation was somewhat surprising since good growth of *S. natans* was obtained by Linde (1913) with both ammonium and nitrate nitrogen in a mineral salts-sucrose medium and by Cataldi (1939) with ammonium nitrogen in a mineral salts-glucose medium and in a mineral salts-citrate medium. Also Lackey and Wattie (1940) obtained fair growth with ammonium and nitrate salts in a mineral salts-glucose medium. Further investigation disclosed, however, the interesting fact that the degree of availability of inorganic nitrogen for our strains was dependent upon the type of organic carbon and energy source supplied in the medium. Excellent growth was obtained with ammonium or nitrate nitrogen when sucrose, glycerol, or succinate was the carbon source whereas very poor growth occurred with glucose. A biochemical explanation for this phenomenon is not apparent.

It can be concluded therefore that *S. natans* can utilize a wide variety of nitrogen sources ranging from inorganic nitrogen salts to individual amino acids, mixtures of amino acids, and complex nitrogenous substances such as peptone and meat extract.

Growth factor requirements. As described above, our strains grew abundantly in chemically defined media which consisted of mineral salts, sugar, and inorganic nitrogen. A number of the strains also were subcultured successfully, serially through several transfers in a mineral salts, glucose, vitamin-free, casein hydrolyzate medium. Similarly, Linde (1913), Cataldi (1939), and Lackey and Wattie (1940) have been able to grow *S. natans* in chemically defined media without the addition of growth factors. This organism, therefore, does not require an exogenous supply of growth factors and presumably has the ability to synthesize whatever growth factors it needs.

Pringsheim (1949*b*) reported that soil extract stimulates growth in dilute beef extract and other media. At the very beginning of our investigations we also found that soil extract was necessary for good growth of *S. natans* in media

containing 0.1 per cent peptone and 0.2 per cent glucose. It was also found that the soil extract could be replaced completely by a mixture of small amounts of $MgSO_4$, $CaCl_2$, and $FeCl_3$; and these three salts were incorporated, therefore, into all media subsequently used. These results suggest that the stimulatory effect of the soil extract is due to the minerals contained in it. The complex nitrogenous materials are relied upon to supply essential minerals as impurities, but the quantities apparently become limiting for growth when dilute beef extract, peptone, and similar media are used, and additional amounts of minerals must be supplied then in the form of soil extract or as pure salts for good growth.

General physiology. Effect of temperature. The medium contained 0.1 per cent each of glucose and peptone and also the usual mineral salts and phosphate buffer. All of the strains of *S. natans* grew in the range of 15 C to 40 C; no growth occurred at 5 C and 46 C. On the basis of both the rate and extent of development, the optimum temperature is about 30 C. Growth is slow at 15 C and especially at 40 C.

These results are in good agreement with those of other investigators. Höflich (1901) obtained growth of *S. natans* between 15 C and 35 C with an optimum at 25 C to 30 C. According to Linde (1913), no growth takes place below 10 C nor above 40 C, and the optimum lies between 30 C and 35 C. Zikes (1915) obtained practically no growth at 5 C and 39 C, and the optimum was between 25 C and 29 C. Cataldi (1939) reported a minimum growth temperature of 15 C and an optimum of 37 C which is somewhat higher than that found by other investigators and also in the present studies.

Effect of pH. The peptone-glucose medium described above was used. The desired pH values were obtained by the addition of appropriate, sterile, phosphate buffer mixtures; and the final concentration of buffer was M/100. This was not very much buffer, but it was sufficient to prevent any appreciable changes in pH levels during growth. No growth appeared at pH 5.5. Slow and not always full growth occurred at pH 5 8. Rapid and full development was obtained in the range of pH 6.4 to pH 8.1. The results of some experiments suggested that growth can be initiated even at pH 9 or pH 10.

Cataldi (1939) obtained growth of *S. natans* in the range of pH 5 to pH 9.8 and reported that the amount of growth increased with an increase in pH value. Lackey and Wattie (1940) obtained growth in the range of pH 5.5 to pH 8.0. It is evident that there is general agreement that *S. natans* cannot tolerate a very acid environment and grows best in a neutral or slightly alkaline medium.

Requirement for oxygen. It is clear from the literature that *S. natans* is an obligate aerobe since it will not grow without oxygen. And this is supported fully by our investigations. But it was not easy to establish, beyond doubt, the aerobic nature of the organism. We obtained growth frequently, although not always, on agar plates incubated presumably under strictly anaerobic conditions in desiccators with pyrogallic acid and K_2CO_3 under an N_2 atmosphere, in oat jars and in Brewer jars. Good growth was always obtained in stab cultures and throughout the depth of the agar medium. This occurred even when the stab cultures were layered with paraffin or when the cotton plugs were saturated with pyrogallic acid and alkali and then sealed off from the atmosphere with paraffined rubber stoppers. In contrast, growth never occurred in liquid media, containing as much as 0.5 per cent glucose, in completely filled glass stoppered bottles or in stab or slant cultures supplied with alkali, pyrogallic acid, and rubber stoppers and refrigerated overnight prior to incubation in order to allow time for the pyrogallol to absorb all of the oxygen present before the organisms could effectively compete for it.

The difficulty of preventing growth of *S. natans* by the usual anaerobic procedures suggests strongly that the organism can grow quite well with very small amounts of oxygen. And such traces of oxygen, which are difficult to remove, were undoubtedly present in many of our supposedly anaerobic systems. Moreover, the cells are not harmed by storage for relatively long periods under strict anaerobiosis. Inoculated plates which showed no visible growth after a week of incubation in Brewer jars became covered with abundant growth of *S. natans* when removed from the Brewer jars and incubated aerobically.

On the other hand, the marked preference of *S. natans* for oxygen is indicated by its predominant surface growth in flasks and tubes of liquid media.

No growth was obtained in anaerobic bottle cultures with media containing 0.2 per cent KNO_3. The latter therefore cannot replace oxy-

gen in the metabolism of *S. natans*. This was also the conclusion of Linde (1913). Nitrates, however, are reduced readily to nitrites by all of our strains.

Oxidative metabolism. Since *S. natans* is an aerobe, experiments were made to obtain information on its oxidative metabolism. The conventional Warburg apparatus and techniques were employed. The cells were grown in a medium consisting of 0.2 per cent each of casamino acids and glucose plus the usual mineral salts and phosphate buffer. Most of the experiments were made with the smooth form of *S. natans*, strain 12, but some of the experiments were repeated with strains 4 and 11. Similar results were obtained with all three strains.

Cells were obtained from cultures grown for approximately 16 hours at 28 C on a shaker. The cells were centrifuged, washed once with water, and resuspended in sufficient M/50 phosphate buffer, pH 7.1, to give a concentration of one mg to two mg of cells, dry weight, per ml of suspension. The cell suspensions were aerated for 3 hours to 5 hours by bubbling a slow stream of air through them in order to reduce their rather high rate of endogenous respiration. This high rate may be due to the large amount of fatty material stored in the cells. Aeration for more than 5 hours tended to destroy the oxidizing capacity of the cells.

Each Warburg vessel received two ml of cell suspension in the main compartment, 0.1 ml of an M/50 neutral solution of substrate or two μM of substrate in the side arm, and 0.2 ml of 10 per cent KOH in the center well for the absorption of CO_2. The gas phase was air, and the bath temperature was about 30 C.

The compounds investigated were mainly the carbohydrates, amino acids, and the other substances which were used in the experiments on the carbon and nitrogen requirements. Data on the rate and extent of oxidation of some of these compounds are given in table 1, and the kinetics of the oxidations of additional compounds are shown in figure 17. In general, the compounds which were utilized as sources of carbon or nitrogen by *S. natans* also were oxidized readily by nonproliferating cell suspensions. It is noteworthy that large amounts of the substrates are assimilated oxidatively and these may be as high as 70 per cent or even 80 per cent in the case of sugars and sugar alcohols. The assimilation process is only slightly inhibited by sodium azide and 2,4-dinitrophenol—compounds which are usually effective in preventing assimilation and synthetic processes in general. When used in suitable concentrations, these poisons stimulated somewhat the rates of oxidation by *S. natans*, but this effect was mainly on the endogenous respiration.

TABLE 1

The rate and extent of oxidation of various compounds by Sphaerotilus natans

SUBSTRATE	Q_{O_2}*	PER CENT OXIDATION
Glucose	53	30
Galactose	37	39
Sucrose	26	19
Mannitol	26	31
Sorbitol	17	29
Benzoate, Na	52	48
Succinate, Na	52	41
Butanol	15	37
Lactate, Na	118	35
Pyruvate, Na	98	38
Acetate, Na	55	50
L-Glutamate, Na	55	33
L-Aspartate, Na	49	75
L-Leucine	29	50
DL-Alanine	49	51
L-Proline	40	50

* μL O_2 consumed per mg (dry wt) of cells per hour; the value for the endogenous respiration, Q_{O_2}27, has been substracted.

In an experiment on the effect of pH on the oxidation of glucose, it was found that as the pH was increased from pH 6.6 to pH 8.1, there was a concomitant increase in the apparent rate of glucose oxidation. But this increase could be accounted for entirely as due to the stimulation of the endogenous respiration because when the higher endogenous rates were subtracted, the rates for glucose were identical at all pH levels.

The oxygen consumption curves for galactose, mannitol, sorbitol, and sucrose showed an initial lag which lasted about an hour. But this lag was not apparent when the cells used were grown with the specific substrate rather than with glucose. These substrates, therefore, are oxidized by adaptively formed enzyme systems. Also benzoate appears to be oxidized by adaptive enzymes. There is a delay of about 40 minutes before there is any appreciable increase in oxygen consumption with benzoate above the level of the endogenous oxygen consumption. Although benzoate is oxidized readily, it could not be used as a source

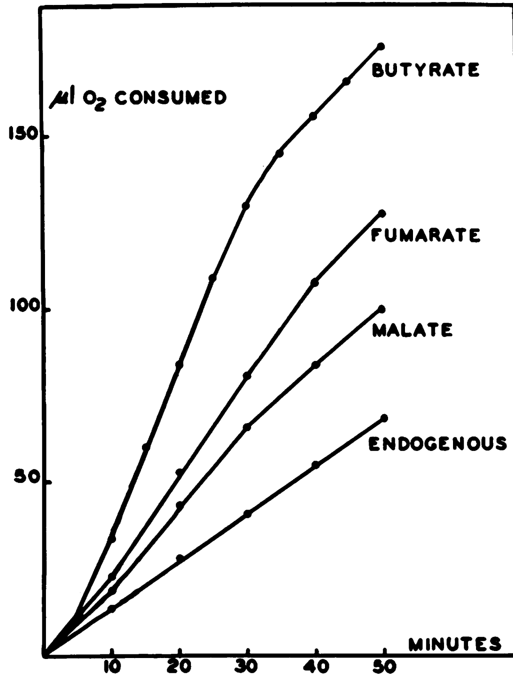


Figure 17. Oxidation of two µM each of Na butyrate, Na fumarate, and Na DL-malate by *Sphaerotilus natans.*

of carbon and energy by *S. natans.* This lack of correlation between growth and oxidation is not surprising since growth is a much more complex process than mere oxidation. It is well known that formate can be oxidized rapidly by many bacteria although few organisms can use it as the sole source of carbon and energy.

Cell suspensions in bicarbonate buffer, under an N_2-5 per cent CO_2 atmosphere, did not form any acid from glucose. This indicates the absence of a fermentation mechanism for carbohydrate decomposition and is in agreement with the finding that the organism cannot grow without oxygen.

DISCUSSION

The distinctive feature of *S. natans* is the occurrence of the cells in an organic tube or sheath. The chemical composition of the sheath is not known, and because it is difficult to see on living cells, little is known about how it is formed. But the sheath is normally a closed tube which closely encases the chains of rod shaped cells, and it must be permeable, therefore, to the nutrients required by the cells since the nutrients must pass through the sheath. In media containing iron, the sheaths readily take up the iron, and although they may continue to be colorless, the presence of the iron can be demonstrated easily by means of the Prussian blue reaction. In this test the iron containing sheaths, on contact with dilute solutions of potassium ferrocyanide and HCl, turn blue. And according to Pringsheim (1949*b*), after long periods of incubation, sufficient iron may be laid down on the sheaths to color them brown and to give them the glassy appearance which is characteristic of and identical with that of the *Leptothrix ochracea* sheaths which are so commonly and abundantly found in natural waters where iron is being deposited.

There is little doubt that *S. natans* is truly an iron bacterium in so far as it accumulates iron from solution in a characteristic and morphologically distinct form. But whether it, or indeed whether any of the other classical filamentous and nonfilamentous iron bacteria can grow at the expense of the energy liberated in the oxidation of ferrous to ferric iron, remains to be rigorously established. The long existing, confusing, and often acrimonious controversies on the possible autotrophy of the iron bacteria have been fully discussed by Molisch (1910), Cholodny (1926), Winogradsky (1949), Pringsheim (1949*a*), and others. There is much strongly suggestive evidence in favor of autotrophy, but incontrovertible proof is still lacking. There can be little doubt, however, that the unusual bacterium, *Thiobacillus ferrooxidans*, recently isolated by Temple and Colmer (1951) from the acid drainage waters of bituminous coal mines, can grow autotrophically with ferrous iron. It develops well at pH 2 or pH 3 in inorganic media with ferrous iron and CO_2, and growth occurs concomitantly with the oxidation of the iron and the fixation of CO_2. At these low pH levels oxidation of the ferrous iron by the oxygen of the atmosphere cannot take place, and therefore the oxidation which occurs in cultures must be due to the metabolic activities of the bacteria. Because *S. natans* and other typical iron bacteria develop only in an approximately neutral environment in which direct chemical oxidation of ferrous iron is rapid and extensive, it becomes difficult to determine what role, if any, the bacteria play in the oxidation process. This is a technical problem which does not appear to be insurmountable although it has not yet been solved in a fully satisfactory manner.

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SUMMARY

Nine pure cultures of the filamentous sheathed iron bacterium, *Sphaerotilus natans*, were isolated from stream, pond, and ditch water. The cultures gave rise to two types of colonies: a rough, somewhat cottony type composed mainly of filaments and smooth, glistening colonies composed principally of single cells. The filaments consist of chains of rod shaped cells in closely fitting tubes or sheaths. The individual cells are from 2.5 to 16 μ long and 1.2 to 1.8 μ wide. They are motile, and each cell has a tuft of spirally coiled flagella which is located usually subpolarly or on the long side of the cell. All of these morphological features are presented in a series of photographs of stained and living cells taken with the ordinary light, phase, dark field, and electron microscopes.

S. natans can grow with a wide variety of organic compounds, including sugars, sugar alcohols, and 4, 3, and 2-carbon compounds as sources of carbon and energy. Ammonium salts, nitrates, individual amino acids, and complexes such as peptone and meat extract can supply the nitrogen needs of the organism. An exogenous supply of growth factors is not required.

All strains developed in the range of 15 C to 40 C, and the optimum temperature is about 30 C. Growth occurred in the range of pH 5.8 to pH 8.1, and the best development was in neutral or slightly alkaline media. *S. natans* cannot grow without oxygen although growth will occur when only small amounts of oxygen are present; nitrates cannot replace oxygen. Nonproliferating cell suspensions oxidize a variety of carbohydrates, amino acids, and related compounds; and for some of these compounds the oxidizing enzymes are adaptively formed. As much as 70 per cent to 80 per cent of carbohydrates may be oxidatively assimilated, and this process is very resistant to inhibition by sodium azide and 2,4-dinitrophenol. Glucose is not fermented by cell suspensions under anaerobic conditions.

REFERENCES

Büsgen, M. 1894 Kulturversuche mit *Cladothrix dichotoma*. Ber. deut. botan. Ges., **12**, 147–152.

Cataldi, M. S. 1939 Estudio fisiólogico y sistemático de algunas Chlamydobacteriales. Thesis, Buenos Aires.

Cholodny, N. 1926 Die Eisenbakterien, Beiträge zu einer Monographie. Pflanzenforschung, **4**, 69–133.

Cohn, F. 1875 Untersuchungen über Bakterien II. Beitr. Biol. Pflanz., **1**, 141–207.

Eidam, E. 1879 Über die Entwickelung des *Sphaerotilus natans* Ktz., sowie über dessen Verhaltnis zu *Crenothrix* und zu den Bacterien. Botan. Zeitung, **37**, 724–728.

Fischer, A. 1895 Untersuchungen über Bakterien. Jahrb. wiss. Botan., **27**, 1–163.

Höflich, K. 1901 Kultur und Entwickelungsgeschichte der *Cladothrix dichotoma* (Cohn). Oesterr. Monatschr. Tierheilk., **25**, 4–23, 49–64.

Knaysi, G. 1951 *Elements of bacterial cytology*. Chapter IX. Second Edition. Comstock Publishing Co., Ithaca, N. Y.

Kützing, F. T. 1833 *Sphaerotilus natans*, eine neue Süsswasseralge. Linnaea, **8**, 385.

Lackey, J. B., and Wattie, E. 1940 The biology of *Sphaerotilus natans* Kützing in relation to bulking of activated sludge. Public Health Repts. (U. S.), **55**, 975–987.

Linde, P. 1913 Zur Kenntnis von *Cladothrix dichotoma* Cohn. Centr. Bakteriol. Parasitenk., II Abt., **39**, 369–394.

Molisch, H. 1910 *Die Eisenbakterien*. Gustav Fischer, Jena.

Pringsheim, E. G. 1949*a* Iron bacteria. Biol. Revs., **24**, 200–245.

Pringsheim, E. G. 1949*b* The filamentous bacteria *Sphaerotilus*, *Leptothrix*, *Cladothrix*, and their relation to iron and manganese. Trans. Roy. Soc. (London), Series B, **233**, 453–482.

Temple, K. L., and Colmer, A. R. 1951 The autotrophic oxidation of iron by a new bacterium: *Thiobacillus ferrooxidans*. J. Bact., **62**, 605–611.

Winogradsky, S. 1949 *Microbiologie du Sol*. Masson et Cie, Paris.

Zikes, H. 1915 Vergleichende Untersuchungen über *Sphaerotilus natans* (Kützing) und *Cladothrix dichotoma* (Cohn) auf Grund von Reinkulturen. Zentr. Bakteriol. Parasitenk., II Abt., **43**, 529–552.

Zopf, W. 1882 *Zur Morphologie de Spaltpflanzen (Spaltpilze) und Spaltalgen*. Leipzig.