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THE FILAMENTOUS BACTERIA *SPHAEROTILUS*, *LEPTOTHRIX*, *CLADOTHRIX*, AND THEIR RELATION TO IRON AND MANGANESE

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CONTENTS

The Chlamydobacteriaceae are a family of filamentous bacteria varying extremely with environmental conditions. The tacit assumption that species can be distinguished by mere microscopic inspection has led to the creation of numerous genera, species, and varieties of doubtful validity. In order to detect reversible modifications and hereditary differences cultures were undertaken.

Threads of *Sphaerotilus natans*, *Cladothrix dichotoma* and *Leptothrix ochracea* were washed in sterile soil extract and transferred to agar plates with a low concentration of meat extract. By repeated plating several strains of each form were obtained in pure culture. All of these were identical, despite their origin from the various 'species' mentioned.

By modifying the cultural conditions each strain could be made to change into the three original forms, as well as into certain others generally believed to belong to distinct genera. Since *Sphaerotilus natans* Kütz. is the oldest name it must be retained for all these forms.

Schwers's *Megalothrix discophora*, which was later referred to other genera and has been grown in culture by various authors, was not obtained from the cultures of *Sphaerotilus natans*. It represents a second related species of *Sphaerotilus* which may be named *S. discophorus*.

Further species of *Sphaerotilus* have been described under the name of *Leptothrix*, but of the remaining socalled genera of the Chlamydobacteriaceae *Crenothrix* alone seems to be well founded.

Conclusions are also reached on the colour of iron-containing deposits. If these appear brown, it is due to admixture of manganese oxides. Otherwise the colour is only slightly yellowish under the microscope and orange-ochre in larger accumulations. The envelopes produced by various organisms may contain much iron without its presence being revealed by their colour, although it can be recognized by the very refractive and brittle character of the envelopes and by micro-chemical reactions.

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I. Introduction

The taxonomy of the Chlamydobacteriaceae (Migula) or sheath bacteria is in a very unsatisfactory state. Cultures, which alone could provide a basis for comparison, are still in great part lacking or insufficient.

The Chlamydobacteriaceae are all iron bacteria in so far as the sheaths covering the filaments usually contain iron, the presence of which is revealed by the yellow to brown colour of larger quantities as well as by chemical analysis. Some authors group the Chlamydobacteriaceae among the 'thread bacteria', which have also been called Desmobacteriaceae or Trichobacteriaceae (Benecke 1912, pp. 188, 202), an arrangement which is followed in various text-books. The Trichobacteriaceae, however, include such genera as *Beggiatoa* and *Thiothrix*, which show no relation whatsoever to the Chlamydobacteriaceae.

Sphaerotilus natans is an important sewage fungus and has been repeatedly studied in relation to water pollution. Iron bacteria have formed the subject of many publications owing to their geological and physiological interest. They are treated in monographs by Rullmann (1904), Molisch (1910), Ellis (1919), Cholodny (1926) and Dorff (1934). Persons directly concerned with their investigation might well believe that the problems concerning iron bacteria were in the main solved (cf. e.g. Henrici & Johnson 1933, p. 278), but the many contradictory statements render further investigation necessary. The search for information in the voluminous literature was laborious, and the experimental results were at first surprising and confusing, but after several years of investigation it has been possible to arrive at relatively simple conclusions.

The first descriptions of sheath bacteria were by Kützing, who gave diagnoses of *S. natans* (1833) and *Leptothrix ochracea* (1843). His data were supplemented by Eidam (1876) and Cienkowski (1877), and especially by Zopf (1882). Meanwhile, Cohn (1870) had published a remarkably exact description of his *Crenothrix polyspora* and (1875) his observations on *Cladothrix dichotoma*.

Cultural work was first performed by Winogradsky (1888), whose experimental skill and physiological penetration shed light on the role of sheath bacteria in the biological oxidation and precipitation of iron compounds. His theory of energy relations in connexion with such chemical transformations attracted wide scientific interest in the group called by him iron bacteria. By adapting bacteriological methods to the requirements of these organisms Büsgen (1894) succeeded in obtaining pure cultures of *C. dichotoma*, and Molisch (1910) of a species wrongly indentified as *Leptothrix ochracea* (Cholodny 1926, p. 17). Since the latter multiplied on organic substrata, Molisch rashly concluded that Winogradsky's autotrophism of iron bacteria did not exist. Lieske (1919) grew a form, identical with that of Molisch, on manganous acetate. He also tested manganous bicarbonate, but without general success, and moreover does not seem to have cultivated his *Leptothrix* in such inorganic media in serial subcultures, so that his experiments are not decisive in favour of Winogradsky's theory.

Since Eidam (1876, 1879) first described *Sphaerotilus natans* from a river polluted by brewery waste, members of the sewage fungus community have found considerable publicity, and numerous genera, species, and varieties of sheath bacteria have been described on an insufficient basis. The most complete study of the group so far published is that by Cataldi

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(1939), which came to my knowledge when my experimental work was finished. She isolated a considerable number of stains with the object of providing correct diagnoses of the species of Chlamydobacteriaceae, a task in which, despite good progress, she was not entirely successful, partly because the conditions under which her strains were grown were too different from those in nature. There is, nevertheless, agreement in many points between Cataldi and myself. While, however, I found that *S. natans*, under certain circumstances, produced growths not distinguishable from those of *Cladothrix dichotoma* and *Leptothrix ochracea*, Cataldi maintains all these species and refers the numerous strains she isolated to five species, while I have been able to recognize only two.

Among a multitude of strains that differ in minor characteristics, there can possibly be distinguished a number of closely related microspecies or varieties, but it is impossible to decide which is the true *Leptothrix ochracea*, the most frequently mentioned iron bacterium. The form described by Cataldi under that name does not correspond to the customary diagnosis, according to which there are no motile stages and the filaments are never attached (Cholodny 1926, pp. 11, 67), but actually such an organism does not seem to exist.

My chief criticisms of the conclusions arrived at by Cataldi are: (1) the author did not realize the importance of iron and manganese respectively in sheath formation in the Chlamydobacteriaceae; (2) she overlooked the similarity between the sheaths described for *L. ochracea* and those formed under certain conditions by *Sphaerotilus natans*; (3) the nature of *Cladothrix dichotoma* was not established; (4) her cultures on inorganic media do not support her conclusion that autotrophy is impossible in iron bacteria since the media she used could not be expected to be suitable; (5) she does not seem to have compared her five species when grown on the same media. A detailed consideration of her results would have involved much further experimental work for which I could not spare the time. I have, however, reinvestigated my strains with the object of reconciling our mutual results, but only obtained new evidence to reinforce my conclusions.

A historical review of the development in this field and a more complete consideration of the literature is given in another paper (Pringsheim 1949), which also deals with other iron bacteria.

II. Nomenclature in the Chlamydobacteriaceae

(1) *General remarks*

Many of the numerous generic and specific names given to Chlamydobacteriaceae are uncertain, and there is much controversy as to taxonomic units. My observations show that there are only a few species, but these are subject to so many changes of appearance that specific distinction can only be secured by determining which features are hereditary and which are liable to modification. It is therefore difficult to give short and adequate diagnoses and to identify the forms described by other authors.

Many, seemingly well-defined forms, have been found in special localities and described as new genera or species, although they belong to one or the other of the few toxonomic units. These can be found again by any one and must therefore be maintained and their status defined. They cannot of course be regarded as taxonomic units, and the question arises what should be their designation?

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In this paper the minor units of a genus are designated as follows:

(*a*) *Collective species*. This is the next entity to the genus. It embraces a number of discernible units differing slightly in hereditary properties.

(*b*) *Elementary species*. This comprises individuals whose differences are determined solely by external influences. The elementary species included in one collective species differ in respect of several hereditarily independent features.

(*c*) *Variety*. This differs from the type, defined by the first description of a species, in one or a few interconnected features and has probably originated by a single mutation.

(*d*) *Forma*. Populations found in special habitats may consist of similar individuals which are different from the type as a result of morphological reaction to local influences. This was first recognized by Naegeli (1877, p. xiv; 1882, p. 129) and styled habitat or nutritional modification (Standorts- oder Ernährungsmodifikation). Every modification is not, however, of the nature of a forma or growth form. These terms will be reserved for instances in which the entire population in a certain locality is influenced in the same way, so that the result resembles a variety or even a higher taxonomic unit. The expression forma should not be used for a less pronounced variety.

(2) *Genera and species of the Chlamydobacteriaceae*

The family Chlamydobacteriaceae Mig. is characterized by its filaments of rod-shaped bacteria-like cells which are enveloped by a sheath, from which reproductive filamentous fragments, composed of one or several cells, escape to give origin to new filaments. The Chlamydobacteriaceae are a striking demonstration of the backward condition of wide domains of microbiology. Forms, to which taxonomic names were given, were habitually described from material gathered in the field, often without sufficient knowledge of related forms.

A closer examination, supplemented by culture experiments, showed that the previously defined boundaries of species do not correspond to those based on hereditary characters. Microbiologists dealing with the Chlamydobacteriaceae will have to make up their minds whether they consider 'natural' material alone suitable for the framing of diagnoses, with the risk that species and varieties so created often represent random groups of forms, or whether they admit that species are not always readily recognizable until their characters have been ascertained by cultural methods. At present, both in the sheath bacteria and in certain other groups, there are two kinds of species, those of the field biologist and those of the bacteriologist.

Sphaerotilus natans has been recorded under at least five generic names, with a considerable number of species and varieties, some of which are very characteristic and occasionally found as almost uniform populations. It is essential to determine the conditions under which the one or other form is produced, using so far as possible pure cultures. Similar work must be carried out with all other species of sheath bacteria, but this cannot be done by a single investigator, and apart from *S. natans* I have only dealt with *S. discophorus*.

The following generic names have been given to members of the Chlamydobacteriaceae (1) *Sphaerotilus* Kütz., (2) *Leptothrix* Kütz., (3) *Cladothrix* Cohn, (4) *Crenothrix* Cohn, (5) *Clonothrix* Roze, (6) *Chlamydothrix* Mig., (7) *Megalothrix* Schwers. The attempt to clarify the nomenclature can only be partially successful because of the vast and scattered literature

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and the often inconclusive descriptions. A list of names and synonyms is given in another publication (Pringsheim 1949). In preparation of a synopsis an attempt has been made to put the names right in order to facilitate future research.

Leptothrix ochracea Kütz. (1843) and *Cladothrix dichotoma* Cohn (1875) are reversible modifications of *Sphaerotilus natans* Kütz. (1833). Since *Sphaerotilus* is the oldest name, it must be retained. *Chlamydothrix* Migula (1900) and *Megalothrix* Schwers (1912) are mere synonyms of '*Leptothrix*' and must be suppressed. *Clonothrix* Roze (1896) is probably another synonym. *Leptothrix discophorus* (Schwers) Dorff (1934) differs from *L. ochracea*, but should also be grouped under *Sphaerotilus*. *Leptothrix trichogenes* Cholodny (1926), the third well-established species (Teichmann (1935), should likewise be referred to *Sphaerotilus*. Like Molisch (1925) I failed to find *Crenothrix* and cannot add anything to a not altogether satisfactory picture, but the genus must certainly be retained.

The Chlamydobacteriaceae thus comprise only two genera, *Sphaerotilus* and *Crenothrix*. The former multiplies by swarmers, while the latter is supposed to produce only non-motile conidia. All the others are either synonyms or inadequately characterized. Most of them are probably nothing more than habitat modifications of the species mentioned.

Of securely established names only the following remain: (1) *Sphaerotilus natans* Kütz. with numerous formae; (2) *S. discophorus* (Schwers) n.comb. with at least four formae; (3) *S. trichogenes* (Cholodny) n.comb.; (4) *Crenothrix polyspora* Cohn.

One of the latest enumerations of species of *Sphaerotilus* is by Lackey & Wattie (1940), according to whom the following have been recorded: (1) *S. natans* Kütz. syn. var. *typica* Butcher 1932, pseudo-dichotomous, with swarmers; (2) *S. paludosus* Smit 1934, lacking pseudo-dichotomous branching and motile cells; (3) *S. tenuis* Beger 1934, slightly narrower; (4) *S. dichotomus* (Cohn) Migula 1900; (5) *S. fluitans* (Migula) Schikora 1899. This calls for the following comments: (1) is not Kützing's type, in which no branching was reported; (2) appears to be near the original type; (3) Beger's description is very scanty and one can only think that his material was a modification due to poor nutrition; (4) Cohn did not recognize the identity of his *Cladothrix* with *Sphaerotilus* because he observed an extreme modification, and according to Migula this form is identical with no. 1; (5) is probably a form occurring in fast rivers admitted by Lackey & Wattie to need further investigation.

Synopsis of the formae or growth forms of

Sphaerotilus natans Kütz. 1833

(1) *S. natans* typ. = forma *eutrophica*.
(2) *S. natans* forma *ochracea*, syn. *Leptothrix ochracea* Kütz. 1843, *Chlamydothrix ochracea* (Kütz.) Migula 1900, *Leptothrix major* Dorff 1934.
(3) *S. natans* forma *dichotoma*, syn. *Cladothrix dichotoma* Cohn 1875.
(4) *S. natans* forma *sideropus*, syn. *Chlamydothrix sideropous* Molisch 1910, *Leptothrix sideropous* (Molisch) Cholodny 1924.
(5) *S. natans* forma *fusca*, syn. *Clonothrix fusca* Roze 1896 (?).

Sphaerotilus discophorus n.comb.

(1) *S. discophorus* forma *eutrophica* similar to *S. natans* typ., produced when there is a rich supply of organic substances.

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(2) *S. discophorus* forma *manganifera*, syn. *Megalothrix discophora* Schwers 1912, *Leptothrix crassa* Cholodny 1926.

(3) *S. discophorus* forma *arachnoidea*, common where organic material and Mn are scanty.

(4) *S. discophorus* forma *ochraceoides*, similar to *Leptothrix ochracea* and caused by low Mn supply.

III. Methods and material

(1) *Cultures*

Winogradsky (1888) devised the method of enrichment with decaying hay and ferric hydroxide as a means of obtaining a good growth of iron bacteria. It has been adopted by most of his successors. Winogradsky, like Zopf (1879), also followed the development of iron bacteria under a cover-slip, a technique which has not subsequently been used although it gave good results.

The first pure cultures of a member of the Chlamydobacteriaceae were made by Büsgen (1894). He isolated *Cladothrix dichotoma* in a dilute beef-extract medium solidified with gelatine. Threads growing centrifugally purified themselves, so that bacteria-free cultures could be obtained. Höflich (1901) used agar with 0·05 % beef extract for the same purpose. Molisch (1910) obtained pure cultures of the 'typical iron bacterium *Leptothrix ochracea*' (actually *Sphaerotilus discophorus*) with the help of a special preparation of peptone with manganese, which had the advantage of producing recognizable brown colonies on the agar medium. Strains of *Sphaerotilus* have since been repeatedly isolated in pure culture (Lieske 1919; Cataldi 1937, 1939; Kalinenko 1940; Lackey & Wattie 1940; Tomlinson, (private communication).

My own technique does not differ much from that of earlier workers. My aim was to compare the characteristics of various forms of sheath bacteria under diverse conditions in pure cultures. In starting cultures it is necessary to ensure that the material contains living filaments, since the brownish or reddish aggregations of iron bacteria often include scarcely any living cells. When in a state of multiplication the growths usually look almost colourless or faintly yellowish.

Healthy filaments of *Sphaerotilus* rapidly multiply on agar with a low concentration of proteins, 0·05 to 0·1 % of beef or yeast extract or proteose peptone Difco being suitable. As already established by Büsgen (1894) and Molisch (1910) purification, prior to plating, facilitates the preparation of pure cultures. This is not so necessary with the dichotomous modification of *S. natans* with its firm sheaths, as with the type form, in which the sheath has a mucilaginous surface to which bacteria adhere. The methods of preliminary cleaning, adopted by earlier workers, are not as efficient as the washing of threads under the microscope with the help of capillary pipettes (Pringsheim 1937, p. 639; 1946*a*, p. 71 *seq.*). The washed threads are transferred to the dry surface of a plate with 0·05 % beef extract and 2 % agar and streaked out with a loop in the usual way. The edges of colonies are generally relatively clean and from them cells can be transferred to another plate with a capillary pipette. Plating has to be repeated to secure pure cultures. Colonies not too close to one another may increase in diameter by about 10 to 12 mm. at room temperature in 24 hr. In liquid media the growths are colourless, attached or free floating and appear like cobwebs or cotton-wool. Old cultures in beef extract media have a faint brownish colour.

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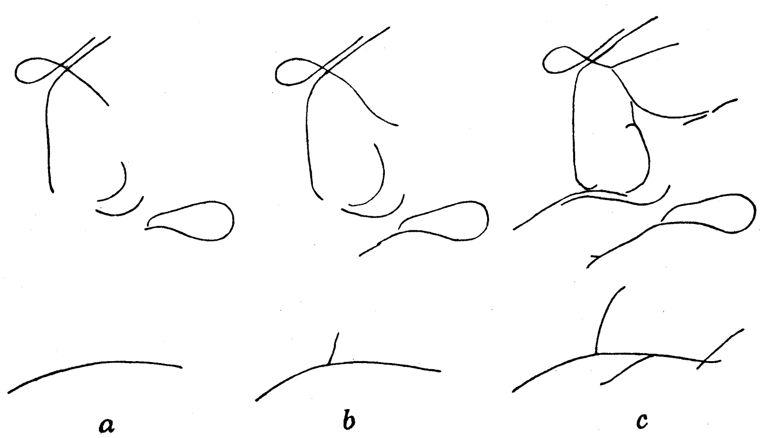
If the material is definitely known to be *S. natans* the technique just described is adequate. If, however, there is a possibility that *S. discophorus* is present, it is necessary to supplement the agar with 0·002 to 0·005 % manganous sulphate. Colonies of *S. discophorus* are then readily recognized by their brown colour, while those of *S. natans* are arrested by manganous salts and, if developing at all, appear colourless.

The few fragments of filaments placed on an agar plate to initiate subcultures, when inspected at intervals (figure 1), are seen to put out irregular side-branches which arise from intercalary cells. A day later one finds a mass of entangled filaments, some of which are relatively straight, but the least obstacle results in bending with the formation of loops or spirals so that eventually there are dense masses of accumulated trichomes (figure 2).



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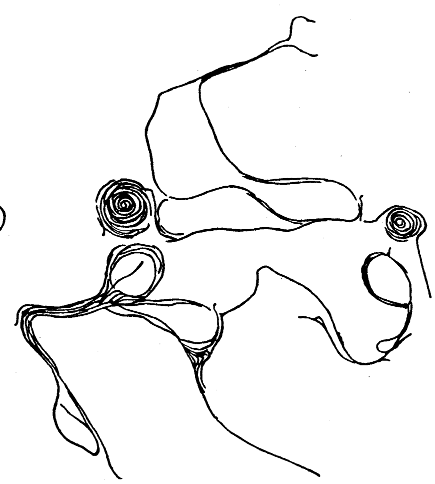

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FIGURE 1. *Sphaerotilus natans*. Growth of a few rods on 0·2 % beef-extract agar: *a*, 11.40 a.m.; *b*, 1.00 p.m.; *c*, 3.00 p.m. No alteration of any recognizable feature. A day later a confused mass of filaments, but some of the loops still recognizable in the same places (×200).

FIGURE 2. *Sphaerotilus natans*. Young colony on agar (×100).

R *S*

FIGURE 3. *Sphaerotilus discophorus*. *R* (rough) and *S* (smooth) colonies on agar with 0·05 % yeast extract (×20).

Colonies of *S. discophorus* generally do not all appear the same. Some are translucent without indication of a filamentous structure, while others are opaque and hairy with an irregular outline (figure 3); the latter acquire a brown colour much sooner. In short there is 'dissociation' into *S* and *R* colonies, known in so many other bacteria, the *S* colonies

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being composed of short rods, the *R* colonies of longer filaments. Subcultures of the two kinds of colonies do not give pure strains and the habit does not seem to be constant. On identical plates, streaked with material of either kind, dissociation again took place. In liquid media also no certain difference could be established. The phenomenon of the appearance of two kinds of readily differentiated colonies can perhaps be explained as due to the exhaustion of some substance by those colonies which develop quickest. The appearance of the colonies is readily influenced by slight differences in the medium. The *S* colonies, in which deposition of the brown manganese compound is delayed and reduced, were at first rather confusing since they were mistaken for those of a contaminating bacterium.

As a source of iron ferrous ammonium citrate was generally used, except of course for experiments on autotrophy. A 1 % solution, though only slightly acid, withstands heating without much alteration. Of manganous sulphate a 0·5 % solution was used. Both stock solutions were autoclaved separately from the rest of the media.

For maintaining pure cultures of *S. natans* on slopes, the agar concentration is reduced to 1 to 1·5%, that of beef or yeast extract raised to 0·2 to 0·3 %, and the pH adjusted to 7 to 7·5. The addition of 0·2 % glucose results in a heavier growth. These media can also be used without agar. *S. discophorus* can be maintained on the same media. For the development of a strong brown colour a medium of dilute soil extract with 0·02 % ferrous ammonium citrate and 0·01 % manganous sulphate with or without agar is especially suitable. Addition of organic nutrients reduces the oxidation of manganous salts to the brown compound. Soil extract favours the growth of both species.

The development on gelatine supplied only with mineral salts is poor until liquefaction takes place. The extent of this varies in different strains of *S. natans*, while it is never strong in *S. discophorus*. The same differences in the amount of protease produced are observed on milk agar. Addition of beef extract, peptone, or glucose increases growth and hence liquefaction on gelatine media.

(2) *Methods of observation*

The growths formed by sheath bacteria are delicate and easily disturbed. If, for instance, *S. natans* is transferred to a slide, the material is distorted beyond recognition owing to its soft and slimy nature. The *Cladothrix* and *Leptothrix forms* withstand such handling only slightly better. Direct inspection of cultures is necessary to give information on the normal appearance and arrangement of the threads. Strong hand-lenses with suitable artificial illumination facilitate observation of growths within test-tubes. The distortion resulting from the curvature of the surface can be reduced by using cover-slips fastened with glycerine, which even admit of inspection with a medium-power compound microscope. The growths covering the inner surface of a test-tube can be seen surprisingly well in this way.

Germlings from swarmers, generally attached just below the surface of the culture fluid, form circular growths of increasing density which fall to the bottom at the slightest shaking. It is only when development is scanty or slow that attached growths appear lower down, either as cobweb-like nets or as radiating colonies. Detached filamentous fragments develop into ball-like or irregular floccules.

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Hanging drop cultures and miniature aquaria (Pringsheim 1946*b*, p. 321) give more detailed information about the growth forms of sheath bacteria than test-tube cultures or agar plates. The hanging-drop culture can be dried and stained with bacteriological dyes. The small aquaria admit of prolonged observation under more natural conditions. Both are suitable for watching individual filaments, their sheath formation and reproduction. It is also instructive to compare sketches made at intervals of young colonies on the agar surface. In this way groupings of filaments were found to persist, showing that no gliding movements are exhibited. Such are also lacking in filaments enclosed in sheaths, in which movements might be inferred from the remarks made by Cholodny (1926, p. 9).

(3) *Sources of the strains*

Sphaerotilus natans. Eleven strains were used. In order to compare the forms in pure culture material of different appearance and from several localities was employed, viz.:

(A) Four strains, identified as *Cladothrix dichotoma* by their regular false branching, from: (1) a ditch at Cherry Hinton near Cambridge, in July; mud with plant debris. (2) a ditch on Coldham Common near Cambridge, in August; black mud and decaying leaves with various algae and *Beggiatoa*; (3) black mud from an excavation on low ground near the paddling pool at Newnham, Cambridge, in October; (4) greyish tassels between algae in a ditch near Fen Causeway, Cambridge, in July.

(B) Two strains, identified as *Leptothrix ochracea* by their thin, refractive, colourless or slightly yellow sheaths giving the Prussian blue reaction, from: (5) ditch with puddles on allotments in June, floating and attached tufts with *Euglena viridis*; (6) detritus from the edge of the pond in the Botanic Garden, Cambridge, in October.

(C) Five strains, characterized by densely naked filaments, slimy feeling and general appearance of the tufts, from: (7) a polluted ditch near Jesus College, Cambridge, in February—the dirty water runs through a kind of weir; (8) material from the cooling tower of a sugar beet factory, in January, mycelia and *Sphaerotilus*; (9) pure culture isolated from the bed of a polluted stream near Watford; (8) and (9) were supplied by the courtesy of Mr T. G. Tomlinson, Water Pollution Research Laboratory, Minworth near Birmingham; (10) pure culture from sewage; and (11) pure culture from the Columbia River, U.S.A., supplied by Mr J. B. Lackey, U.S. Public Health Service, Cincinnati, Ohio, to whom I am much indebted for his co-operation.

S. discophorus. Growths, immediately recognizable as *S. discoporus*, were found quite as frequently as those of *S. natans*. Owing to the greater difficulty in obtaining pure cultures only three strains were raised, but there can be no doubt that the results are generally applicable. This species has such characterstic sheaths and reacts so uniformly to various influences that mistakes are unlikely. The strains were collected as follows: (1) From the same ditch as no. 5 of *S. natans*. After 2 months material, placed in a beaker with decaying hay and ferric hydroxide, produced brown growths near the surface mainly composed of *S. discophorus*. (2) From the same ditch as no. (2) of *S. natans*, found as a brown growth just below the surface of the vessel after standing a few days in the laboratory. (3) Reddish mud from a wet, boggy place at Caldbeck, Cumberland, in August.

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IV. *Sphaerotilus natans* and its variability

(1) *Appearance of the growths*

Pure cultures of strains originating from growths determined as *S. natans*, *Cladothrix dichotoma* and *Leptothrix ochracea* respectively all had the same appearance. In agar cultures with a relatively rich supply of nutrient substances, the surplus of food may, however, suppress differentiating features (as it does the differences between *Sphaerotilus natans* and *S. discophorus*), while these might become more apparent in media affording conditions similar to those in the original habitat.

Abundant development was obtained using agar or liquid media containing various mixtures and concentrations of several brands of peptone, beef extract, yeast extract, and glucose, without much difference. No special biochemical peculiarities were revealed. In liquid media composed of glucose with the necessary inorganic salts and nitrate or ammonium salts as sources of nitrogen there was only a poor growth while contamination with bacteria was sometimes beneficial. On the same media, but with agar which contains sufficient organic nitrogen to supply the modest needs of this organism, the growth is better. In neutral liquid media with 0·2 to 0·5 % peptone, beef extract or yeast extract the growth is plentiful, the floccules being slimy and clinging together, like that styled sewage fungus by water pollution experts. Such natural growths of *S. natans*, mainly composed of more or less parallel soft elongate threads reach their fullest development only in polluted running water (Kolkwitz 1909, p. 145). These circumstances were not completely realized in my cultures which showed a less marked parallel arrangement and shorter threads.

In order to elucidate the nature of the isolated strains, they were exposed to conditions imitating those obtaining in their natural habitats, and which might be expected to reproduce the growths known as *Cladothrix dichotoma* and *Leptothrix ochracea* respectively.

The *Cladothrix* form can be obtained with a medium consisting of garden loam covered with water and heated in a steam chamber, the growth being richer if a small grain of barley is placed beneath the soil (Pringsheim 1946*c*). The sheaths of the threads, though almost colourless, give a definite Prussian blue reaction. If several barley grains are used, the growth is more like that of typical *Sphaerotilus natans*. These experiments show that a low content of organic matter causes the regular, almost dichotomous false branching to which Cohn's specific name refers.

Cladothrix is commonly found in water with decaying leaves. Extracts of dead leaves as a medium for pure cultures give the *Cladothrix* form, provided that they do not contain much toxic tannin material like the leaves of *Quercus* and *Populus*. In a dilute neutralized decoction of green hay the characteristic branching is very evident but higher concentrations produce the *Sphaerotilus* form in the same way as beef extract, yeast extract, or peptone. By diluting these media or bacterial broth the branched form is again obtained.

A concentration of 0·02 % beef extract is about the optimum for this purpose, especially with the addition of a little soil extract. 0·02 to 0·05 % proteose peptone Difco solution likewise results in a dichotomous filamentous growth which is recognizable with a hand lens, the threads being delicate and not giving in this case the Prussian blue reaction. With the addition of 0·01 % ferrous ammonium citrate, however, the development is improved and the sheaths, although scarcely tinged, contain iron. In higher iron concentrations the old

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sheaths acquire a faint brownish colour. Manganous sulphate is harmful to *S. natans*, sometimes even in concentrations as low as 0·005 %, so that media which are suitable for *S. discophorus* (cf. p. 460) are unsuitable for *S. natans*. A definite threshold concentration cannot be given. It depends on the composition of the medium. Concentrations which are not toxic do not give a brown colour to the sheaths of *S. natans* as they do to those of *S. discophorus*.

All the strains, whether originally identified as *Cladothrix*, *Leptothrix* or *Sphaerotilus*, gave rise to *Cladothrix*—growths in solutions of low nutritive value, those sent me by water pollution experts being no exception. The specific identity of *Sphaerotilus natans* and *Cladothrix dichotoma* is thus established.

It proved more difficult to determine the conditions under which the relatively thick, brittle sheaths, characteristic of *Leptothrix ochracea* are produced. These appear to be composed of ferric compounds,.but addition of ferrous salts did in the beginning afford the desired result. This is not surprising. *L. ochracea* is described as being almost devoid of living cells and producing no swarmers, suggesting that deposits conforming to the diagnosis represent the final phase in the development of the organism rather than a thriving growth.

A soil-water culture, with ferrous ammonium citrate from an experimental series started six months previously, gave a clue to the solution of the problem. In media similar to those which produce the *Cladothrix* form, but containing a relatively large amount of ferrous salts, the threads deposit more and more ferric hydroxide until they eventually assume the condition known as *Leptothrix ochracea*. It is possible that this change is not entirely biological but partly due to mere physico-chemical causes, as claimed by Molisch (1910, p. 49) and Cholodny (1926, p. 45). This view is supported by the fact that the *Leptothrix* form contains only a few living cells, which could scarcely be responsible for the large amount of the deposits.

The form known as *L. ochracea* can be made to arise from all the various strains of *Sphaerotilus natans*. Decoctions of hay and dilute solutions of beef extract, both with 0·01 to 0·02 % ferrous ammonium citrate, are suitable for this purpose. Even more bulky sheaths are obtained in soil-water cultures with grains of cereals and addition of iron. The growths produced after several months in undisturbed cultures kept cool are not distinguishable from natural deposits. The sheaths differ from those of *S. discophorus* in being narrower and smoother and not brown, this colour being only produced by admixture of manganese which is not found in the sheaths of *S. natans*. The undisturbed growth is composed of straight threads tapering very slightly. They may sometimes be twisted in a screw-like manner with a remarkable regularity. The thick, brittle, and highly refractive sheaths, appearing like short lengths of glass tubing, found in the well-known deposits of *Leptothrix ochracea*, represent the accumulated remains of an aged vegetation.

(2) *Description of the forms*

(*a*) *Sphaerotilus*

The true *S. natans* var. *typica* has been frequently described, most clearly by Kolkwitz (1909, p. 144). The diagnoses agree regarding the tufts of irregularly arranged filaments with delicate mucilaginous and sticky sheaths, the production of lophotrichous swarmers and the scarcity of false branching. According to Kolkwitz (1909, p. 145) false dichotomy

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is produced if nutriment is deficient. The biology of *Sphaerotilus* has often been investigated (Butcher 1932; Lackey & Wattie 1940) but little is known of its biochemical characteristics.

The appearance in the natural habitat is conditioned by running water with organic substances (cf. p. 462), aeration and low temperature favouring the growth in competition with putrefying bacteria, since it is not much affected by winter conditions (Kolkwitz 1909, p. 145). In the absence of contaminating bacteria higher temperatures up to about 30° C accelerate development.

In liquid media with peptone and beef extract the growth of *S. natans* begins as a ring of germlings just beneath the surface. Later, floating, cotton-wool-like floccules and tufts are formed which float in the fluid but are easily deformed and cling together when shaken. The threads produce plentiful swarmers which in part germinate on the filaments and give the appearance of irregular branching. The cells within the soft sheaths divide to form groups, some cells of which escape through ruptures in the sheaths and grow out into filaments, adding to the confusion of heaps and tangles.

In similar media solidified with agar a thick, slimy growth is formed which may not betray a filamentous character, except perhaps at the edges and that only in media of lesser concentrations. In rich growths the cells gradually disappear as a result of self digestion, but subculturing remains possible for several weeks.

(*b*) *Cladothrix*

Cohn's (1875, p. 185) original description does not mention the deposition of iron in the sheaths of *Cladothrix*. Zopf (1882, pp. 6 seq.), who gives the best available drawings, observed a direct relation between threads of *Cladothrix* and those with the appearance of *Leptothrix*. He also states (p. 2) that inspection of the original specimens of *Leptothrix ochracea* Kütz. shows them to consist of iron-containing *Cladothrix* filaments so that the former species should be eliminated. Winograsky (1888) disputes this, while Molisch (1892, p. 350) concludes that *Cladothrix dichotoma* is actually a collection of at least three species which also differ in their physiological behaviour. Two of them are, according to him, not iron bacteria at all and, though morphologically similar, can at once be distinguished by their much more delicate sheaths. According to Dorff (1934, p. 43) quite a number of different species or even genera have been confused. But how could they possibly know without cultures?

The view that *Cladothrix* is unsuitable for investigations on iron bacteria has repeatedly been advanced. It is based on the failure to recognize iron deposits when not brown and on regarding various growth forms as distinct taxonomic units. A close affinity between *Cladothrix* and *Sphaerotilus* was suspected by Migula (1900) because of the existence of intermediate forms and the successive appearance of the two kinds of growths in the same locality as a result of changes in the impurities present. Neither Migula, nor Kolkwitz (1909, p. 148) who holds the same view, include both forms in the same species. The latter compares the diagnoses of *Cladothrix* given by six authors, which differ in certain details. That by Mez (1898) is of interest because *Cladothrix* is stated to differ from *Sphaerotilus* in its non-mucilaginous threads, and in showing its characteristic features only in undisturbed samples, not in nature.

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(*c*) *Leptothrix*

L. ochracea should be the best known of all sheath bacteria, since it is the one most frequently described. Yet it is scarcely possible to obtain a clear picture of it.

Kolkwitz (1909, p. 138) gives the following diagnosis: cells arranged in filaments, without the sheath about 1 μ wide. Sheath thin when young, mostly colourless, later thickening and becoming yellow or brown, rigid or gelatinous. Reproduction by non-motile ovoid cells, occasionally perhaps also by fragments of filaments. May deposit manganese compounds in the sheath. Of all iron bacteria the commonest. Kolkwitz's figures partly show *Sphaerotilus discophorus* which is also included in the diagnosis. Measurements for the thickness of the sheath are not given. The only objections to this diagnosis are that motility is not mentioned and that it gives no idea of the development.

Cholodny (1926, pp. 8, 67) gives the following description: cotton-wool-like masses of ochre colour, composed of a multitude of small cylindrical tubes, 2 to 3 μ wide and firmly twisted together. Inner diameter about 1 μ. In spite of their great length the tubes are generally of even width throughout. They have a smooth surface, are never branched and consist of a strongle refractive, homogeneous and translucent substance which disappears completely in dilute hydrochloric acid. Ferrous potassium cyanate in the presence of hydrochloric acid gives them an intense blue colour. The threads are always free floating. Propagation so far not exactly known.

Cholodny believes that the reproduction may be similar to that of *Sphaerotilus*, but if there were swarmers, they would probably settle on some substratum so that attachment of young filaments should occur. Kolkwitz (1909, p. 140) saw such attached threads and rightly suspected them to be germlings or stunted specimens of *Sphaerotilus*, although Migula (1900, p. 1033) described them as a separate species, *Chlamydothrix epiphytica*. *Leptothrix parasitica* Kütz. (1843, p. 238) at least in part consists of similar germlings of *Sphaerotilus*.

The uncertainty about *Leptothrix ochracea* is due to the restriction of the name to aged stages of development of the organism concerned (cf. p. 463). When other stages, such as filaments with thin sheaths producing swarmers, or germlings attached to some foreign body were found, they were referred elsewhere because they did not conform to Kützing's diagnosis. Most authors (Cholodny 1926, p. 8; Dorff 1934, p. 32; Teichmann 1935) are agreed that usually only empty sheaths of *L. ochracea* are found, occasionally enclosing a few cells. Naked filaments have seldom been observed and, in the absence of cultures, could not certainly be assigned to this species. Those referred to *L. ochracea* by Winogradsky (1888), Molisch (1910) and Lieske (1919) were actually filaments of *Sphaerotilus discophorus* (cf. p. 458), so that Cataldi (1939) was the first to recognize such uncoated filaments of *Leptothrix ochracea*.

The following survey of the taxonomic characters will make the position clearer: (*a*) According to Dorff (1934, p. 35) his *L. major* is 1·4 μ wide, *L. ochracea* only 1 μ, but in clone cultures of the latter the width is variable and includes the dimensions given for the former. (*b*) The length of the filaments, which is used by Cholodny (1926, p. 9) and Dorff (1934, p. 30), is not a suitable specific character, since it depends on momentary conditions and moreover cannot be accurately measured when the threads are bent and exceed a certain length. (*c*) The stated absence of attached threads and of zoospores in *L. ochracea* can be explained by the firm sheaths which prevent the escape of swarmers, while the soft

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ones of *Sphaerotilus natans* typ. swell up and allow them to emerge. (*d*) False branching may be plentiful, scarce, or lacking according to conditions; it may well be absent in old brittle deposits, but in cultures threads indistinguishable from the true *Leptothrix ochracea* include portions with characteristic branching (cf. figure 7*c*, p. 469), although this is really scarcer in threads with thick sheaths. Zopf (1882, p. 5) has already stated that branching is hampered by sheaths encrusted with ferric hydroxide. (*e*) According to Cholodny (1926, p. 67) there is no difference between base and apex, but how could this have been recognized in fragments of even width?

This analysis of the main items in the diagnosis of *Leptothrix ochracea* shows it to be incomplete, and a real picture of the organism is in fact only obtained when its relation to *Sphaerotilus natans* and *Cladothrix ochracea* is recognized. I would not, however, like to maintain definitely that there is only one species of *Sphaerotilus* capable of producing the *Leptothrix ochracea* state. It may well be that there is a second form, perhaps one adapted to lower temperatures and therefore not growing in my cultures; certain observations and comments in the literature, as well as Teichmann's results at a low temperature point in this direction. Further investigations may show how the latter can be brought into agreement with Cataldi's experiments carried out in a warm climate.

(3) *Morphology*

(*a*) *The filaments*

Young vigorous growths of *Sphaerotilus natans* appear as filaments composed of cylindrical cells. Germlings, originating from swarmers, are attached by a special base (figure 4). The filaments resemble those of other bacteria, for instance *Bacillus subtilus* or *Lineola longa* (Pringsheim & Robinow 1947). On closer inspection there are differences, one of them being the sheath of *Sphaerotilus* which is lacking in the other bacteria.

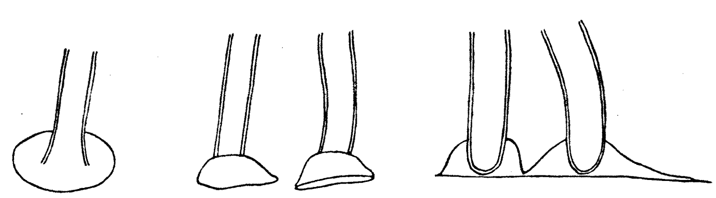

FIGURE 4. *Sphaerotilus natans*. Basal parts of filaments attached to surface of medium (×2500).

In the luxuriant growths caused by a rich nutrient supply the arrangement of the cells may become very irregular (figure 5). The origin of the rows of cells lying more or less parallel to one another could not be established, but this arrangement may be due to longitudinal division of the cells or, what seems more probable, displacement after separation.

The length of the cells is about equal to or double their width, but the limits of the cells are not always readily recognized. Long filaments, seemingly without cross-walls, are called 'giant cells' by Cholodny (1926, p. 9), but like many bacterial rods hitherto regarded as single cells (Robinow 1944) they are actually multicellular. Staining of such filaments with Giemsa after hydrolysis revels nucleoid structures similar to those found in other bacteria. The individual cells are cylindrical with rounded ends, the terminal cell

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being hemispherical or elongate. There are shallow constrictions between the cells so that the filaments may appear moniliform and in these there is a tendency to break up into short lengths or even single cells. This can happen also inside old refractive sheaths, in which the cells are often separated from one another. The cells never give the Prussian blue reaction shown by refractive sheaths, and Gicklhorn's (1920, p. 16) erroneous statement must be due to inadequate optical equipment. Molisch (1892, p. 69) did not find iron in the cells, although he later makes a contrary statement (1910, p. 49).

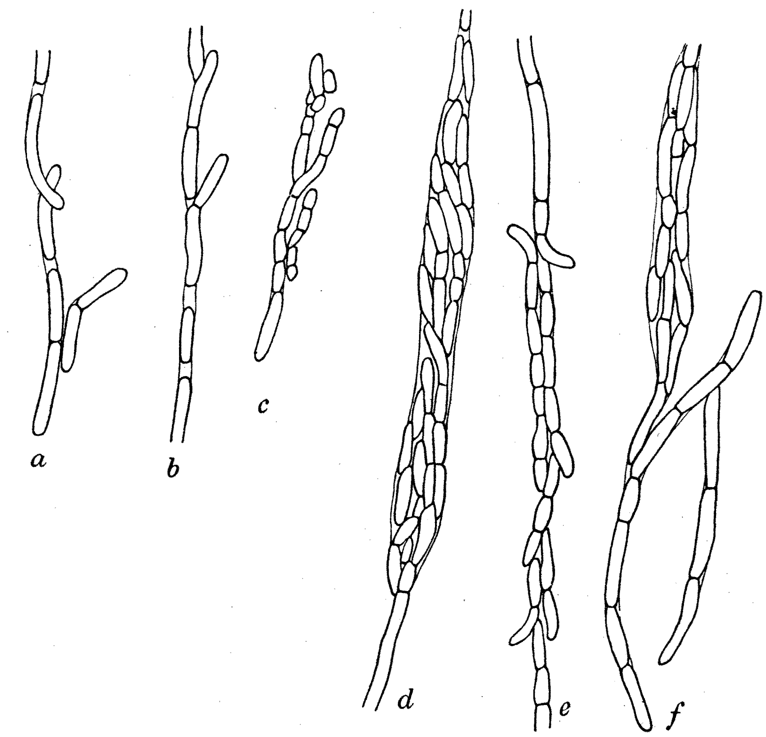

FIGURE 5. *Sphaerotilus natans*. Arrangement of cells in growth with soft sheaths devoid of inorganic deposits; *a* to *c*, contact between the cells loosening; *d* to *f*, displaced cells kept together by irregularly widened sheath (× 1250).

The cytoplasm in the cells of young thriving filaments is generally translucent and without evident inclusions. Older cells contain oil droplets and volutin (Fischer 1897, p. 111), which are so refractive that they might be mistaken for sulphur drops or spores. Dark-ground illumination is helpful in discerning details.

(*b*) *The sheaths*

It is useful to distinguish between the primary sheath, its secondary coating with inorganic gel-like compounds, and the mucilage excreted in the absence of such rigid deposits.

Cholodny (1926, p. 11) maintains: 'A gelatinous sheath composed of organic substance does not exist. The material, secreted by the cells of this species (viz. *Leptothrix ochracea*) and deposited on their surface as a more or less voluminous layer, is from the first exclusively composed of ferric compounds completely soluble in hydrochloric acid.' The old brittle

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refractive sheaths are, it is true, mainly composed of inorganic substances, but a sheath (Ellis 1919, p. 72, figure 1) exists prior to such deposition or even when there is none (cf. figures 6 and 8). These primary sheaths are extremely delicate and only recognizable where cells of young filaments are displaced by pressure, or when there are gaps between the cells as generally occurs in older threads. This shifting of cells proves also that the sheath is distinct from the cell-wall.

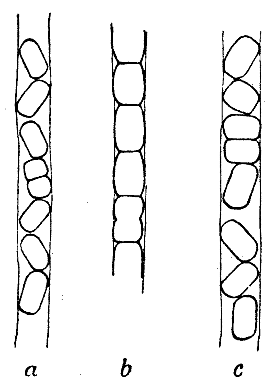

FIGURE 6. *Sphaerotilus natans.* Cells within primary sheath beginning to separate; culture in hay decoction (× 2500).

When there is no inorganic deposit the sheath can still be either tough or soft dependent on nutritional conditions. In rich media it may take up water or even be altogether transformed into mucilage, the cells becoming heaped up and exposed so that they can be dispersed. The presence of the sheath can be demonstrated by dark-ground illumination or by mounting in Indian ink or nigrosin. Moreover, the separate bacterial rods could not retain their mutual position if they were not connected by the almost invisible, elastic sheath. No stain has so far been found that colours the primary sheath preferentially or more than slightly but it can be demonstrated with a mordant dye.

The thicker, more readily visible sheaths are believed by Molisch (1892, p. 70; 1910, p. 49) to consist of an organic gelatinous substance impregnated with iron compounds. Actually thickening of the primary sheath is not due to impregnation, but to surface deposition. Molisch's comparison of the gelatinous substance with a filter retaining ferric compounds is not appropriate. If deposition were due to reaction between a gelatinous medium and inorganic compounds, one would speak of adsorption. There does not, however, seem to exist such a primary mucilage. The thin original sheath persists as and where it was, since treatment with dilute acids causes the deposited substance to disappear, while the inner layer remains. It can also be recognized within the secondary coating under dark ground illumination. Further evidence of the true relations can be found in growing ends of threads where the various stages of development lie close together. The primary sheath can there be traced from the 'naked' terminal cells into the secondary layers coating the older parts.

The relation between the organic primary sheath and its inorganic deposits can also be demonstrated by varying the culture medium. In a 0·05 % solution of beef extract the sheaths are barely visible in ordinary illumination, but if 0·05 % ferrous ammonium

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citrate is added, distinct sheaths, giving the ferric reaction, are formed. In hay decoction the differences are still clearer. Without iron the filaments, though branched, are relatively soft and devoid of iron deposits, while addition of a ferrous compound produces the typical *Cladothrix* form with rigid, yellowish sheaths. The Prussian blue reaction is faint towards the apex, but marked towards the base which may be the only part stained when ferrous compounds are deficient.

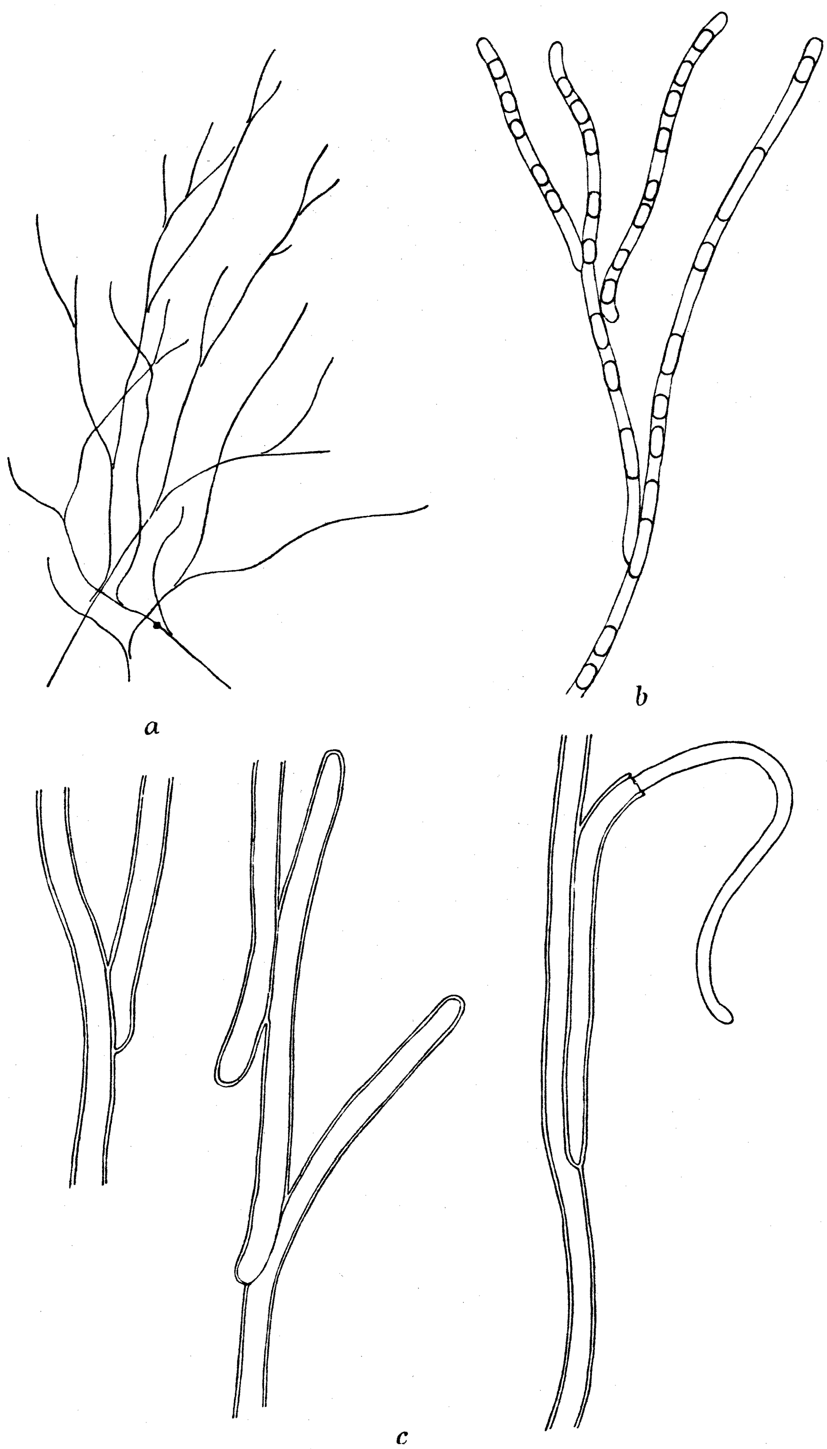

FIGURE 7. *Sphaerotilus natans*. *Cladothrix* form. *a*, false branching and bifurcation (× 150); *b*, the same, at a higher magnification (× 1000); *c*, false branching, with sheaths (× 2000).

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In soil-water cultures, in which branching is a characteristic feature (cf. p. 462), the sheaths always possess a ferric coating derived from the iron content of the garden loam (figure 7). The threads are then wider near the base and narrower towards the apex, where the iron content is less. This form resembles that described as *C. fusca* (cf. Molisch 1910, p. 20, figure 4). The occurrence of false branching seems to depend, not only on the low concentration of organic nutrients, but also on the presence of a sheath of a certain rigidity.

There is no mutual exclusion between false branching and the possession of a firm sheath, as the diagnoses of *Leptothrix ochracea* (cf. p. 465) seem to indicate. Migula (1904–7, p. 56, plate I, figure 10) already figures such branching. The protrusion of the cells to form lateral branches must take place before the sheath is heavily coated, but unless such initials of side branches had been kept in position by the sheath they would have escaped and grown out separately into distinct filaments as actually occurs in the *Sphaerotilus* form. The trichomes in the latter are covered with a layer of mucilage as can be demonstrated with Indian ink. This is especially well developed in media with glucose.

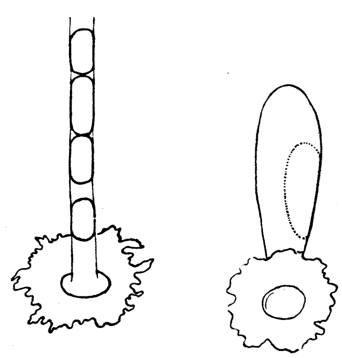

FIGURE 8. *Sphaerotilus natans*. *Leptothrix sideropous* form and germling of the alga *Tribonema* from the surface of a ditch, showing similar bases impregnated with iron. After detachment of the filaments both haptera could be mistaken for *Siderocapsa*, a unicellular iron bacterium (×2500).

When iron is in poor supply, it is soon used up by the germlings, particularly by the holdfasts and the neighbouring parts of the filaments (figure 8). Such conditions arise in media composed of dilute hay-extract or 0·05 % beef extract with up to 0·01 % ferrous ammonium citrate. With methylene blue the basal parts are stained pink, with ferrous potassium cyanate and hydrochloric acid deep blue. The encrusted region around the base is discoid to hemispherical (Zikes 1915, p. 536) or irregularly shaped, and from this then extends upwards an encrusted cylindrical region, while the upper part of the filament may remain uncoated. This is the appearance of *Chlamydothrix sideropous*, as described by Molisch (1910, p. 10).

The old, refractive sheaths of '*Leptothrix ochracea*' have often been described. The primary sheath is not evident in them. The surface is smooth, while the colour is faintly yellow with a slight brownish tinge, especially, it seems, when humus substances are present. Manganese has no effect on the colour. These sheaths are resistent and often accumulate in considerable quantity. Where hydrogen sulphide is produced, the sheaths become grey owing to the formation of ferrous sulphide.

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(*c*) *Reproduction*

Reproduction is not so well defined in *Sphaerotilus natans* as in many other lower organisms, since every cell or fragment of a filament can grow out into a new thread. If there is already a firm sheath, only the uncoated apex plays a role in reproduction. This takes place by detachment of the terminal cells which either immediately multiply by division or swim away with the help of flagella-like ordinary bacteria. When the sheaths are soft, whole threads may dissolve into separate rods or give rise to irregular groups of cells, which behave in the same way.

The designations swarmers and conidia (Cholodny 1926, p. 11) for the reproductive rods perhaps lay too much emphasis on the difference from other cells. They may either consist of single cells or of short chains, not quite 1 μ wide and usually 5 to 10 μ long, but sometimes considerably longer (Molisch 1910, pp. 20, 40). Fischer's (1895, p. 122) figure of the motile stages of *Cladothrix*, which has often been reproduced, shows unusually short rods and almost ovoid cells. He describes and figures these stages (cf. also Höflich 1901 and Linde 1913–14) as bearing tufts of flagella on one side, while Ellis (1919, p. 83) records them at the ends as in *Spirillum*; Lackey & Wattie (1940, pp. 978–9) observed one to several subapically inserted long flagella. Preparations, which Dr W. J. Dowson very kindly stained in various ways, showed only a single subpolar flagellum (figure 9). This has already been recorded in *Sphaerotilus natans* by Zikes (1915, p. 538), who regarded this feature as a distinction from *Cladothrix dichotoma*, in which he found several flagella. Various strains therefore seem to differ in this respect.

The polarity displayed by the flagellar insertion no doubt also determines which end of the swarmer becomes attached to the substratum and forms the gelatinous hapteron which later becomes encrusted with ferric hydroxide.

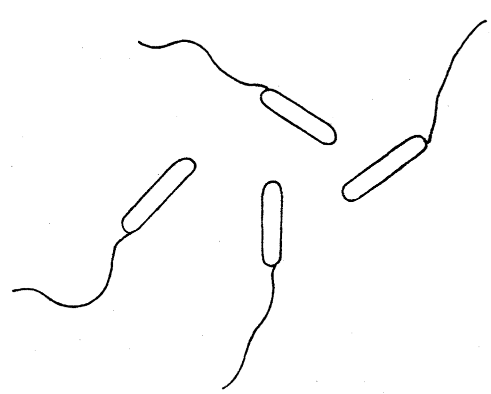

FIGURE 9. *Sphaerotilus natans*. Motile stages from 4-day-old culture on 0·2 % yeast extract agar, pH 7·5. Those of *S. discophorus* quite similar (× 2000).

V. *SPHAEROTILUS DISCOPHORUS* AND ITS VARIABILITY

(1) *Appearance of the growths*

Judging by the descriptions of *Leptothrix ochracea* and *L. discophora*, these species should be easily distinguished, but that is so only when the sheaths are well developed. Such stages of *Sphaerotilus discophorus* have been clearly figured already by Zopf (1882, plate I, figure 4) under the name *Cladothrix dichotoma*. *Sphaerotilus discophorus* has been far less often re-

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corded and might be thought to be much rarer than *S. natans*. This would, however, be an incorrect impression since all the cultures grown by Winogradsky (1888), Molisch (1910), Lieske (1919), and Cataldi (1937) under the name *Leptothrix ochracea*, were actually *Sphaerotilus discophorus*. Apart from cultures of *Sphaerotilus* and *Cladothrix*, Cataldi (1939) seems to have been the first to grow the true *Leptothrix ochracea*.

The reason is probably to be found in the conspicuous brown sheaths of *Sphaerotilus discophorus*, while when *S. natans* has developed pronounced sheaths they include few living cells, so that the material does not readily afford cultures. Both organisms are actually common and can easily be grown from waters with decaying plant material, especially those with a bottom of black mud. When such water is kept in the laboratory for a few days, unmistakable threads of *S. discophorus* are usually to be found in the surface film, while *S. natans* in the easily recognizable *Cladothrix* form floats below the surface. Single filaments of the two species without sheaths are not distinguishable.

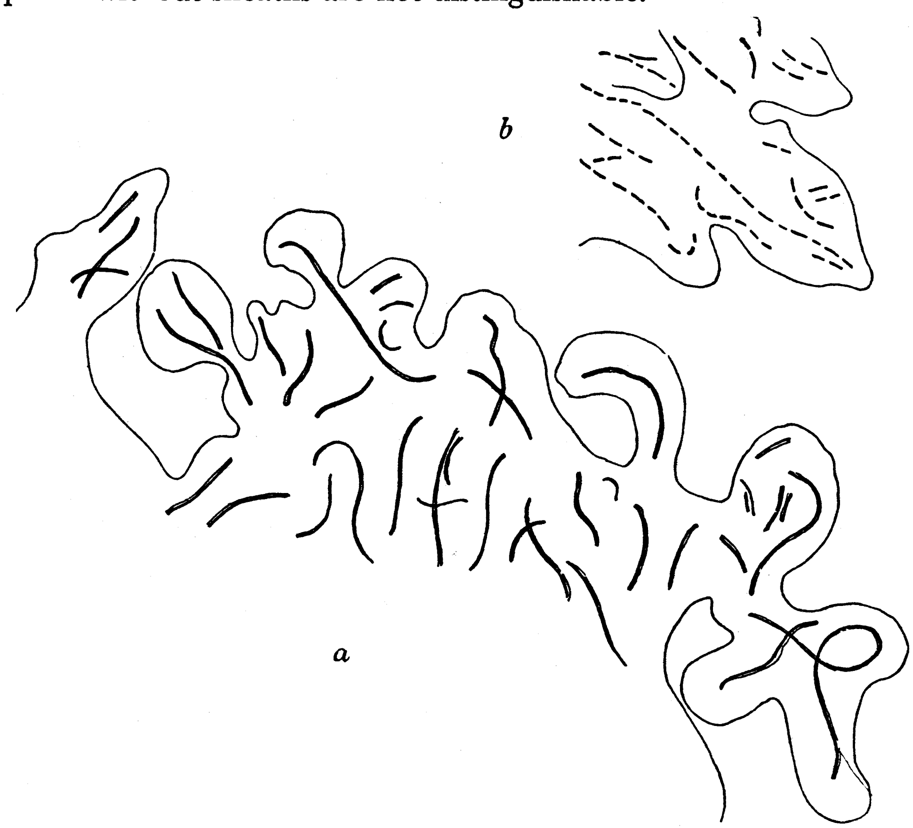

FIGURE 10. *Sphaerotilus discophorus*. Marginal portion of floating growth: *a*, filaments embedded in an amorphous, brown, colloidal precipitate; *b*, part of *a* at higher magnification, after treatment with hydrochloric acid (×200).

The most striking feature of typical growths of *Sphaerotilus discophorus* are the golden brown sheaths, which are often confluent and sometimes constitute a coherent though brittle film in which the filaments are embedded (figure 10). The later are then bent and interwoven so that distinct threads are only found at the edges of the films. Such growths include many other small organisms so that they are not suitable for starting pure cultures. Separate threads are, however, obtained in a day or two after gentle shaking, and from these cultures can be prepared.

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(2) *Ecological modifications*

S. discophorus shows much the same variability as *S. natans*, although there are two points of difference. It does not multiply as copiously in rich media nor does it form long threads, so that there is no form parallel to *S. natans* typ., and it does not occur as 'sewage fungus'. Further it rarely exhibits false branching, and there is no dichotomous form.

Four modifications are distinguishable:

(*a*) *S. discophorus* forma *eutrophica* is the commonest. It forms threads of limited length which readily break up into rods. The latter are motile with the help of polar flagella (Cataldi 1939, plate VII, figure 2) and are not distinguishable from the motile stages of *S. natans* or indeed from those of many other bacteria. When cultivated on routine bacteriological media this modification differs so much from the typical growth of a sheath bacterium that it might be mistaken for a species of *Pseudomonas*. The characteristic hairy colonies (cf. p. 459) are not produced in such cultures. Long chains of cells are only formed in dilute media and even then are not as long as those of *Sphaerotilus natans*, though otherwise very similar and with the same delicate elastic sheaths.

The 'bacterial' habit develops in neutral media with 0·3 to 0·6 % peptone (Bacto, proteose, etc.) and 0·3 to 0·6 % beef or yeast extract, with or without agar.

(*b*) *S. discophorus* forma *manganifera* is the modification described by Schwers (1912) and Cholodny (1926, pp. 14 *seq.*) and shown in Cataldi's photograph (1939, plate 3). It is the only modification which is easily identified. Its appearance depends on the simultaneous presence of small amounts of both ferrous *and* manganous compounds and a very low concentration of organic substances.

The most striking development of this form is obtained in either very dilute hay decoction or soil extract or else soil-water cultures, to all of which 0·01 % ferrous ammonium citrate and 0·01 % manganous sulphate has to be added. The iron concentration may be a little higher, the manganese concentration somewhat lower, without an appreciably different result. In soil-water and soil-extract media the addition of iron is usually not absolutely necessary since traces are present. In hay decoction or in media with yeast extract or peptone, with manganous but without ferrous compounds, however, the characteristic continuous sheath is not produced, the manganese deposit then consisting of fine granules or amorphous masses (figure 11). Molisch (1910, pp. 39–40) obtained well-developed sheaths with his manganese peptone agar medium when prepared with Prague tap water, but observed only precipitations when the medium was prepared with Vienna water from a mountain spring which no doubt contained insufficient iron. In iron-deficient media the growth is poor and there is no Prussian blue reaction.

The manganiferous form may cover the surface of the medium with a coherent film as described on p. 472, but it may also produce various aberrant growths, some of which are shown in figure 12.

(*c*) *S. discophorus* forma *arachnoidea* is conditioned by media of a lower nutritive value than those producing modification (*a*), for example, dilute hay decoction or beef extract. In these there is no surface film nor any kind of dense aggregate, the organism forming fine cobweb-like growths on the wall of the glass vessel. It is probably frequent in nature although not readily observed.

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If the medium contains a certain low concentration of ferrous and manganous compounds the growths become deep brown and form scattered radiating colonies and single threads. The difference from growth form (*a*) is only one of density, but the tendency to spread over the inner surface of the vessel is rather characteristic.

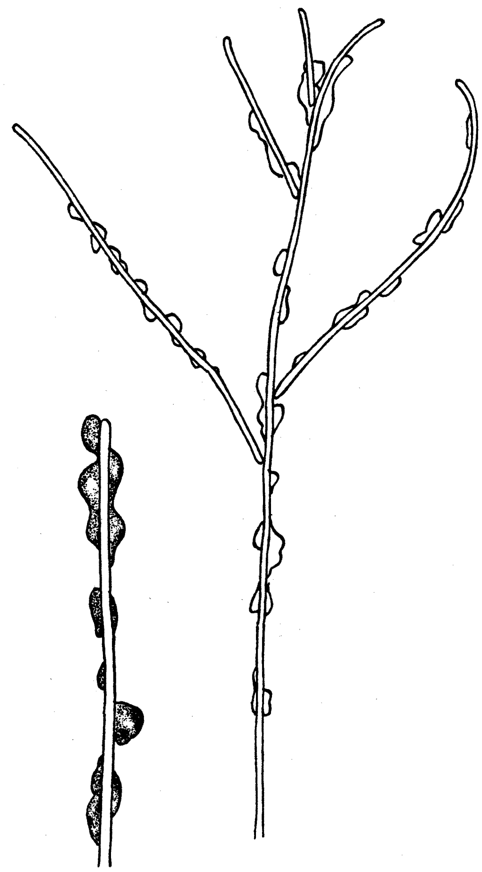

FIGURE 11. *Sphaerotilus discophorus*. Growth in a medium with manganese, but lacking iron. Beijerinck's solution + hay decoction, diluted to 1/10, with 0·01 %$MnSO_4 . 7H_2O$. The granular deposits do not give the Prussian blue reaction (×700; ×500).

(*d*) *S. discophorus* forma *ochraceoidea* is somewhat similar to *S. natans* forma *ochracea*, although the sheaths are wider, less sharply outlined and less refractive. It is observed in media without sufficient manganese but containing a fair amount of iron. It is not possible to state definite concentrations because the limits alter with the composition of the medium. This modification is often found together with others. Its sheaths give a strong Prussian blue reaction.

Sometimes a film of the *manganifera* form, in which the filaments only appear as colourless tracks in a brown mass, produces at its edges threads with relatively thin sheaths of the *ochraceoides* form. In such instances manganese has evidently been used up before the conditions became too unfavourable for a further development. Cholodny (1926, p. 16) refers to relatively long cylindrical threads observed under certain, not defined, circumstances and recalling the threads of *S. natans* ('*Leptothrix ochracea*'), which may have belonged to the form just mentioned.

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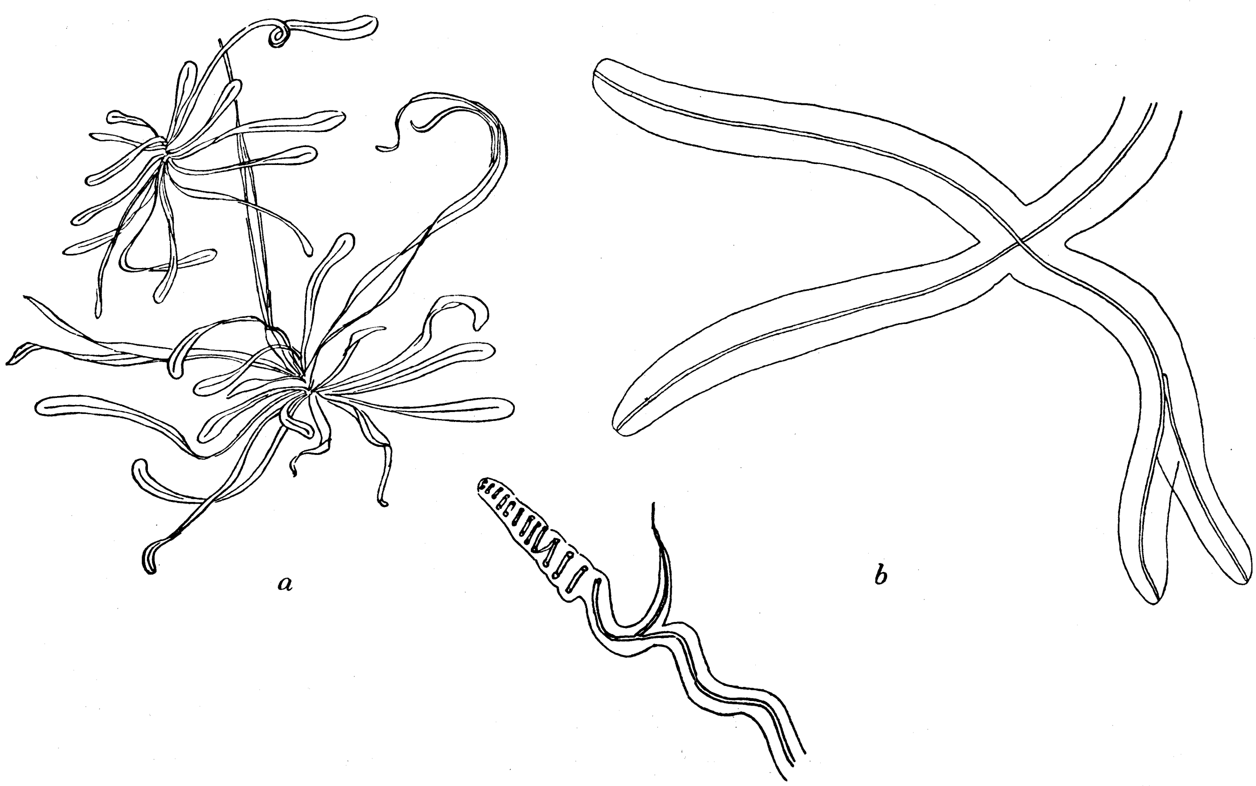

FIGURE 12. *Sphaerotilus discophorus*. *a*, colonies of club-shaped threads grown in a dilute mineral solution with soil extract, 0·02 % ferrous ammonium citrate and 0·01 % manganous sulphate (×200); *b*, filaments kept together by well-developed sheaths (×500); *c*, unusual spiral form from the surface film (×500).

(3) *Morphology*

The filaments of *Sphaerotilus discophorus* vary in the same way as those of *S. natans* and on the whole are so similar to them that they can scarcely be distinguished. Their width is the same. Short rods and longer, probably multicellular filaments are also found, although the latter are rare (Cholodny 1926, p. 16). The delicate primary sheath is just as difficult to observe as in *S. natans*. Nor is there any difference in the holdfasts of germlings which, when developed at all, are irregularly discoid and stain pinkish with methylene blue.

The mature sheath, as described by Schwers (1912), Cholodny (1926, pp. 14 *seq.*), and Cataldi (1939, p. 61), are much wider than those of *S. natans*; according to Cholodny they are 10 to 15μ wide, while Cataldi gives 16μ as the upper limit. They may actually reach a diameter of up to 23μ under favourable circumstances, for instance, growth in an infusion of over-wintered grass leaves with 0·02 % ferrous ammonium citrate and 0·01 % manganous sulphate. Unlike the sheath of *S. natans* that of *S. discophorus* tapers towards the end where the filament projects, or part of it is encased in a markedly narrower sheath and only the very end is without deposits and merely enclosed in the primary sheath (figure 13).

The thick secondary layers precipitated on the original sheath do not consist of vitreous homogeneous matter like those of *S. natans* forma *ochracea*, but of a less refractive, more watery substance, with a slightly granular appearance and an uneven surface. The sheath is orange to golden brown and less translucent than that of the other species.

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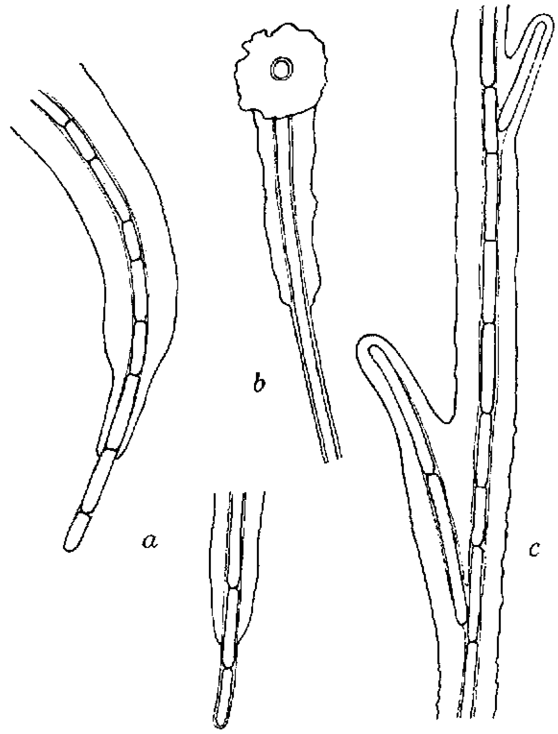

FIGURE 13. *Sphaerotilus discophorus*. *a*, ends of two filaments with deposits on the sheaths; *b*, filament attached to water surface with a thicker basal coating; *c*, common sheath surrounding parent filament and side branches (× 1250).

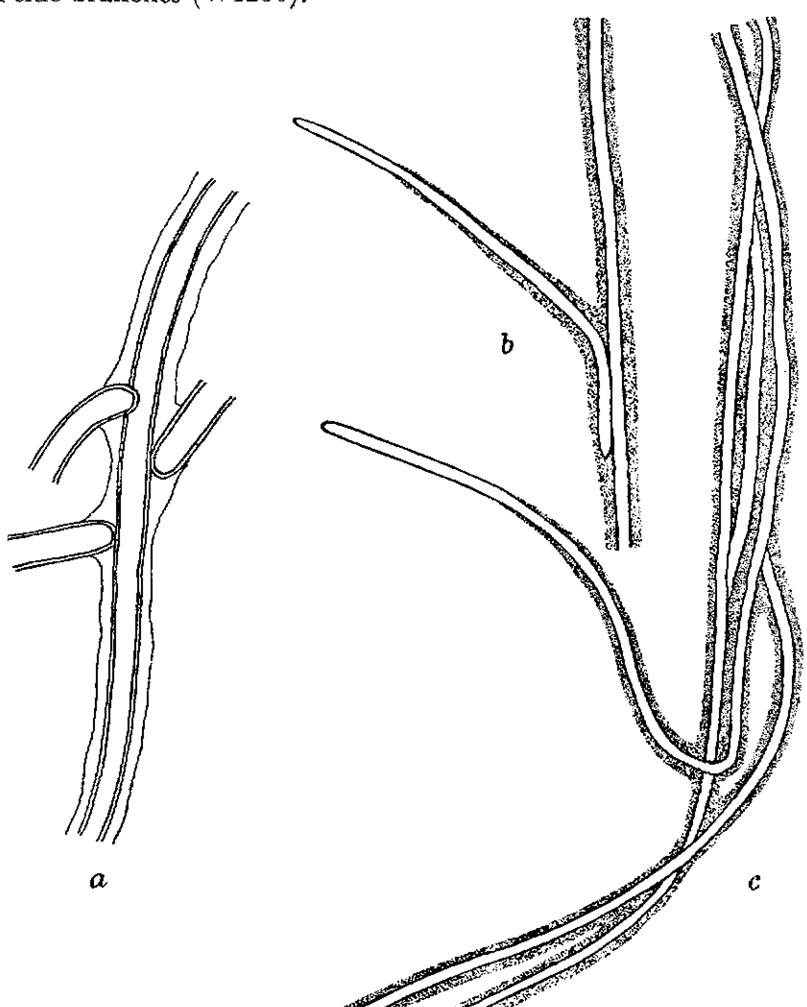

FIGURE 14. *Sphaerotilus discophorus*. *a*, germlings attached to older thread, the sheath of which surrounds their bases (× 2000); *b*, False branching (× 1000); *c*, filaments with confluent sheaths (× 1000).

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An appearance of ramification due to adherent germlings is frequent, although false branching is rare. The germlings are often surrounded by a deposit continuous with that of the parent sheath (figure 14). Irregular composite structures are thus formed which spread over the surface of the medium as a more or less continuous film of confluent sheaths and appear sepia brown in appropriate cultures.

The innermost part of the secondary sheath is deeper brown and denser than the rest and often persists after the outer part has been destroyed by bacterial action. It is also not as quickly dissolved by dilute acids as the outer part. These inner portions, when isolated, are similar to the sheaths of forma *ochraceoidea* in lacking the outer coating which has been destroyed whereas in that form it has never been deposited. They are not produced by impregnation of the primary sheath, but by deposition on the surface, followed by the formation of a more substantial outer layer.

Reproduction of *S. discophorus* resembles that of *S. natans* and takes place by mechanical fragmention, by detachment of filaments and rods, and by the formation of motile stages. Filaments provided with a well-developed sheath can only reproduce from their free ends, but broken threads often show filaments growing out at either end. Plentiful nutrients favour breaking into short fragments and rods, owing to the soft envelope, and production of motile stages is then abundant. On agar with a good supply of nutrients the growth consists almost entirely of rods which would immediately assume mobility in water. Such growths are more readily obtained in *S. discophorus* than in *S. natans*. The swarmers resemble those of *S. natans*, being long rods, 5 to 10μ or more in length, and bear one, relatively thick, flagellum.

Differences between *Sphaerotilus natans* and *Sphaerotilus discophorus*

S. natans	*S. discophorus*
sheaths generally 3μ, rarely up to 5μ in diameter, refractive, yellowish, surface smooth	sheaths 10 to 20μ wide, less refractive, golden brown with a differentiated inner portion, surface uneven
false branching often distinct and regular; every thread with a separate sheath	ramification by adherent germlings; false branching rare; numerous threads often enclosed in mass of confluent sheaths
secondary sheath consists of ferric hydroxide; adsorption of manganese very doubtful	manganese strongly adsorbed, so that growth becomes sepia brown
sheaths for the most part of even width, rarely any difference in one and the same thread; filaments not protruding, but some of them naked	sheath tapering towards the end, which is delicate and colourless, with the filament protruding
readily isolated in pure culture, the filaments growing beyond surrounding bacteria on agar plates	difficult to isolate from bacteria owing to the lack of long filaments; repeated plating necessary
colonies on agar of low nutritive value composed of looped or spirally arranged filaments, with a greater food supply circular, smooth, mucilagenous	colonies either smooth or rough, edges even or frayed, when luxuriant thick, drop-like; on agar with manganese *R* colonies deep brown, *S* colonies slowly darkening; without manganese translucent, mucilaginous

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VI. Discussion

Strains, identified as *Sphaerotilus natans*, *Leptothrix ochracea*, and *Cladothrix dichotoma* respectively, gave rise to identical cultures, all of which responded to certain treatments by undergoing similar modifications. Among the forms so produced were some which every expert would describe as typical examples of the above-mentioned 'species'. Since *Sphaerotilus natans* Kütz. is the oldest name, it must be retained for this organism.

Strains identified as *Leptothrix discophora* (Schwers) Dorff belong to a different species, but are so similar to *Sphaerotilus natans* that this species should be renamed *S. discophorus* (Schwers) n.comb.

Pure cultures of the two species grown on a routine bacterial agar, or better on a dilute neutral beef-extract glucose agar, are similar in appearance, but can be distinguished by the structure of the colonies. The easiest way to differentiate them is, however, to use a manganous sulphate agar, as little as 0·001 % of the salt often sufficing. The colonies of *S. discophorus* then become brown as a result of the oxidation of manganous salts to higher oxides, provided there is only a very low concentration of organic substances present, those of the *R* form doing so sooner than those of the *S* form. *S. natans* colonies, if growing at all (cf. p. 459), are colourless. This species also grows more quickly and luxuriantly than *S. discophorus*.

In suitable liquid media, if not too dilute, *S. natans* covers the fluid as a thickish slimy layer, while *S. discophorus* produces merely a ring near the surface. In very dilute media and in soil-water cultures *S. natans* grows as the *Cladothrix*—modification with abundant branching of the stiff filaments, while *Sphaerotilus discophorus* appears as delicate, cobweb-like nets and floccules.

Typical *S. natans* cannot be obtained in static cultures, since it is confined to flowing polluted waters. It consists of slimy tufts of long threads attached to solid objects. The growth in large culture vessels with a shallow layer of a rich medium, however, approaches the typical form. *S. discophorus* is never found in such extensive masses, either in cultures or in nature, where it forms a slight greyish fur attached to leaves, algae, and the like, or occurs as single filaments between other organisms in the surface film of pools.

The *Leptothrix*—modification of *Sphaerotilus natans* is found in waters poor in organic substances but containing a certain amount of ferrous compounds. It occurs in small quantities in situations where ferric iron is undergoing reduction by bacterial processes in the underlying mud, or ferrous sulphide is being oxidized to ferrous sulphate by oxygen, either atmospheric or formed by algal photosynthesis. In both cases anaerobic conditions prevail near, and for the most part beneath, the region where the *Leptothrix* grows. Larger quantities of this form appear at the edges of bogs and marshes where waters rich in carbon dioxide or humus substances or both dissolve iron in the lower state of oxidation and convey it to situations where it becomes oxidized. The products of this biological oxidation form yellowish red masses, which have long been used as a source of iron ore because of the accessibility and purity of the material. Its special value is due to the absence of other metals, especially manganese, which is not precipitated by *Sphaerotilus natans*.

'*Leptothrix ochracea*' was one of the organism which suggested to Winogradsky the well-known theory of the utilization of chemical energy derived from the oxidation of inorganic

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substances. Other 'inorgoxidants', as Winogradsky (1922) has called them, have long been shown to behave in accordance with his theory, but as regards iron bacteria opinions differ considerably. While Winogradsky himself, as well as Pfeffer (1897, p. 531), Lieske (1911, 1919), Jost & Benecke (1924, p. 392) and Cholodny (1926) take the chemo-autotrophism of iron bacteria as probable or established, others (Cataldi 1937; Kalinenko 1940) follow Molisch (1910) in denying it. The first point of view is supported by the following considerations: (1) observations in nature are in agreement with Winogradsky's theory; (2) Molisch's demonstration that *Leptothrix* can grow on organic substances does not speak against the probability that under certain circumstances it could grow autotrophically, while his observations on the effect of iron and manganese compounds (1910, pp. 34–5) indicate their importance; (3) Winogradsky's and Lieske's results with *Leptothrix* and *Gallionella*, though not quite conclusive, suggest that success might be achieved with improved methods.

The difficulty in the way of growing iron bacteria without the presence of organic substances is probably an experimental one. Lieske's attempt to overcome the difficulty of ferrous salts being too readily oxidized, by using manganous instead of ferrous salts, could not meet with complete success because *Sphaerotilus discophorus*, his experimental organism, although oxidizing manganous compounds, does not grow in the absence of ferrous salts (cf. p. 474). Had he really used the true *Leptothrix ochracea* (= *Sphaerotilus natans*) he would not even have obtained this degree of success, since the threshold for a toxic effect of manganous salts in this species is much lower. This is also the reason why this modification of *S. natans* failed to grow on Molisch's media.

Autotrophy has thus not been proved in these iron bacteria, although utilization of the energy derived from the oxidation of inorganic substances is probable. This could only be demonstrated by exact experiments combined with chemical analyses. The evidence already suggests that considerable growth can be obtained with only a minute quantity of organic matter, while the presence of oxidizable manganous and ferrous compounds increases considerably the amount of cell material formed. This result supports the probability that inorganic oxidation energy is used and that the influence of small amounts of organic substances required is based on their power to keep iron in the reduced state. The organisms under discussion are, however, not exclusively adapted to live on inorganic substances, since they can thrive on media employed for the culture of ordinary saprotrophic bacteria, especially if these are less concentrated. They will grow on beef extract and yeast extract, on peptone, and on sugar plus asparagine as a source of nitrogen.

Both kinds of nutrition are indicated by their occurrence in nature, the heterotrophic one by their presence among fungi and bacteria characteristic of mesotrophic waters. In competition with these organisms good aeration is necessary so that *Sphaerotilus natans* typ. is plentiful only in running waters. When transferred to the laboratory the organism soon exhausts the oxygen and undergoes decay. *Sphaerotilus* is adaptable to an extraordinary range of environments and varies remarkably in rapidity of growth and morphological appearance.

Owing to the abundance of the organisms discussed in this paper, their significance in biochemical processes in rivers must be great, but comparatively little is known about their nutritive needs, their metabolism and their enzymatic systems.

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Species of micro-organisms are often considered to be restricted to certain, well-defined conditions under which they thrive best and can withstand competition most effectively. These conditions are supposed to prevail in their natural habitats and the particular state and appearance of the organism observed there are regarded as characteristic or 'natural'. In the opinion of field botanists diagnoses should therefore be based on material found in such habitats. This principle is for the most part sound, but observation of small organisms both in nature and in culture leads to the conclusion that the matter is not always so simple. A convincing example has already been furnished by *Anthophysa* (Pringsheim 1946*b*). The experimental results have been confirmed by observations in Sweden (Skuja 1948, p. 312). It is impossible to decide which of its modifications is the 'typical' one.

The natural state of delicate organisms can often not even be ascertained by investigations on the spot (cf. p. 460). Small forms can often only be found after the natural mixed assemblage has rearranged itself and recovered from disturbance. The changes, which have meanwhile taken place, remain unknown. Material from a healthy culture in a state of multiplication is often more suitable for investigation.

These considerations apply quite particularly to the species of *Sphaerotilus* where the distinction between typical and modified forms is entirely problematic. A considerable number of different modifications of *S. natans* have been described as so many genera, no one of which has the prerogative of being regarded as 'the type' or of being either the prevalent form or representing an especially healthy state under natural conditions. Kützing's name has to be used solely on grounds of priority. Such a marked capacity for modification may be infrequent, but similar examples are likely to be found among the remaining Chlamydobacteriaceae and may also be expected in various groups of flagellates and algae.

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* Not seen.

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