ON THE DECOMPOSITION OF CELLULOSE BY AN AEROBIC ORGANISM (*SPIROCHAETA CYTOPHAGA*, N. SP.).

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THE processes leading to the decomposition of cellulose and related substances in the soil present many features of considerable theoretical and economic interest. As is well known, cellulose displays a remarkable inactivity towards the simpler solvents, but under the action of strong acids it gives rise to the formation of less complex carbohydrates, sugars, such as cellobiose, dextrose, etc. On the other hand, certain organisms possess the faculty of attacking cellulose not only with great rapidity, but they also appear to do so with the formation of products which are not encountered in the purely chemical reactions. From the standpoint of soil fertility, the question acquires considerable importance in view of the enormous quantities of cellulose and its derivatives which, year by year, find their way into the soil in the form of plant residues and organic manures. As our acquaintance with the various soil processes extends it is becoming more and more evident that the inter-relations between carbon and nitrogen are not only exceedingly complex, but are also vital in their effect on the crop producing power of the soil.

The practical significance of some of these changes has already been discussed in an earlier paper[1] in which it was shown that by supplementing the amount of readily decomposable organic matter in the soil quite appreciable changes in the nitrogen content and in the fertility of the soil may be induced. In the present paper we propose to confine ourselves to the consideration of the processes whereby an inactive carbon

[1] Hutchinson, H. B., This *Journal*, 1918, **9**, 92–111.

compound—in the form of cellulose—becomes potentially active by its resolution into simpler products.

Although for many years the typical fermentation of cellulose was regarded as being an essentially anaerobic one, and due to the methane and hydrogen producing organisms described by Omelianski[1], it is nevertheless an indisputable fact that many of the changes in nature indicate the operation also of an aerobic process.

In this connection may be mentioned the known differences in behaviour of coarse sandy, and of clay, soils in their capacity to retain reserves of organic matter and humus. The former are reputedly "hungry," since decomposition changes proceed in them at the maximum. On the other hand, close textured or clayey soils present conditions which are less suitable for the degradation of organic matter. Apart from the physical constitution of the soil, the presence of excessive moisture, by the exclusion of air, also makes for less rapid decomposition; soils in districts of high rainfall, or those subject to flooding by surface springs, are generally characterised by a high content of undecomposed matter or crude humus. From the fact that cultivation and aeration of a soil lead to a reduction of organic matter, and that the recent work of Russell and Appleyard[2] shows that the composition of the free soil atmosphere differs only slightly from the normal, some direct relationship between decomposition and air supply may be deduced.

The first observations on the aerobic decomposition of cellulose appear to have been made by C. van Iterson[3], in Delft, who found that when a medium consisting of paper and mineral salts was distributed in shallow layer, inoculated with ditch mud, and incubated at 28°–35°, an energetic decomposition of the paper took place. In addition to the various organisms, sporogenous bacteria, spirilli and infusoria, that developed under these conditions, two others were encountered, the one being a very small rod-shaped organism, to which the name *Bacillus ferrugineus* was given, and the other a large micrococcus which was regarded as being unable to decompose the paper itself but which facilitated the decomposition in some manner not precisely indicated.

Decomposition of filter paper was further demonstrated by placing, in a glass dish, two pieces of Swedish filter paper between which some powdered ammonium magnesium phosphate had been introduced and

[1] Omelianski, W., *Compt. rend.* 1895, **121**, 653–655; 1897, **125**, 970–973, 1131–1133. *Arch. d. Sci. Biol.* 1899, **7**, 411–434. *Cent. Bakt. Par.* II. 1902, **8**.

[2] Russell, E. J. and Appleyard, A. This *Journal*, 1915, **7**, 1–48.

[3] Iterson, C. van, *Cent. Bakt. Par.* II. 1904, **11**, 689–698.

moistened with a 0·05 per cent. solution of di-potassium hydrogen phosphate. The paper was infected by means of a suspension of garden soil, ditch mud, humus or dry leaves, and incubated at 24°–28°. After four to five days yellowish brown spots were produced on the paper which, at a later stage, became pulpy owing to the growth of the two organisms referred to above, until finally the individual fibres of the paper were enveloped in a "micrococcus mucilage." Van Iterson states, however, that decomposition of the paper could never be obtained by the use of such pure cultures as he was able to isolate from decomposing paper. His reference to the "micrococcus mucilage" encountered in crude cultures is interesting in its relation to our own results, which are given below. Van Iterson was also able to demonstrate the occurrence of a considerable number of filamentous fungi which are capable of utilising filter paper as a source of carbon.

At a later date Christensen[1] suggested the use of the cellulose decomposing power of a soil as an index of soil fertility. According to the amount of change undergone by strips of filter paper when reposing on the surface of different soils, the latter were placed in one of five grades—from 4 to 0—of biological potency.

The first systematic investigations on aerobic cellulose decomposition were those initiated by Kellerman and McBeth[2] and subsequently continued by McBeth and Scales[3]. The former paid particular attention to the technique of cultivation and in this connection suggested the use of a number of special media such as cellulose agar, potato agar, starch agar, and an agar containing dextrose, mineral salts and ammonium sulphate. By the preliminary use of "elective" cultures, and plating out on the above media, they succeeded in isolating a number of organisms of which three, *Bacillus rossica*, *Bacillus amylolyticus* and *Bacterium flavigena*, were derived from cultures of anaerobic cellulose decomposing organisms obtained from Omelianski. McBeth and Scales examined soils from widely separated regions of the United States and isolated therefrom eleven species of bacteria while an additional four species were obtained from other sources. All these organisms are morphologically and physiologically distinct from Omelianski's hydrogen and methane organisms and grow well on ordinary gelatine media, although continued cultivation on such media is rapidly followed by loss of cellulose destroying power. None of these species produced gas

[1] Christensen, H. R., *Cent. Bakt. Par.* II. 1910, **27**, 449–451.

[2] Kellerman, K. F. and McBeth, I. G., *Ibid.* 1912, **34**, 485–494.

[3] McBeth, I. G. and Scales, F. M., U.S. Dep. Agr., Bureau Plant Ind., *Bull.* 266, 1913.

in peptone-carbohydrate broth, but the majority (four exceptions) formed more or less acid from sugars, starch and higher alcohols. With some species the principal by-products were found to consist of formic and acetic acids, while others only gave rise to traces of fatty acids. None of the solutions examined was found to contain any trace of aldehydes, ketones, alcohols, or of carbohydrates capable of reducing Fehling's solution.

Concurrently with these American investigations, the question of cellulose fermentation had been taken up by Löhnis and Lockhead[1], who employed an agar medium consisting of mineral salts, sodium nitrate and 0·3–0·5 per cent. chemically pure cellulose. The paper partakes of the nature of a preliminary communication; so far as we are aware, a description of the causative organisms of fermentation has not been published.

Experimental.

Preliminary experiments to demonstrate the presence of aerobic cellulose decomposing organisms in Rothamsted soils presented practically no difficulty, the usual procedure being to prepare flat bottomed 300 c.c. flasks containing 100 c.c. of mineral salt solution[2], 0·25 grm. sodium nitrate, and 1·0 grm. filter paper. Before sterilisation, the latter was orientated so that it reposed in direct contact with the sides of the flask, with its upper portion protruding above the level of the culture liquid. Inoculation of these flasks was carried out by the introduction of about one gram of field or garden soil. After incubation at 25° for upwards of 4–6 days, the filter paper at, or slightly above, the level of the liquid assumed a yellowish or yellowish-brown colour and gradually lost its consistency. Although it was possible to reproduce this change repeatedly by transference of some of the decomposing paper to further flasks, such elective culture failed to yield a culture which possessed any high degree of purity, and it was decided, therefore, to attempt some method of plating out.

Prior to the publication of Kellerman and McBeth's paper, two methods were in general use. The first of these consisted in the use of an agar medium composed of mineral salt solution, with 0·25 per cent.

[1] Löhnis, F. and Lockhead, G., *Cent. Bakt. Par.* II. 1913, **37**, 490–492.

[2] The stock mineral salt solution used was the nitrogen-free solution given by Meyer (*Practicum d. botan. Bakterienkunde*, Jena, 1903, p. 15) and had the following composition: 1·0 grm. KH_2PO_4, 0·1 grm. $CaCl_2$, 0·3 grm. $MgSO_4 + 7H_2O$, 0·1 grm. NaCl, 0·01 grm. Fe_2Cl_6, 1000 grm. H_2O.

sodium nitrate and 1·5 per cent. agar; this stock agar was tubed in portions of 10 c.c. and sterilised in the autoclave. A number of such sterile portions were then poured into a like number of Petri dishes and, after consolidation of the medium, a circular piece of sterile filter paper was laid and pressed upon the surface of the agar. Fresh portions of the melted agar were then inoculated with various dilutions of the culture to be plated out and were then poured in as thin a layer as possible over the surface of the paper. In this manner plates were obtained which on incubation for upwards of a week showed quite strong bacterial growth, the paper became covered with bright yellow spots (colonies), and eventually became transparent owing to the complete dissolution of the paper (Plate I, fig. 1).

Although otherwise satisfactory growth of the causative organisms could be obtained in this manner, this method of preparing plate cultures was abandoned; in the first place, because the colonies uniformly gave what were regarded as impure growths, and, secondly, on account of the fact that the colonies resulting from the inoculation of any one plate appeared to bear little relation to the number of organisms actually used as inoculant.

The second method consisted in the preparation of a suspension of cellulose by grinding up filter paper with coarse sand and water. By this means a quantity of finely divided cellulose was obtained and made into agar similar in composition to the preceding one. The preparation of cultures with this medium gave, after upwards of 8–10 days, plates showing a number of zones in which, by the growth of the organism, the cellulose had become partially or wholly dissolved (Plate I, fig. 2). Microscopical examination of plates from crude cultures revealed the presence of numerous surface and embedded colonies which, however, had no obvious connection with the zones of change, and which on transference to filter paper tubes did not result in characteristic breakdown of the paper. Microscopical examination of the agar of the zones was made and showed the presence of two chief cell forms which have also been invariably found in cultures capable of inducing active and typical decomposition. One of these forms, usually referred to as the "thread" form, was found in abundance in young cultures on agar or on paper, and consisted of a fairly long thin filamentous and frequently curved cell, that was stained with difficulty by ordinary methods. The second form was that of a large "coccus" which, particularly in old cultures, occupied a predominant position.

From time to time we were able to obtain colonies on cellulose agar

plates which, under the microscope, appeared to consist wholly of the filamentous organism, but inoculation to fresh medium and incubation for a few days resulted, without exception, in the incidence of the coccus form.

Despite the preparation of a considerable number of subcultures and the use of widely different media[1], we have been unable to effect a permanent separation of these two forms, nor has the employment of the media suggested by Kellerman and McBeth, whose paper appeared about this time, been followed by any greater measure of success.

Various attempts were made to set up conditions which might conceivably favour one of the forms at the expense of the other. Experiments were carried out, for example, in which tubes containing filter paper and mineral salts were inoculated with a culture of the two forms and then subjected to a range of temperatures varying by 2° from 40° to 62°. After seven days, growth occurred in all tubes subjected to temperatures of 40°–56° for ten minutes, while after nine days growth was also evident in the tube heated to 58°. None of the tubes showed a pure culture either of the thread or coccus form.

Similarly, attempts to obtain simplification of the flora by differential reaction of the media yielded negative results. Treatment of crude cultures with volatile antiseptics was equally ineffective in bringing about separation of the two forms. Chloroform, toluene, and carbon bisulphide all had the effect of destroying both the filamentous and the coccus forms. On the assumption that, on plating out, simple coherence of the two forms occurred, mechanical dissemination of the cultures by means of an atomiser and exposure of cellulose agar plates to the spray was tried. In this manner we obtained characteristic colonies, but none of these was found to be pure. We were, therefore, inclined to regard the mutual relationship of these two forms as strictly symbiotic —neither of the forms being able to grow in the absence of the other. It was obviously impossible under these conditions to ascertain which of the forms was the one actually responsible for the observed changes in the filter paper, and the investigation was, consequently, temporarily abandoned.

[1] The media tested and found unsuitable for the growth of the cellulose decomposing organisms, or which failed to allow of separation of the two forms, include nutrient gelatine and nutrient agar with and without dextrose, sodium nitrate-mineral salt-cellulose agar, sodium nitrate-dextrose agar, soil extract-cellulose agar, with the addition of 1·0 per cent. of the following sources of nitrogen—asparagin, ammonium citrate, peptone, sodium ammonium phosphate, potassium nitrate: Kellerman and McBeth's cellulose agar, dextrose agar, starch agar, and the numerous solutions suggested by Meyer (*loc. cit.* p. 24) for diagnostical purposes.

Subsequent work with cellulose agar plates resulted in the production of colonies which agreed macroscopically with those previously obtained, but microscopically appeared to consist solely of the "thread" form. Inoculation of cellulose agar slant tubes and incubation for five days was, however, followed by the occurrence of the coccus form again. Hence it was assumed that isolated cells of the latter form had eluded search under the microscope.

Having occasion at a later date (1915) to prepare tubes and plates of the so-called mixed culture, it was evident that growth in liquid culture with filter paper was not only more vigorous but arose in much less time than was the case on agar plates. It was accordingly decided to prepare subcultures by the dilution method, and to this end a set of test tubes was prepared, each tube containing a piece of filter paper 15 × 60 mm. in size, and 5 c.c. of stock mineral salt solution containing 2·0 grm. of sodium ammonium phosphate per litre. In this as in all subsequent sets the reaction of the mineral salt solution was first brought to the neutral point with phenolphthalein and then to each 100 c.c. of solution 1·0 c.c. of N/10 sodium hydroxide solution was added.

The requisite attenuations were made in physiological salt solution and ranged down to 1×10^9. By means of sterile pipettes one drop ($\frac{1}{20}$th c.c.) of a particular attenuation was introduced into each of six tubes set aside to receive this attenuation.

After incubation for four days at 30°, growth was evident in all tubes which received attenuations up to 1×10^7. Of the six tubes receiving the next higher dilution, viz. 25–30 inclusive, tubes 26, 27, 28 and 29 showed the characteristic colour and growth on the filter paper, while examination of tube 29 showed it to contain what was apparently a pure growth of the thread form. As an alternative to incubating the culture for a further period, thus giving opportunity for the development of any occasional cell of the coccus form that might be present, it was decided to prepare a fresh dilution set on similar lines. This was completed in less than an hour and, in addition, the culture was transferred to ordinary filter paper tubes. Incubation for a further period of four days served to induce growth in the dilution tubes as high as tube 23 but it was then evident on microscopical examination that all the tubes contained a mixture of the "thread" and "coccus" forms. In view of the fact that the inoculant used for this set of cultures contained apparently a pure thread form and could most certainly not have contained anything approaching the number of coccus forms indicated by this second dilution set, viz. 40,000,000 per c.c., the conclusion was

unavoidable that there existed a vital and intimate connection between the two forms. Subsequent dilution cultures as well as direct examination under the microscope confirmed this view, and resulted in observations which are given in detail below. On account of the peculiar developmental cycle through which the organisms appear to pass, as well as on other grounds, we are unable to regard it as a representative of the true Bacteriaceae. In form (especially when grown in liquid media), lack of flagella, and perfect flexibility of cell, it approaches more closely to the Spirochaetoideae and we suggest therefore the name *Spirochaeta cytophaga*. While doing so we recognise that the organism under consideration exhibits a number of features which have not hitherto been observed in the spirochaetes, features which however appear to indicate a more complex development than that of the true bacteria.

Morphology of *Spirochaeta cytophaga*.

Young cultures of *S. cytophaga* invariably exhibit the predominance of long thin frequently incurved cells, which consequently do not permit of measurement with any degree of accuracy. In stained preparations the mean dimensions of the cells have been found to lie in the region of 3μ along the major axis, while the mean diameter is from $0{\cdot}3$–$0{\cdot}4\mu$. Under certain cultural conditions extremely long twisted filaments are obtained, the length of which may extend to upwards of 40μ. The form assumed by these filaments depends largely on the method of fixing which is adopted; if the smears are allowed to dry at room temperature before being fixed in the flame the majority of the cells will be found to be much curved and to have taken up the shepherd's crook, **S** and **U** forms shown in Plate I, fig. 3. If, on the other hand, the film be dried by a short exposure to the flame the filaments are as a rule less bent and are frequently perfectly straight. Cultivation of the organism in liquids in which the cellulose is completely immersed appears to induce the highly undulating sinuous form shown in Plate I, fig. 4.

In older cultures, a predominant position is occupied by the ovoid or spherical form, the dimensions of which are approximately $1{\cdot}5\mu$ diameter. Owing to the fact that this form cannot be regarded any longer as an infection form it is considered desirable to abandon the use of the term "coccus," which might conceivably lead to a certain amount of confusion. Our observations show that this spherical form possesses little analogy with the true bacterial spore while, on the other hand, the use of the term "cyst" might imply more than is perhaps warrantable

at the present time. We therefore propose to refer to it for convenience as a "sporoid" stage until more information respecting the affinity of this to other organisms is obtained.

Motility. Evidence of independent movement of the filamentous form may be obtained from observation in hanging drop, although care must be taken to avoid appreciable changes in temperature. Organisms which are attached to portions of cellulose fibre display a slow rotatory movement, while occasional cells proceed with a sinuous action through the culture liquid. Occasionally, on the extreme edges of the drop the central portion of the filament remains stationary but the ends are reflexed so that, according as to whether they move in the same or opposite directions from the major axis, the outline of the cell assumes an **O** or **S** shape. The filamentous form, therefore, possesses perfect flexibility. Up to the present, we have been unable to demonstrate the presence of flagella.

Staining reactions. S. cytophaga generally takes up the conventional bacteriological stains with comparative tardiness. Methylene blue, while failing to give any satisfactory result with the filament form, gives faint staining of the sporoids. The organism reacts negatively towards Gram's stain and does not respond well to the action of dilute fuchsin. The most satisfactory results obtained hitherto have been by the use of hot carbol fuchsin, for upwards of a minute, without subsequent differential treatment. Bold preparations may also be secured by a "deposition" stain, i.e. either flooding the film with weak alcoholic fuchsin, and after tilting the slide to remove the superfluous stain, allowing the remaining solution to dry on the film or, alternatively, by placing a cover-glass on the film, flooding the intermediate space with the stain, and then quickly raising the cover-glass from one side. By this means small quantities of solution are left in the immediate vicinity of the cells, on which the residual stain is ultimately deposited. The staining capacity of the organism varies greatly, however, with the cultural conditions and occasionally even the most intensive methods fail to yield satisfactory results.

Spore formation. Spore formation in the ordinary sense of the term, i.e. the production of a stage which possesses greater powers of resistance to external factors, does not appear to take place with *S. cytophaga.* Any other interpretation of the function of a spore as, for example, the contention that spore production is nothing more or less than the adoption of a resting stage, is difficult of application to the organism under consideration. Although there is abundant evidence that the

filamentous form predominates in the early stages of growth, and the spherical form occurs more abundantly in older cultures, it is quite unjustifiable to deduce from this that the "sporoid" constitutes a resting stage or that it is in any degree physiologically less active than the earlier form.

Aerobism. *S. cytophaga* is essentially aerobic in character, and practically the only decomposition of cellulose occurs at or slightly above the level of the culture solution. In those cases where the cellulose sinks to the bottom of a comparatively shallow layer of liquid as, for instance, occurs with culture solution containing precipitated cellulose, growth proceeds with extreme slowness.

Developmental cycle of S. cytophaga. The construction of the developmental cycle of *S. cytophaga* is associated with difficulties which up to the present we have not been able entirely to overcome. In the first place, the most satisfactory method of procedure is admittedly the adoption of the hanging-drop culture method in which continuous observations of the development of any specific organism may be made. Unfortunately, and in spite of a considerable amount of work with a large number of media, we are still without a synthetic medium on which the organisms can be well isolated and which permits of growth observations. The use of cellulose, either as fibre or in the form of a precipitate, is obviously unsuitable on account of physical interference with the image of the organism. From time to time attempts have been made to employ the Indian ink method, but without any degree of success. On the other hand the diminutive size not so much of the cell itself but of the cell structures—which apparently undergo a series of changes—renders it difficult even with the use of the highest magnifications to obtain any definite idea of the exact sequence of the changes which proceed in active cultures.

Under these circumstances the cycle of development outlined below is only submitted tentatively and is therefore subject to such revision as future experience may indicate. As far as possible photomicrographs have been secured of typical intermediate stages between the thread and the sporoid forms and some of these are given on Plates I–III. It may also be added that in those instances where a particular cell exhibits noteworthy features, the photographs are so arranged that such cells are below the centre and in line with the vertical axis of the plate.

Two types of growth of *S. cytophaga* appear to be distinguishable. The first may be termed the direct, or purely vegetative, growth whereby new cells are formed by transverse fission, and which proceeds extensively

in young cultures. The second may be referred to as indirect and possibly generative growth in which both cell and cell contents appear to undergo a definite series of changes.

The predominating form in young cultures will generally be found to consist of simple, sinuous filaments the length of which is about ten times the diameter. Carbol fuchsin is fairly readily and uniformly taken up and shows the slightly tapering ends of the cell. With increasing age there occur a number of cells which begin to show differentiation of the cell structures, the terminals becoming less intensely stained, while the cell contents assume the form of a densely stained equatorial band (Plate I, fig. 6) or may take up the spherical form (Plate II, figs. 2 and 5). The nuclear substance then presumably takes up a transverse position and finally undergoes division which is accompanied by a constriction of the cell wall. During the latter phases the cell itself has become appreciably thicker and less filamentous, while owing to almost complete indifference to the action of the stain, the cell wall is perceptible as little more than a "shadow" form (Fig. 1 below). After further constriction the two halves of the cell apparently become detached, the resultant oval or ovoid cells each containing a plate or disc of nuclear substance which remains attached to the cell wall when the spherical or sporoid stage is eventually reached. Finally, the sporoid—which up to this has exhibited comparatively slight staining capacity—becomes evenly and intensely stained, presumably owing to a dispersion of nuclear substance, and gives rise to the thread form. This, therefore, must be accepted as being merely our interpretation of the differences of cell form and cell contents which may be observed in stained preparations of cultures of different ages. It is, of course, fully recognised that heat fixation is objectionable on account of the risk of distortion of nuclear figures or of chromatin substance, and that all structures which take up fuchsin are not necessarily of the nature of nuclear substance. It may be mentioned, however, that identical appearances are to be obtained by formaldehyde-alcohol fixation and the use of stains such as those of Leishman and Geimsa.

In the figure on p. 154 the main intermediate stages between the filamentous and the sporoid forms of *S. cytophaga* are diagrammatically represented, the most frequently occurring types being arranged in horizontal order from left to right, while a few of many divergent examples are grouped both above and below.

Of these variations from the normal may be mentioned the tendency towards the production of highly sinuous forms in liquid media, while

cultivation under unfavourable conditions is frequently followed by intense granulation of the thread form. This is evident, for example, in cultures containing an excess of nitrogen or, in fact, of soluble organic substances generally—nutrient beef agar, 1·0 per cent. peptone, 0·5 per cent. asparagin, and also in dilute solutions of phenol, etc. The most pronounced changes of this type were observed in cultures which, in error, had been subjected to a temperature of 36°–37° for some days. The culture that actually underwent this exposure was characterised by low stain receptivity, the majority of the cells showing phases such as are represented in Plate II, figs. 2 and 3. On transference of this

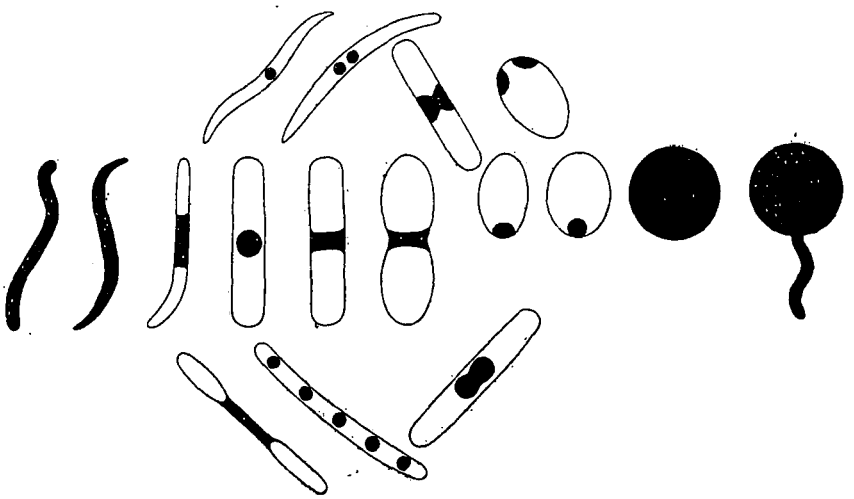


Fig. 1.

culture to a lower temperature (25°) and especially on inoculation to fresh medium, almost all the filamentous forms exhibited strong granulation, and this characteristic persisted for at least two generations after the culture had ceased to be under unfavourable conditions (Plate III, fig. 6). Well-marked granulation is of interest morphologically, in that it imparts to the cell an appearance somewhat similar to that found by Leishman[1] to be possessed by spirochaetes giving rise to the formation of "coccoid granules." There is no evidence however that these granules of *Spirochaeta cytophaga* have any special significance.

A further divergence from the normal course appears to be the formation of a pre-sporoid stage possessing either double granules or a band of nuclear substance (Plate II, figs. 3 and 4). The general for-

[1] Leishman, W. B., *Trans. Soc. Trop. Med. Hyg.* 1910, **3**, 97.

mation and persistence of sporoids tend to become accentuated with increasing age of the culture until, at the end of two to three weeks, the whole of the bacterial mass and also of the partially decomposed cellulose fibres appear to consist exclusively of micrococci. This was, no doubt, the phase observed by van Iterson, who refers to the fibres as being embedded in "micrococcus mucilage."

Until the organism has been subjected to further study by the use of special cytological methods it would be premature to express any opinion as to the significance of the changes which the organism undergoes. It is hoped that sufficient evidence has been already adduced to indicate the complexity of these changes.

PHYSIOLOGICAL.

On account of the difficulty of separating the filamentous and the sporoid forms of the organism and, incidentally, of establishing their relationship to one another, we recognised at an early date the desirability of obtaining some synthetic medium suitable for the preparation of hanging-drop cultures and on which continuous observations of the organisms would be possible. With this end in view it was decided to investigate systematically the relative value of various forms of nitrogen and carbon.

As is shown by the results of the experiments reported below, *Spirochaeta cytophaga* is markedly specific in its nutritive requirements; at the same time it displays extraordinary sensitiveness towards the presence of compounds which it is apparently unable to utilise but which with the general run of bacteria have been found to serve as sources of nitrogen or carbon or both—peptone, amino-acids, sugars, salts of organic acids, etc. Since the organism invariably grows well in mineral salt solution with cellulose, the general method adopted in systematic tests was to prepare a range of test tubes having the same basal mineral salt solution, but with different, and sometimes increasing, amounts of carbon or nitrogen compounds. According as to whether cellulose was also present or absent, it was possible to ascertain (*a*) inhibitive effects, and (*b*) nutritive values of the compound tested.

In the summary of these experiments recourse is had to tabular representation of the results; the individual cultures are arranged as a rule in order of gradually increasing concentration, while the amount of growth or, where present, the destruction of cellulose is indicated as $-$ = lacking, $-+$ = very faint, $+$ = distinct, $++$ = fairly strong, and $+++$ = vigorous.

Relations to Sources of Nitrogen.

Early attempts to obtain pure cultures of *Spirochaeta cytophaga* by means of the conventional media sufficed to demonstrate the unsuitability of such media as nutrient agar and nutrient gelatine with and without the addition of dextrose. The invariable result was that while any organism resembling *S. cytophaga* failed to grow, various contaminating forms were secured which, on transference to cellulose media, were found to be incapable of bringing about any dissolution. The absence of cellulose from these media might, of course, have contributed materially to these negative results, but later experience indicated that ack of growth was more probably attributable to (*a*) the concentration of soluble compounds and (*b*) the unsuitability of the more complex nitrogen compounds as sources of nitrogen.

Although the use of nutrient agar had, therefore, to be abandoned, various attempts have been made to induce growth of the organisms on this medium, and in this respect the following may be mentioned. Negative results were obtained when agar plate cultures were made from dilutions of 1/200, 1/40,000 and 1/4,000,000 of active culture, the organisms being evenly distributed in the usual manner. Equally bad results followed the transference of *loopfuls* of active culture to the surface of nutrient agar plates. When *masses* of decomposing filter paper were transferred to the surface of nutrient agar plates, slow growth of the organism or, at least, continued enzymic action was evident. The paper was gradually resolved and gave rise to a semi-translucent bacterial mass, yellow in colour and of mucilaginous consistency. The bacterial filaments became highly granular and apparently suffered in vitality. Transference of portions of this bacterial mass to fresh nutrient agar was not followed by growth, thus showing that the capacity of growth without cellulose had not been acquired.

The relations of the organism towards peptone have been investigated and the results are given in Tables I and II. In the preliminary set (Table I) a short range of concentrations was tested, in all of which growth occurred with the exception of 1·0 per cent. solution. As it was possible that this solution had not received its proper amount of inoculant, several reinoculations were made with active culture from the next lower concentration but these also failed to give rise to growth. There was thus no acclimatisation to higher concentrations.

The second series included a set of tubes without cellulose to test the capacity of the organism for growth on peptone.

TABLE I.

Growth of S. cytophaga *in Peptone Solution with Cellulose.*

	Concentration of Peptone Solution (per cent.)				
	0·0001	0·001	0·01	0·1	1·0
Growth	+ +	+ +	+ +	+ +	–

TABLE II.

Growth of S. cytophaga *in Peptone Solution with and without Cellulose.*

Growth after 8 days	Concentration of Peptone Solution (per cent.)								
	—	0·005	0·010	0·025	0·050	0·10	0·25	0·50	1·0
Without cellulose	–	–	–	–	–	–	–	–	–
With cellulose and ammonium salts	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+	– +	–	–

The unsuitability of peptone as the sole nutrient is well brought out, the organism failing to show growth in any of the various concentrations tested. In the presence of filter paper strong growth occurred up to the 0·025 per cent., but marked inhibition set in with 0·25 per cent. solution.

Comparison of simpler nitrogen compounds. Throughout the cultural work in connection with *S. cytophaga* the superior value of inorganic forms of nitrogen has been well demonstrated, and a comparison of these is afforded by Table III. Proceeding from a basis of normal mineral salt solution and filter paper, tests were made with increasing amounts of sodium ammonium phosphate, ammonium phosphate, sodium nitrite and sodium nitrate.

TABLE III.

The Relative Value of Inorganic Nitrogen Compounds.

Growth after 6 days	Concentration of salt solution (per cent.)									
	0·004	0·009	0·018	0·037	0·075	0·15	0·31	0·62	1·25	2·50
Sod. Amm. Phosph.	–	–	– +	+	+ +	+ +	+ +	+ +	+	–
Amm. Phosph.	–	– +	+	+ +	+ +	+ +	+ +	+	–	–
Sod. Nitrite	– +	+	+	+	+ +	+	– +	–	–	–
Sod. Nitrate	–	+	+ +	+ +	+ +	+ +	+ +	+ +	+	–

The results obtained after three days' incubation were on the whole similar to those given above, except that with longer incubation the growth was stronger and that the critical concentration showed a general displacement to the higher levels. It may be assumed that the

lower limits of growth are probably associated with minimum nitrogen requirements, since the more highly nitrogenous salts give better growth than sodium ammonium phosphate. On the other hand, the upper limits of growth do not appear to be determined by nitrogen concentration, since sodium nitrate gives as good results as sodium ammonium phosphate, although it contains about 2½ times as much nitrogen. It is also to be expected that the favourable effect of sodium nitrate is a complex one, i.e. with increasing abstraction of nitrogen the reaction of the solution would be better maintained owing to the formation of sodium carbonate or of neutral salts of acid by-products.

A supplementary set of experiments included the use of a number of the simpler organic nitrogen compounds which, from their constitution, might be expected to serve as sources of nitrogen. Two concentrations were tested, viz. 0·5 and 0·05 per cent., the general method being to add the test solution to the slightly alkaline mineral salt solution after sterilisation, thus reducing the risk of decomposition of the compound. The results are given below (Table IV).

The majority of the compounds are thus suitable for growth, although their values are widely different. The behaviour in urea is peculiar and requires further investigation, but from the paucity of growth in the initial stages and the abundance of growth after 40 days it might be assumed that acclimatisation to the compound took place, or that the power of hydrolysing the urea was eventually acquired. The results with hydroxylamine sulphate and hydrazine sulphate are in conformity with the frequently observed intolerance of *S. cytophaga* of any reducing substances. Acetamide and asparagin give poor results in low concentration but fairly good effects when present to the extent of 0·5 per cent. Formamide, on the other hand, is apparently of value only in low concentrations (0·05 per cent.), and acts inhibitively in strengths approaching 0·5 per cent.

Relations to various Sources of Carbon.

The carbon requirements of the constituent species of the soil flora may be met by a considerable range of compounds—from carbon dioxide through the paraffins, alcohols, fatty and hydroxy-acids, and the various monoses and bioses. Of these, such compounds as glycerol and mannite, malic, citric, succinic and tartaric acids, dextrose, maltose and saccharose are of the most general utility. At the same time several of the species exhibit a high degree of specificity as, for example, the nitrifying organisms and some of the sulphur bacteria. On this account, and in

TABLE IV. *The Relative Value of some Simple Organic Nitrogen Compounds.*

Time	Concentration	Sod. Amm. Phosphate	Ammonium tartrate	Hydrazine sulphate	Hydroxylamine sulphate	Sodium nitrate	Ethyl nitrate	Formamide	Acetamide	Succinamide	Carbamide	Asparagin	Tyrosin
5 days	0·05 %	+	+	−	−	+	+ −	+ −	−	+	−	+ −	−
	0·50 %	+	−	−	−	+	−	−	+	+	−	+ +	−
10 days	0·05 %	+ + +	+ +	−	−	+ + +	+ −	+	−	+	−	+ −	−
	0·50 %	+ +	−	−	−	+ +	+	−	+	+ +	−	+ + +	−
15 days	0·05 %	+ + +	+ +	−	−	+ + +	+ −	+ +	−	+ +	−	+ −	−
	0·50 %	+ + +	−	−	−	+ +	+ +	−	+	+ +	−	+ + +	−
40 days	0·05 %	+ + +	+ +	−	−	+ + + +	+ −	+ + +	−	+ + +	+ +	+	−
	0·50 %	+ + +	+ +	−	−	+ + + +	+ +	−	+	+ +	+ + +	+ + + +	−

TABLE V. *The Relative Effects of Carbohydrates, etc. on Growth of* S. cytophaga.

Growth after 12 days	Concentration	Mannite	Dulcite	Adonite	Arabinose	Dextrose	Levulose	Galactose	Saccharose	Maltose	Lactose	Raffinose	Starch	Dextrin	Inulin
Without cellulose	0·10 %	+	+	+	−	−	−	−	+	−	+ −	+	+	+ −	+
	1·00 %	+	+	+	−	−	−	−	+	−	−	+	+	+	+
With cellulose	0·10 %	+	+	+	−	−	−	−	+	−	+ −	+	+	+ −	+
	1·00 %	+	+	+	−	−	−	−	+	−	−	+	+	+	+

view of the lack of success with nutrient dextrose agar and with dextrose mineral salt agar, it was considered desirable to ascertain what position *S. cytophaga* would assume in this respect.

The first series of experiments included a range of higher alcohols and carbohydrates some of which have been found useful for general diagnostical work and possess a high nutritive value for bacterial growth. Tests were made in the first instance with two concentrations, viz. 0·1 and 1·0 per cent. Cultures were made with and without cellulose in order to give (*a*) inhibitive, and (*b*) nutritive effects. The results of this series are given in Table V.

From these, a number of interesting points emerge. Of the fourteen compounds tested, none appears to be capable of meeting the carbon requirements of the organism either in 0·1 or 1·0 per cent. concentration. While unsuitable for growth, they evidently differ greatly in inhibitory power, since decomposition of the cellulose proceeded normally in some, but was completely absent in others. This disparity which, at first sight, might appear to be somewhat inexplicable, permits of an explanation on the basis of the chemical behaviour of the various compounds. All those compounds which possess reducing properties, e.g. which induce reduction in Fehling's solution, are shown to exert marked inhibitory effects on the organism. The behaviour towards carbohydrates is, therefore, similar to that previously observed with nitrite, hydrazine and hydroxylamine. Whether this effect is directly connected with an interference of the aerobic requirements of the organisms or of some specific enzymic change must be left open, but it may be mentioned that the contrary effect—stimulation of the anaerobic organisms—has been observed on the addition of such reducing substances as dextrose, sodium formate, pyrocatechin, sodium hyposulphite, etc. to nutrient media[1].

In our experiments saccharose, raffinose, starch, inulin and the higher alcohols are non-toxic even in 1·0 per cent. solution; subsequent tests with gum arabic gave the same result. Dextrine is toxic in high, but non-toxic in low, concentrations, while all the rest with the exception of lactose cause inhibition even in 0·1 per cent. strength. Since the two concentrations chosen for the experiment were more or less arbitrary, two further tests were carried out. The first consisted in the use of dextrose in the presence of cellulose and indicates that inhibition occurs when the concentration exceeds 0·05 per cent.

[1] Kitasato, S. and Weyl, T., *Zeitsch. Hyg.* 1890, **8**, 41. Beijerinck, M. W., *Verhand. d. konink. Akad. Wetensch.* Amsterdam, 1893.

TABLE VI.

Inhibitory Effect of Dextrose on the Growth of S. cytophaga *in the Presence of Cellulose.*

Growth after	Concentration of solution (per cent.)								
	Control	0·005	0·01	0·025	0·050	0·10	0·25	0·50	1·00
3 days	+ +	+ +	− +	− +	−	−	−	−	−
8 days	+ + +	+ + +	+ + +	− +	− +	−	−	−	−
12 days	+ + +	+ + +	+ + +	+ + +	+ +	−	−	−	−

A further series was designed to show the difference in inhibitory power of two bioses, viz. saccharose and maltose, in the presence of cellulose. In the following table the records refer to actual growth but not to intensity of growth as in the other series.

TABLE VII.

Relative Inhibition of Growth by Saccharose and Maltose in the Presence of Cellulose.

		Concentration of solution (per cent.)										
		0·004	0·009	0·018	0·037	0·075	0·15	0·31	0·62	1·25	2·50	10·00
Saccharose	4 days	+	+	+	+	+	+	+	−	−	−	−
	6 „	+	+	+	+	+	+	+	+	+	−	−
	9 „	+	+	+	+	+	+	+	+	+	−	−
Maltose	4 days	−	−	−	−	−	−	−	−	−	−	−
	6 „	+	+	−	−	−	−	−	−	−	−	−
	9 „	+	+	+	−	−	−	−	−	−	−	−

The striking difference in effect of a reducing and a non-reducing sugar is well brought out, the critical concentration of maltose being in the region of 0·018 per cent., and of saccharose approximately 1·25 per cent., i.e. a ratio of 1 : 70. So far as we are aware, this specific susceptibility is quite unique.

Having determined the behaviour of the organism towards representatives of the carbohydrates, it was decided to proceed to an examination of the value of a number of organic acids as sources of carbon.

The general plan was similar to that adopted in the case of the carbohydrate series, i.e. cultivations were attempted in the absence and presence of cellulose. As we were in possession of a number of calcium salts of certain mono- and di-basic acids it was most convenient to carry out the tests with these. Unfortunately, the use of calcium salts introduces a serious complicating factor in that, as the solubility

of certain of the salts is very low, they tend to show only slight inhibitory powers.

TABLE VIII.

Relative Effects of Organic Calcium Salts.

Growth after 9 days			Concentration	Calcium formate	Calcium acetate	Calcium propionate	Calcium butyrate	Calcium oxalate	Calcium malate	Calcium tartrate	Calcium citrate
Without cellulose	...	...	0·1 %	–	–	–	–	–	–	–	–
			1·0 %	–	–	–	–	–	–	–	–
With cellulose	...	...	0·1 %	+	+	–	–	++	++	++	++
			1·0 %	–	–	–	–	–	–	+	++

From these experiments the conclusion appears justified, therefore, that representatives of the lower fatty acids, and of di- and tri-carboxylic acids, are equally unsuitable as sources of carbon as are the carbohydrates, since the organism completely failed to grow in any of the solutions in the absence of cellulose. In regard to their inhibitory power, both calcium formate and acetate are only slightly inimical in 0·1 per cent. solutions and this also appears to be the case with malate, oxalate, tartrate and citrate. This might at first be explained on the basis that the maximum solubility of the latter compounds is lower than the amount of the salt actually added, but this assumption makes it difficult to account for the obvious inhibition in the 1·0 per cent. solutions. It should be remembered, however, that the possibility of a slight amount of interaction with the constituents of the mineral salt solution and the formation of more soluble alkali salts is not entirely excluded. Both calcium propionate and calcium butyrate show inhibitory effects in 0·1 and 1·0 per cent. solutions.

The above work on the nutrition of the organism may be summarised as follows. The nitrogen requirements of *S. cytophaga* may be fulfilled by a range of nitrogen compounds—ammonium salts, nitrates, amides, amino-acids and peptone—provided that these, and especially the organic compounds, are not present in other than low concentrations. With peptone this is particularly the case, the critical concentration having been found to lie in the region of 0·25 per cent. Ammoniacal and nitric nitrogen are, on the whole, markedly superior to the other forms tested and may be supplied in much higher concentrations without inhibitory effects being introduced. *S. cytophaga* appears to be specific in its relation to carbon sources and its requirements are only met by cellulose. Not only are other compounds—higher alcohols, organic acids

and various carbohydrates—unsuitable for nutrition of the organism, but some, namely those compounds possessing marked reducing properties, have been found to be toxic even in very low concentrations, e.g. 0·018 per cent. maltose or 0·050 per cent. dextrose.

As regards the distribution and action of the organism in nature, it might be thought that, by virtue of its sensitiveness to cultural conditions, *S. cytophaga* would play a somewhat subordinate rôle. It is not improbable, however, that the conditions obtaining in the soil—aeration, presence of ammoniacal and nitric compounds and of plant residues, as well as the absence of appreciable quantities of soluble organic compounds —constitute ideal conditions for the growth of the organism.

In this relation to soil biological changes it may be of interest to set up a comparison, between *S. cytophaga* and the two other soil forms possessing monotropic relations to carbon compounds—the nitrous and nitric organisms. Such a comparison is made in Table IX, the data respecting the nitrifying bacteria being obtained from Winogradsky and Omelianski's paper[1].

TABLE IX.

Comparison of the Behaviour of Nitrosomonas, Nitrobacter and Spirochaeta cytophaga *towards Soluble Organic Compounds.*

	Inhibitory concentrations (per cent.)				
	Nitrosomonas		Nitrobacter		*S. cytophaga*
	min.	max.	min.	max.	max.
Sodium acetate	0·5	>1·5	1·5	3·0	>0·10*
Sodium butyrate	0·5	>1·5	0·5	1·0	>0·10*
Asparagin	0·05	0·3	0·05	>1·0	>0·50
Carbamide	>0·20	?	0·50	>1·0	>0·50
Peptone	0·025	0·20	0·8	1·25	0·25
Dextrose	0·025	0·20	0·05	0·2–0·3	0·05
Maltose	?		?		>0·018
Saccharose	?		?		>1·250

* Calcium salt used.

S. cytophaga is considerably more sensitive than either of the nitrifying bacteria, practically the sole exception being the high sensitiveness of Nitrosomonas to asparagin. The three organisms have certain common features such as their sensitiveness towards dextrose and their greater intolerance of butyrates than of acetates. It is somewhat remarkable that these three soil forms, two of which obtain their carbon as carbon

[1] Winogradsky, S. and Omelianski, W., *Cent. Bakt. Par.* II. 1899, **5**, 436.

dioxide and the other which appears to rely on the most resistant carbohydrate, should exhibit this pronounced intolerance of soluble organic compounds.

General Aspects of the Cultivation of *S. cytophaga*.

Mention has already been made of some early attempts to secure elimination either of the thread or of the sporoid form of the organism by means of differential treatment. Although the object of the experiment was not achieved, the data thus obtained possess a few points of cultural interest. They relate to the effect of (*a*) reaction of the medium, (*b*) temperature, and (*c*) disinfectants.

Relations to reaction of the medium. Two series of experiments have been carried out, and the results secured indicate a fairly wide range of resistance to acid and alkaline conditions. The gradations in the reactions of the two sets are not identical and this is reflected in a certain amount of divergence in the results. In the first series there appears to have been reluctance to grow in concentrations exceeding N/80 acid and N/160 alkali; in the second series a tolerance was found ranging from N/50 acid to N/55 soda or, expressed in the terms in common bacteriological use, from − 2° to + 2°. In view of the fact that liquid, and not solid, medium was employed, this range of resistance is somewhat noteworthy.

Table X.

Influence of the Reaction of the Medium on Growth.

Reaction of the culture solution											
HCl						NaOH					
N/20	N/40	N/80	N/160	N/320	N/640	N/640	N/320	N/160	N/80	N/40	N/20
−	−	+	+ +	+ +	+ +	+ +	+ +	+ +	−	−	−
N/25	N/50	N/100	N/130	N/300	N/720	N/800	N/160	N/100	N/55	N/25	
−	− +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ +	− +	−	

An approximately neutral reaction of the nutrient solution may, of course, be secured by the addition of calcium or magnesium carbonate, between the respective values of which there is little difference, but general experience shows that in the presence of these compounds the breakdown of the cellulose is less *apparent* than when the reaction of the medium is due to alkaline carbonates.

Relations to temperature. The inquiries under this head are divisible into two distinct lines, viz. the determination of (1) the optimum

temperature for cellulose decomposition, and (2) the thermal deathpoint of the organism.

In pursuance of the first of these objects six portions of 2 grams each of pure cellulose fibre were placed in flat bottomed cultivation vessels and to each was added 100 c.c. of mineral salt solution with 0·25 per cent. sodium nitrate, the solution having been rendered slightly alkaline to phenolphthalein. The flasks were then inoculated with 1 c.c. of a culture of the organism and incubated at 20°, 25° and 30° for 14 days. At the end of this period the resulting cultures were acidified slightly, filtered, and the residues dried and weighed. The losses of cellulose at the different temperatures were found to be as follows:

20° (*a*)	Cellulose decomposed		0·334 grm.
20° (*b*)	,,	,,	0·302 grm., mean 0·318 grm.
25° (*a*)	,,	,,	0·370 grm.
25° (*b*)	,,	,,	0·414 grm., mean 0·393 grm.
30° (*a*)	,,	,,	0·414 grm.
30° (*b*)	,,	,,	0·446 grm., mean 0·430 grm.

The most vigorous decomposition thus took place in the region of 30°. Owing to limited incubator capacity at that time it was not possible to carry out a more extensive series, but subsequent experience has shown that although decomposition does proceed at 35°, this is not so rapid as at 30°. Moreover, the organisms exhibit certain abnormal features and have also been found to display a marked reluctance to grow in high dilutions at the higher temperature.

The determination of the thermal deathpoint has been carried out on a number of occasions, and the results of two series are given.

Series I. Length of Exposure—5 minutes.

Growth after	48°	50°	52°	56°	58°	60°	62°	62·5°	66°
6 days	+ +	+ +	+ +	+ +	+ +	+	−	−	−
15 days	+ +	+ +	+ +	+ +	+ +	+ +	+ +	−	−

Series II. Length of Exposure—10 minutes.

Growth after	40°	42°	44°	46°	48°	50°	52°	54°	56°	58°	60°	62°
7 days	+	+	+	+	+	+	+	+	+	−	−	−
9 days	+	+	+	+	+	+	+	+	+	+	−	−

In the two series the organism succumbed after exposure to a temperature of 62° for 5 minutes or a temperature of 58° for 10 minutes. In its thermal deathpoint *S. cytophaga* thus resembles the majority of the non-sporogenous organisms, as compared with the sporogenous

bacteria whose deathpoint is generally very much higher. It is, therefore, probably safe to assume that the formation of the sporoid stage does not confer on the organism any increased resistance to high temperatures.

Behaviour towards disinfectants. The susceptibility of *S. cytophaga* to volatile antiseptics was noted in some of the early experiments. Treatment of crude cultures with toluene or toluene vapour invariably resulted in the destruction of *S. cytophaga* and the persistence of contaminating sporogenous forms which, of themselves, were incapable of inducing typical decomposition of cellulose. Hence, it might be inferred that the power of resistance to disinfectants would be similarly low, and this is brought out by experiments with phenol. The tests were carried out in sodium ammonium phosphate-mineral salt solution with cellulose.

TABLE XI.

Effect of Phenol on S. cytophaga.

	Concentration of phenol (per cent.)							
Growth after	0·004	0·008	0·017	0·035	0·070	0·15	0·31	1·25
5 days	+	− +	−	−	−	−	−	−
9 days	+ +	+	−	−	−	−	−	−

Microscopical examination of the culture with 0·008 per cent. of phenol showed the presence of both the thread and sporoid forms and a decided tendency to the production of highly granular filaments. The organisms were evidently already suffering from the effects of the phenol without, however, either of the forms being entirely suppressed.

Destruction of cellulose, etc. Under favourable conditions as to nitrogen and oxygen supply, the decomposition of filter paper, cotton wool, parchment paper and gun cotton proceeds with fair rapidity and, in fact, limitation of the breakdown changes frequently arises solely through exhaustion of the nitrogen supply of the medium. In this connection the results of two experiments may be given. In the first experiment a culture was made in a 2000 c.c. Erlenmeyer flask containing 200 c.c. of normal solution, i.e. with 2·0 grms. sodium ammonium phosphate per litre, together with 5·0 grms. of filter paper, the absolute dry weight of which was 4·66 grms. After eight days at 25° the culture was boiled, filtered through a tared paper, washed with hot water, dried and weighed. The weight of this residue was found to be 4·28 grms. thus indicating a loss of 0·38 grm. of cellulose in eight days. Taking into consideration the fact that there is generally no apparent growth

of *S. cytophaga* in less than three to four days after inoculation, the above loss of cellulose is by no means inconsiderable.

A second experiment shows the relation between cellulose breakdown and nitrogen supply. A number of flasks were prepared each with 2·0 grms. air-dry cotton wool and 100 c.c. of neutral nitrogen-free mineral salt solution. After sterilisation, four of the flasks received a sterile solution of sodium ammonium phosphate so that the concentration of the latter in the different culture flasks was equal to 0·5, 1·0, 2·0 and 4·0 grms. of the salt per litre of culture solution. After inoculation and incubation for 21 days at 25° the culture in each flask was treated with dilute (3·3 per cent.) hydrochloric acid, boiled, filtered, and the residue washed with distilled water and dried. The various data are given in Table XII.

TABLE XII.

The Relation of Nitrogen Supply to Cellulose Decomposition.

Nitrogen supplied	Wt. of cotton wool (dried at 100°) after 21 days	Loss	Ratio of nitrogen supplied: cellulose decomposed
nil	1·810 grms.	nil	—
3·51 mgrms.	1·712 „	0·098 grms.	1 : 27·5
7·14 „	1·580 „	0·230 „	1 : 32·2
14·28 „	1·415 „	0·395 „	1 : 27·7
28·56 „	1·460 „	0·350 „	1 : 12·3

The ratios for the three lower concentrations of nitrogen show good agreement among themselves, the amount of cellulose destroyed being about 30 times the quantity of nitrogen originally present. This consistency may be taken as indicating that the whole of the nitrogen had been utilised. With the most concentrated solution, the rapidity of the action appears to have been checked; it is also possible that the whole of the nitrogen has not been completely utilised, but unfortunately an actual determination of the residual nitrogen was not made.

By-products of growth of S. cytophaga. Active cultures of *S. cytophaga* have three main characteristics which may be stated to be (*a*) pigment production, (*b*) the absence of obvious gas formation and (*c*) resolution of the cellulose into a glistening mucilaginous mass.

Pigment production. Growth of the organism on filter paper or cotton wool, and especially when the reaction of the medium is slightly alkaline, gives rise to the formation of a brilliant yellow pigment. It may be extracted with ease from the liquid or dried cultures by means of alcohol, the extract giving on concentration a dark yellow or orange coloured oily residue. The pigment goes into solution with the ordinary

fat solvents, petroleum ether, benzol, chloroform, carbon bisulphide, ether, etc. The first three yield a bright yellow solution, whilst that with carbon bisulphide is orange yellow, and that with ether canary yellow, in colour. This colour is intensified by alkali and destroyed by weak mineral acids.

The pigment gives reactions approaching those of the carotin group; the production of a blue colouration on exposure to the action of concentrated sulphuric acid is somewhat feeble, but strong hydrochloric acid gives a deep dirty green colour. Hence it resembles the lipochrome substances which are formed by many of the bacteria such, for example, as *Sarcina lutea* and *aurantiaca* and *Staphylococcus pyogenes aureus*. Up to the present we are unable to adduce any evidence as to the physiological importance of the pigment.

Acid products. During the decomposition of cellulose the reaction of the medium undergoes a gradual change so that after 8–10 days the liquid is distinctly acid to litmus. This is not due, as might be supposed, to the abstraction of the ammonium radicle from the phosphates supplied, and the formation of acid phosphates, since the same change proceeds in culture solutions with sodium nitrate. On the other hand, there are indications of the production of small quantities of volatile fatty acids. Old cultures of the organism, when heated with concentrated sulphuric acid, give rise to a distinct smell of butyric acid, and by the addition of ethyl alcohol a smell resembling that of ethyl butyrate is evident. In this connection two cultures were prepared, each with 100 c.c. of mineral salt solution containing 0·287 grm. of precipitated cellulose, in one case with, and in the other without, calcium carbonate. At the end of eight days each culture was steam-distilled and gave a distillate slightly acid to litmus. On titration 2·5 and 3·1 c.c. of N/10 sodium hydroxide solution were required by the respective distillates; this acidity if entirely due to butyric acid would be equal to approximately 7–9 per cent. of the original cellulose. By analogy with other carbohydrate-fatty acid fermentations, it is not improbable that other fatty acids are produced, but their identification and estimation lies outside the scope of the present inquiry.

Mucilage. By the continued growth of *S. cytophaga* for a few days, cellulose material at, or slightly above, the level of the culture solution becomes distinctly mucilaginous and this property is also gradually acquired by the culture solution. The latter is difficult to filter when cold, but presents no difficulties when brought to the boil or after being slightly acidified. It was originally supposed that this mucilaginous

substance might be of a dextrine nature and possibly the precursor of saccharine compounds. Examination of numerous cultures of different ages has failed, however, to indicate the presence of any breakdown product capable of reducing Fehling's solution or possessing any optical activity. The non-dextrin character of the mucilage is shown by the results of an experiment in which a quantity of the substance was exposed to the action of Taka-diastase. After treatment according to the routine method the mucilage solution "failed to show even the slightest trace either of sugar or dextrin." A further attempt was made to obtain hydrolysis of the mucilage by means of fuming hydrochloric acid. Under these conditions cellulose gives rise to the production of dextrose; after digestion for three hours with hydrochloric acid, mucilage solution was utterly lacking in optical activity[1]. Hence a fundamental difference exists between the nature of the mucilage and of cellulose. On the other hand, the mucilage possesses certain points of resemblance to the pectin or pectic acid group of compounds. On various occasions, cultures of the organism have been reduced to dryness *in vacuo* and extracted in alcohol to remove the pigment. On treatment of the residue with cold water a slight, and with hot water a still greater, amount of mucilage was brought into solution. After a partial concentration of the extract the mucilage was thrown down with alcohol and filtered. The residue, when treated with cold water, yielded a thick viscid fluid which was extremely difficult to filter. On being heated, but without being brought to the boil, the solution could be filtered with ease.

It is, of course, probable that such a solution contains a whole range of degradation products. Tannic acid fails to effect any precipitation. On the addition of hydrochloric acid the solution is converted into a thick jelly which on shaking and being allowed to stand, or on heating, assumes the form of a light semi-transparent membranous precipitate. Precipitation also results from the addition of barium and calcium hydroxide, basic and neutral lead acetate, barium and calcium chloride, silver nitrate and magnesium sulphate.

The solution prepared in the above manner is neutral to litmus. Treatment of the mucilage with hot dilute hydrochloric acid gives a precipitate which is insoluble in water, but is readily soluble in ammonia, from which it may again be thrown down on acidification. We thus have a number of reactions which are also exhibited by pectin derivatives,

[1] We desire to express our indebtedness to Mr W. A. Davis and Mr E. Horton, of this Laboratory, for kindly carrying out the Taka-diastase and acid conversion tests respectively.

but further work is required before the chemical character of the mucilage can be definitely established. As far as soil conditions are concerned, it is not improbable that an extensive production of mucilage from plant residues would exert some action on the physical behaviour of the soil; from the chemical standpoint, and on account of its insolubility in acids and solubility in ammonia, the mucilage would, without doubt, appear in the "crude humus" fraction in the conventional soil analysis.

The Relation of Cellulose-decomposing Organisms to Nitrogen Fixation.

Since this, and a preceding investigation on nitrogen fixation, arose from the necessity of accounting for the observed gains in nitrogen in soils that had been allowed to revert to prairie conditions, we may be permitted to refer briefly to the relation between cellulose, as distinct from crude plant residues, and the assimilation of atmospheric nitrogen. In the first instance it was supposed that although cellulose is not directly available to such nitrogen-fixing organisms as Azotobacter, the products of decomposition would include sugars such as are formed on the hydrolysis of cellulose by acids, and that these compounds would serve as sources of energy for Azotobacter. It has already been seen, however, that the formation of sugars during decomposition is highly problematical, but that there are indications of the formation of volatile fatty acids, and the value of some of these for nitrogen-fixation has frequently been observed[1]. Moreover, there is definite evidence that the cellulose breakdown products do increase nitrogen-fixation and in this connection the following results are submitted.

Two sets, each of six flat bottomed flasks, were prepared; one set (A) received 50 c.c. of mineral salt solution containing 0·1 per cent. mannite and, in addition, 1·0 grm. each of cellulose and calcium carbonate. The second set (B) received the same additions with the exception that in this case the mannite was replaced by a supply of sodium nitrate equal to 0·010 per cent. nitrogen, the main object of this variation being to ascertain whether the associative action could be initiated equally well by nitrogen-fixation (set A) or cellulose decomposition (set B). From the results given below it will be seen that this was not the case and set B actually lost nitrogen—possibly by the action of such denitrifying cellulose-decomposing bacteria as were observed by van Iterson[2].

[1] Mockeridge, F. A., *Biochem. J.* 1915, **9**, 272–283.

[2] Iterson, C. van, *loc. cit.* 690.

TABLE XIII.

Associative Growth of Nitrogen-fixing and Cellulose-decomposing Organisms.

	Mean total N after 4 weeks		
	Control	Azotobacter alone	Azotobacter and crude culture of cellulose organism
Set A (mannite)	0·735 mgrms.	0·91 mgrms.	2·835 mgrms.
Set B (sod. nitrate)	7·17 ,,	6·44 ,,	4·69 ,,

The presence of 0·05 grm. of mannite failed to give any increase of nitrogen over the control, but the addition of 1·0 grm. of cellulose together with cellulose-decomposing organisms gave what may be regarded as a definite increase.

A similar experiment was also carried out, but in this case the solution contained 1·0 per cent. mannite; 50 c.c. solution and 1·0 grm. cellulose were again taken for each flask. The results are:

	Total nitrogen in flasks after 3 weeks		
	Control	Azotobacter alone	Azotobacter and cellulose organisms
(*a*)	1·12 mgrms.	4·20 mgrms.	5·81 mgrms.
(*b*)	1·82 ,,	3·01 ,,	7·42 ,,

The results thus show a fixation of 4·27 mgrms. nitrogen per gram of mannite, and a supplementary fixation of 3·01 mgrms. when cellulose was also supplied.

In a third experiment the amount of cellulose lost during the fermentation was ascertained prior to the determination of total nitrogen. The mean nitrogen content of the respective flasks was found to be 1·19, 2·78, and 5·74 mgrms.: the loss of cellulose during fermentation amounted to 0·153 grm. From these data it may be calculated that the fixation of nitrogen per gram of mannite *supplied* was equal to 3·18 mgrms., while that per gram of cellulose *actually decomposed* was no less than 19·3 mgrms. All three experiments provide definite indications of the value of cellulose breakdown products for the assimilation of atmospheric nitrogen. Somewhat analogous results have also been obtained by the combined growth of anaerobic cellulose-decomposing and anaerobic nitrogen-fixing bacteria[1].

[1] Pringsheim, H., *Cent. Bakt. Par.* II, 1909, **23**, 300–304; 1910, **26**, 222–227.

Summary.

From the foregoing account the following summary may be given:

1. Examination of Rothamsted soils on different occasions has revealed the presence of an organism capable of breaking down cellulose with comparative ease.

2. This organism presents a number of features of morphological and physiological interest. Morphologically, the organism appears to possess greater affinities with the Spirochaetoideae than with the bacteria and the name *Spirochaeta cytophaga* is, therefore, suggested.

3. While the spirochaet is capable of considerable vegetative growth as a sinuous filamentous cell, it also appears to pass through a number of phases which terminate in the production of a spherical body (sporoid) which differs in a number of respects from the true spores of the bacteria. Germination of the sporoid again gives rise to the filamentous form, which possesses perfect flexibility and is feebly motile. The latter does not apparently possess flagella.

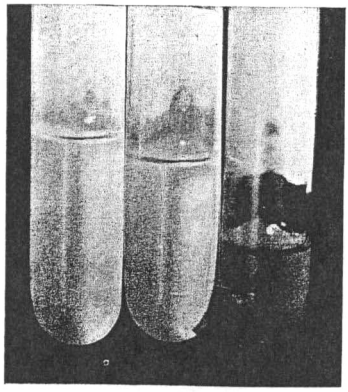
4. *Spirochaeta cytophaga* is essentially aerobic; its optimum temperature is in the region of 30°. Both the thread and sporoid stages are killed by exposure to a temperature of 60° for ten minutes.

5. The nitrogen requirements of the organism may be met by a number of the simpler nitrogen compounds—ammonium salts, nitrates, amides and amino-acids. Peptone is also suitable in concentrations up to 0·025 per cent. Stronger solutions, e.g. 0·25 per cent., lead to marked inhibition of growth. The organism fails to grow on the conventional nutrient gelatine or agar.

6. Comparative experiments with a number of higher alcohols, sugars and salts of organic acids show that none of these is capable of meeting the carbon requirements of the organism. Cellulose is the only carbon compound with which growth has been secured.

7. Although none of the monoses, bioses and other carbohydrates tested is able to support growth, many of them exert an inhibitive action on cellulose decomposition if present in other than very low concentrations. This may be correlated with the reducing properties of the carbohydrate. Maltose, for example, has been found to be approximately 70 times more toxic than saccharose.

8. Of the various by-products of the action of *Spirochaeta cytophaga* may be mentioned (*a*) a pigment possessing relations to the carotin



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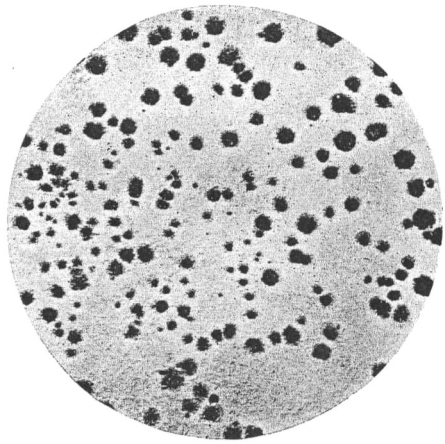

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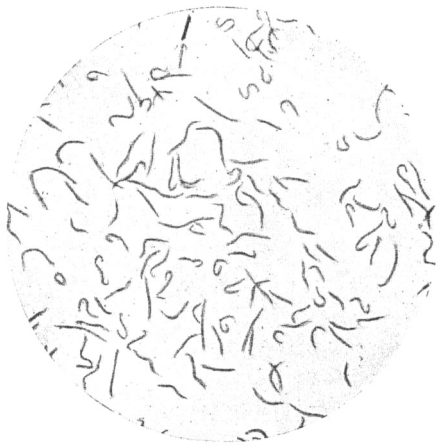

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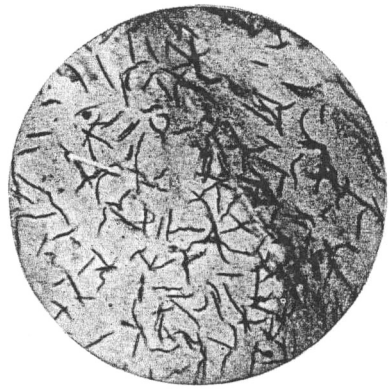

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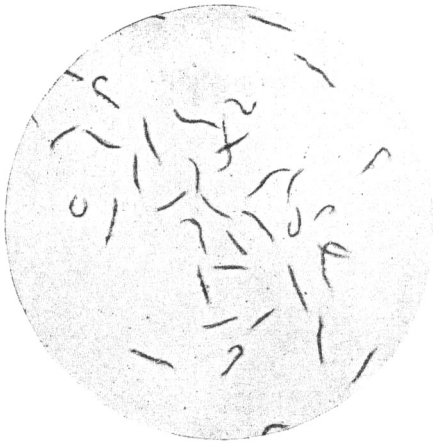

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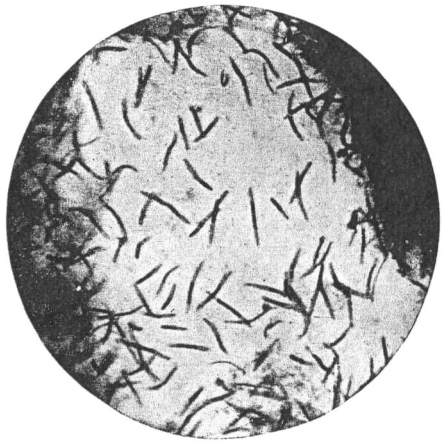

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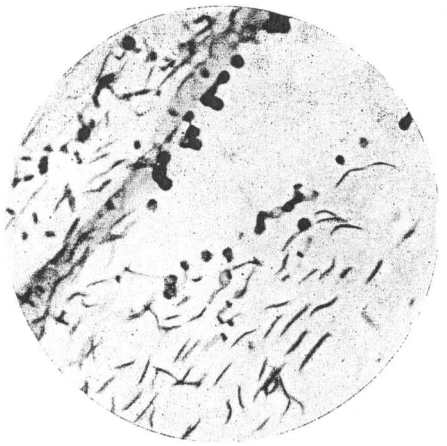

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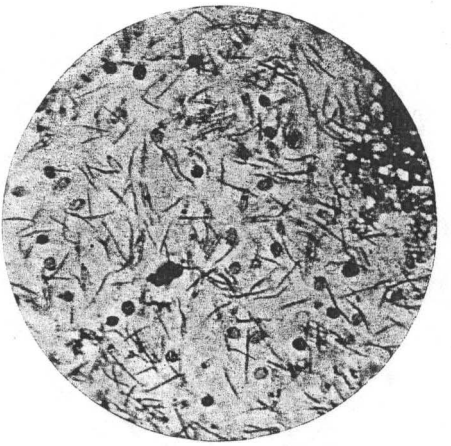

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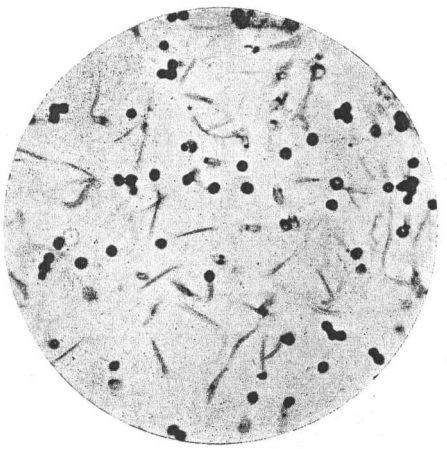

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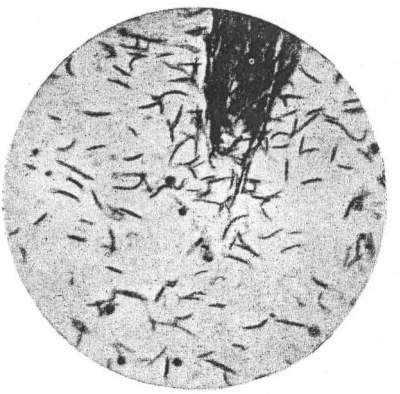

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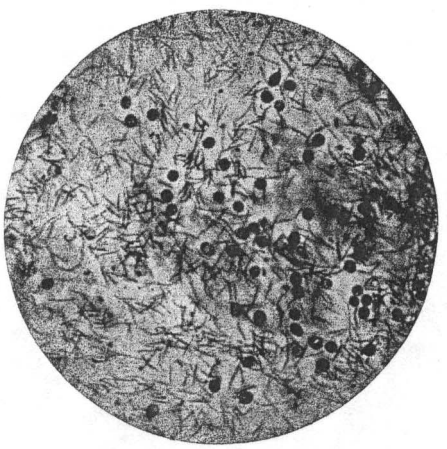

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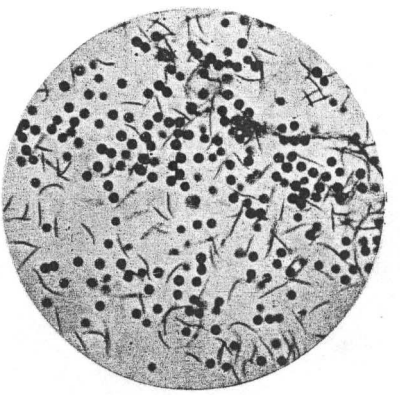

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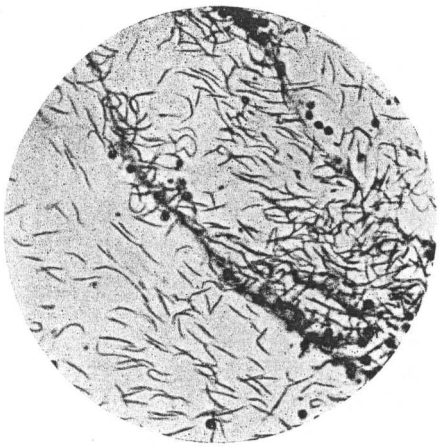

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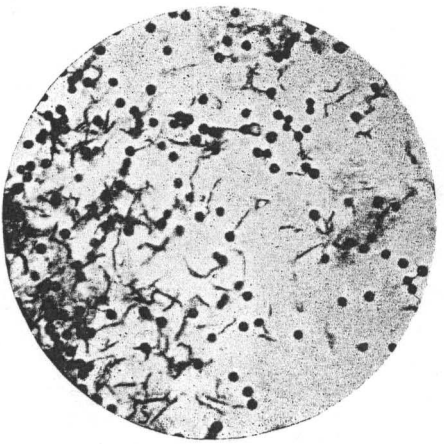

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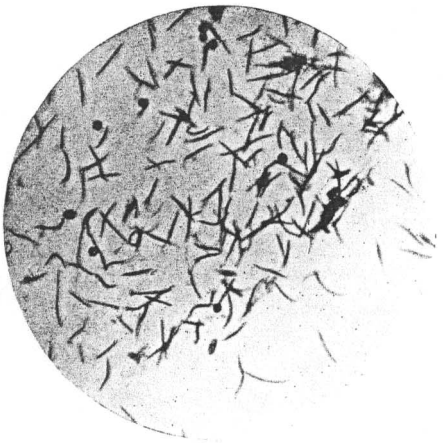

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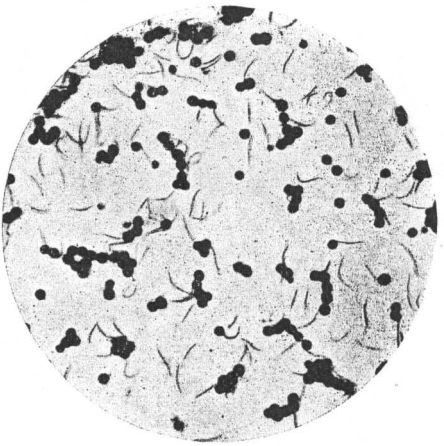

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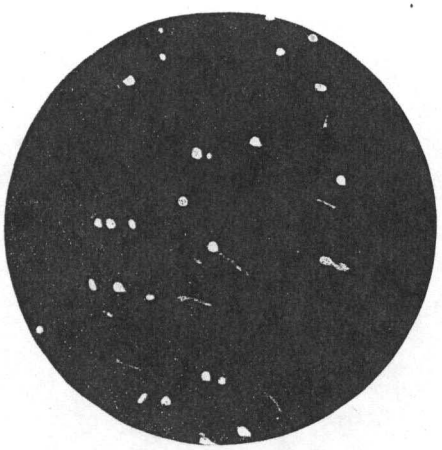

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group, (*b*) mucilage which does not give rise to optically active compounds on hydrolysis and (*c*) small quantities of volatile acids.

9. Evidence is also adduced to show the relation of cellulose decomposition to the assimilation of atmospheric nitrogen.

DESCRIPTION OF ILLUSTRATIONS ON PLATES I–III.

(Photomicrographs—magnification 1000 diameters.)

PLATE I.

Fig. 1. Test tube cultures with mineral salt solution and filter paper; 4, 7, and 12 days old.

Fig. 2. Petri dish culture on sodium nitrate-mineral salt agar with filter paper superimposed. Showing holes in paper by the growth of *S. cytophaga* (nat. size).

Fig. 3. Photomicrograph of young culture of *S. cytophaga* in filter paper tube. Showing typical incurvation of thread form.

Fig. 4. Photomicrograph of young culture of *S. cytophaga* in liquid culture with precipitated cellulose. Showing well-marked sinuous forms.

Fig. 5. Photomicrograph of *S. cytophaga* in *mass culture* on nutrient agar. Enfeebled forms exhibiting initial granulation of cells.

Fig. 6. Photomicrograph of young culture showing equatorial or polar segregation of chromatin substance. "Deposition" stain with alcoholic fuchsin.

PLATE II.

Fig. 1. Photomicrograph of culture on oat plant residue showing cells with chromatin "bridge" (below centre) and also sporoids.

Fig. 2. Photomicrograph of culture on filter paper, incubated at 35°. All stages from the thread to the final sporoid are represented.

Fig. 3. Photomicrograph of same culture at a later stage, showing formation of a pre-sporoid stage with double granules (or band).

Fig. 4. Photomicrograph of culture showing (below centre) cells with (*a*) band, (*b*) bridge, and (*c*) double granules of chromatin. Also adjacent cell with internal coccoid structure.

Fig. 5. Photomicrograph of sporoids of different ages and, below, several apparently devoid of chromatin substance.

Fig. 6. Photomicrograph of older culture showing predominance of sporoids.

PLATE III.

Fig. 1. Final stage in the dissolution of a cellulose fibre.

Figs. 2, 3, and 4. Photomicrographs showing emergence of thread form from the sporoid. Preparation for Fig. 3 stained with alcoholic fuchsin.

Fig. 5. Photomicrograph of Indian ink preparation with sporoid and thread form.

Fig. 6. Photomicrograph of culture shown on Plate II, figs. 2 and 3, second generation after transference from 35° to 25°. Showing intense granulation of cells.

(*Received November 30th*, 1918.)