VIII. DECOMPOSITION OF CELLULOSE BY *CYTOPHAGA*. I

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(*Received 29 September 1937*)

Introduction

The decomposition of cellulose by micro-organism may be brought about by (*a*) aerobic, (*b*) anaerobic, (*c*) thermophilic bacteria or by (*d*) fungi. For a general account of the subject the reader is referred to the works of Thaysen & Bunker [1927], Waksman [1931], Pringsheim [1932] and to the more recent monograph by Khouvine [1934]. In this paper we are only concerned with aerobic decomposition and more particularly with that first described by Hutchinson & Clayton [1919]. To the organism employed, which was isolated from the soil, these authors gave the name *Spirochaeta cytophaga* (n.sp.). The principal features of the changes wrought by this organism are the formation from cellulose of a mucilage of complex structure, together with a yellow pigment. An important point is that cellulose is the only source of carbon which the organism will use. There have been several studies of this and closely related organisms subsequent to the publication of Hutchinson & Clayton, but these have been mainly concerned with the life-cycle, morphology and distribution of the organisms. Doubtless even earlier workers had investigated a similar type of bacterial activity, e.g. van Iterson [1904]. We have concerned ourselves principally with the metabolism of the organism and a preliminary account of the work has already been given [Walker & Warren, 1934]. The work is by no means complete but is being published because one of us has now been obliged to withdraw from participation and because many points have arisen which may take some time to settle.

Identity of organism

The organism used was isolated by us from the local soil and purified after the manner described by Hutchinson & Clayton, which is a dilution method and was further purified by streaking filter paper embedded in silica-gel salt medium, after the manner of Winogradsky [1929]. The simplicity of the medium (inorganic salts + cellulose) constitutes a deterrent to the growth of contaminating bacteria. The metabolic products, too, are not readily susceptible to bacterial attack. It may perhaps be mentioned that our organism has been maintained in artificial culture for more than four years now. The problem of identifying the organism precisely has been rendered more difficult by the divergences of opinion expressed by those who have worked in this field of late years. In general features our organism resembles that described by Hutchinson & Clayton and called by them *S. cytophaga*, although the coccoid form described by them seems to be absent from our culture. Winogradsky in a series of papers culminating in a comprehensive memoir [1929] disapproves the life cycle proposed by Hutchinson & Clayton for their organism and claims that there are many different species of kindred cellulose-decomposing organisms. He examined some dozen different species and for classification purposes he proposed three new genera, *Cytophaga*,

Cellfalcicula and *Cellvibrio*; the particular species is designated by some appropriate suffix. Hutchinson & Clayton's organism appears to be renamed by him *Cytophaga Hutchinsoni*. The important point, and indeed the criterion of membership of this genus, is that the organism should attack cellulose and cellulose only, with the formation of a mucilaginous substance, provisionally called oxycellulose, and a pigment. The pigment is usually yellow and there should be no other metabolic products. The present position is, unfortunately, not quite so clear as when Winogradsky left it. Soon after the latter's contribution, came that of Bokor [1929]; he, too, dismisses the life cycle of *Spirochaeta cytophaga*; in fact, he sweeps away this organism and creates a new one, *Mycococcus cytophaga* (n.sp.), which, metabolically at least, is hard to distinguish from *Spirochaeta cytophaga*. In a brief footnote devoted to Winogradsky's contribution, Bokor expresses disagreement with that author concerning the morphology of the organisms. Since then, Krzemieniewska [1933] has compared *S. cytophaga* (Hutchinson & Clayton) with *Cytophaga Hutchinsoni* (Winogradsky) and comes to the conclusion that they are different and proposes for the former the name *C. myxococcoides*.

All these and ourselves are dealing with what is essentially the same type of organism; i.e. having the same biochemical characteristics which have already been mentioned. Such differences as exist are based on interpretation; in particular, the occurrence of more than one form in a culture raises the question as to whether it represents a stage in the life cycle, or a contaminant or a symbiotic organism. Our own culture shows predominantly the flexuous, filamentous type of organism, which seems to us similar to that described by Hutchinson & Clayton. But it must be admitted that there is invariably present a rod-like form, although this is never conspicuous. We are of opinion that this is another closely related species, probably of the *Cellvibrio* genus (cf. Winogradsky's *Cellvibrio flavescens* and *C. ochracea*). It is certainly not a contaminating organism in the usual sense of the word. Like others, we have attempted to separate the two forms and lately have had some success; if complete, this will form the subject of a later communication. For the time being we will refer to the organism as *Cytophaga Hutchinsoni*, in view of the conflict of opinion recorded above.

Culturing

The organism has been grown on a simple salt medium with cellulose as the sole source of carbon. The medium used was that of Hutchinson & Clayton and had the following composition:

$NaNH_4HPO_4,4H_2O$	2·0 g
KH_2PO_4	1·0
($CaCl_2$	0·1)
$MgSO_4,7H_2O$	0·3
NaCl	0·1
$FeCl_3$	0·01
Water	1000

The *p*H was adjusted finally to 7·5 by the addition of *N* NaOH; usually 5 ml. were required per litre of medium. All the salts used were Kahlbaum's or were specially purified. The water used was distilled in glass from alkaline permanganate. For cellulose, our standard supply has been filter paper (Whatman's No. 1), usually in the amount of 1 g. per 100 ml. medium. We have used other sources of cellulose at various times and these will be referred to in recounting

the results. We have also experimented with the above medium, omitting one constituent at a time and we find that calcium chloride is the only substance that can safely be left out. Since this substance frequently brings about precipitation in the medium we have discarded it from our stock medium. To what extent this constitutes an absolute lack of calcium it is not possible to say; it can only be recorded that both our medium and the cellulose employed fail to give any qualitative reaction for calcium; there can thus only be traces of calcium present. A somewhat similar result has been reported by Rippel & Stoess [1932] for various other micro-organisms.

For general maintenance purposes the above medium is set up in 7 ml. amounts in pyrex test tubes, a strip of filter paper, approximately 6 × 1 cm., introduced, the tubes plugged with cotton-wool and the whole sterilized by steam heating on three successive days. In the quantitative experiments described later, cellulose was supplied in various forms in the proportion of 1 or 2 g. per 100 ml. medium

A strip of cellulose inoculated in the usual manner shows no obvious change for three days. Thereafter, bright yellow patches develop on the paper above the water line, with most growth at the actual air-water interface. The cellulose, where attacked, is gradually transformed into a glistening, mucilaginous mass, impregnated with yellow pigment. In 7–10 days the strip is "eaten through" at the surface, or transformed into a translucent form which gives this appearance. No decomposition takes place below the surface; consequently the greater part of the cellulose is unattacked. Sub-culturing has been carried out fortnightly, although successful inoculations have been made on occasion at much longer intervals.

*p*H. In some preliminary experiments specially carried out for the purpose, we judged the *p*H limits of growth to be 6·5 and 9·0, with an optimum at 7·5. In the test tube this *p*H does not change appreciably, but on the large scale, when a much greater proportion of cellulose is decomposed, the *p*H is found to have shifted to the neighbourhood of 7·0 by the end of the experiment.

Oxygen. The aerobic nature of the organism is manifest from its growth at the surface of the medium and since it was desired to accelerate the decomposition of the cellulose, it was natural to try the effect of increasing the oxygen supply. Four flasks were set up each containing 100 ml. of medium and 1 g. of cellulose (chopped filter paper). Flask 1 was left un-inoculated, whilst each of the others was inoculated with one drop of an active suspension. Flask 2 had a slow stream of nitrogen bubbled through it, flask 3, a slow stream of oxygen and flask 4 was untreated. Residual cellulose was estimated at the end of 21 days. Table I shows the results.

Table I

Flask	Experiment	Residual cellulose g.	Degree of decomposition %
1	Control	0·97	(3)
2	Nitrogen	0·90	10
3	Oxygen	0·64	36
4	Air	0·84	16

The beneficial effect of oxygen is thus evident. Henceforward it became our usual practice in quantitative experiments to pass oxygen through the culture.

Temperature. All cultures have been grown at 29°.

Large-scale experiments. Both for the purpose of obtaining useful amounts of metabolic products and for greater accuracy in quantitative work most of our experiments have been conducted on a scale of 2 l. of medium, containing 20 g. of cellulose. Such cultures were grown in large, squat pyrex flasks, of 4 l. capacity, and a slow stream of oxygen was bubbled through the medium. The oxygen was supplied from a cylinder and was fed to the culture after passing through a solution of permanganate. The duration of an experiment was usually 28 days. In Table II are recorded the results of various attempts to increase the amount of

Table II

	Proportion of cellulose decomposed
Normal	33 %
Halving the cellulose	31
Doubling the ammonium salt	12
Doubling incubation period	38

cellulose decomposed. The normal value in the above table represents the mean value obtained in thirty-two different experiments, recorded later (Table IV). Doubling the nitrogen supply (microcosmic salt) gave an unexpected result; but the matter was not pursued further as we were only interested in increasing the decomposition. Doubling the incubation period naturally had a favourable effect, but not to an extent commensurate with the extra time taken.

Forms of cellulose. Filter paper is the standard type of cellulose which we have used. This is very convenient in test tubes, but less so for flask work because it usually sinks to the bottom of the medium; or, if chopped, adheres to itself or the flask. We have investigated various types of cellulose with a view to finding the most suitable for this kind of work. Eventually we chose cellulose wadding, following the suggestion of Dr R. B. Fisher of this Department. This is easily disintegrated, yet it is of such a texture that it floats readily, a desirable property where aerobic organisms are concerned; we have used it in practically all our experiments. Later, seeking a finer form of cellulose with a view to speeding up the decomposition, good results were obtained using cigarette paper.[1] But it has certain drawbacks; notably, frothing during aeration was excessive and the residual unchanged cellulose was too powdery for convenient handling. Cellophane is readily decomposed by the organism, but suffers from the same drawbacks as chopped filter paper and to a more marked extent. It presents certain interesting points of difference from filter paper cellulose. Thus pigment formation is less conspicuous; and the mucilage formed sinks to the bottom of the medium, whereas ordinarily it floats. Possibly this is a reflection of the difference in micellar structure, or rather the absence of such organized structure in cellophane.

Other forms of cellulose which we have found are attacked by the organism in the usual way are cotton, linen and parchment paper—the latter made by steeping filter paper in 66 % sulphuric acid and washing. Cellulose acetate is not attacked. The exclusiveness with which cellulose, and cellulose only, is attacked makes it not inconceivable that the organism could be used for the detection of cellulose.

Other carbon compounds. The failure of the organism to grow on any other carbohydrate was first established by Hutchinson & Clayton and these authors

[1] Kindly obtained for us through the agency of Mr A. E. Wilhelmi.

also tested a number of lower fatty acids (as calcium salts), with negative results. We have been equally unsuccessful in inducing growth on anything but cellulose and slightly hydrolysed cellulose (parchment paper). Any further hydrolysis of the cellulose results in products incapable of sustaining growth.

We have also tried whether the attack on cellulose could be facilitated in any way by the presence of various lower fatty acids. The following were supplied in the form of their sodium salts, in a strength of 0·1%, along with cellulose: maleic, fumaric, lactic, pyruvic, succinic, citric, tartaric and oxalic. In no case was there any enhanced growth; in the case of oxalic, there was complete inhibition. Other substances which were tried for adjuvant action were furfuraldehyde, furfuryl alcohol and pyromucic acid; all without effect. A partial inhibition was observed with glycerol present in a concentration of 0·3%

An important omission from the list of carbohydrates tested by Hutchinson & Clayton is cellobiose. We have tested this and find that alone it is incapable of supporting growth and in the presence of cellulose it exercises an inhibiting effect at a concentration of 1·0 but not at 0·1%.

We have confirmed the observations of Hutchinson & Clayton that dextrose is inimical to the growth of the organism; we found a marked effect at 0·05% concentration and almost complete inhibition at 0·1%. Potassium cyanide, we noted, had a similar effect in low concentration. In both cases, where inhibition was only partial, such growth as took place was at an appreciably higher level above the surface of the medium than usual.

Metabolic products

The principal products of the action of *Cytophaga* upon cellulose are (1) a mucilaginous material, described by Hutchinson & Clayton as akin to pectin and by Winogradsky as oxycellulose, and (2) a pigment, usually yellow, although a variety of colours is obtainable from kindred types, according to Winogradsky. Again, according to the latter, there are no other metabolic products. Hutchinson & Clayton reported small amounts of volatile fatty acids, but these were attributed by Winogradsky to contamination of their cultures. None of these authors makes any mention of carbon dioxide, which is a conspicuous product of our cultures; indeed it accounts for the greater part of the carbon lost. Possibly its production is to be taken for granted. With regard to other products, we have not been able to detect any volatile fatty acids, but have observed traces of what is considered to be higher fatty acid and, in small amount, relatively simple but non-reducing carbohydrates; the latter are probably penultimate degradation products of cellulose. Finally, we have noticed that large scale cultures have a faint but distinct odour, which implies the production of some volatile carbon compound.

For the purpose of studying the metabolic products a considerable number of large-scale experiments has been carried out. The general technique of these has already been described (p. 34). Many variations in the procedure for working up the material have been practised, either for the purpose of increasing the yield or for obtaining a product of greater purity. These are too numerous to mention individually and only the most satisfactory procedures will be described here. The unchanged cellulose is filtered from the culture, using a piece of fine, unbleached linen. It is squeezed as dry as possible and is then thoroughly extracted with hot water, alcohol and ether in turn, after which it is allowed to dry in the air and is subsequently weighed. The alcoholic extracts are reserved for working up the pigment. The filtered culture medium, together with the aqueous expressate, is treated with three times its own volume of 97–99%

alcohol containing 1 ml. of concentrated hydrochloric acid for every litre of medium to be treated. This brings about the separation of the mucilage. These preliminary operations occupy some 2–3 hr.; the activities of the organism probably cease earlier with the separation of the cellulose and its extraction with hot water. After being allowed to stand for at least 24 hr., the mucilage is obtained by siphoning off the fluid portion and finally by centrifuging. The siphonate is neutralized and allowed to stand a further 24 hr., which results in the separation of most of the salts. It is filtered and concentrated almost to dryness under reduced pressure. The residue is extracted with hot 99 % alcohol, united with the first alcoholic extract of residual cellulose (which carries a good deal of the pigment with it) and filtered. This constitutes the *crude* pigment in alcohol.

Mucilage. The crude mucilage is not easily purified. It is insoluble in water, although it swells up considerably in this medium and retains water of imbibition tenaciously. It is soluble in alkalis, giving a viscous solution, which is difficult to filter and froths readily. We have dissolved the mucilage in dilute (*N*/5–*N*/50) soda or ammonia, using 200–250 ml. for the mucilage from each litre of medium. The ammonia solution seems to be more easily filtered, but even with this salt it is desirable to filter hot, first through linen, then through filter paper. It may be necessary to change the filter paper more than once in order to complete the filtration in a reasonable time. A useful variant is to filter through a thick piece of cellulose wadding. The filtered solution is then acidified with hydrochloric acid, dialysed in cellophane or collodion sacs against distilled water for 7–10 days, separated by siphoning, washed and dehydrated with alcohol. Separation of the mucilage by centrifuging is only effective in an alcoholic medium; in water the mucilage tends to rise to the surface. We have, on occasion, separated it from aqueous medium by saturation with ammonium sulphate, but this has not been a general procedure. Although colourless after the final precipitation with alcohol, the ultimate product gradually darkens and shrivels as it dries and in the end may be almost black. Nevertheless, on soaking in water a sufficient length of time, it will undergo imbibition and assume a colourless, mucilaginous appearance, and if a little alkali be added it will be dispersed to give an opalescent and colourless solution. The amount of mucilage obtained in any given experiment is not large, its bulky appearance when first precipitated being misleading. Under the ordinary conditions of our experiments, quantitative data show that from 1 l. of medium, containing initially 10 g. of cellulose, about 1 g. of mucilage may be expected; in practice, it was appreciably less than this. We have endeavoured to prepare various salts of the mucilage, but these have not been very helpful from the purification point of view, being of variable composition and often very little different from the original mucilage in appearance. It is possible to obtain a white fibrous salt by precipitation with barium salts from alkaline solution, but this has been of variable composition; values of 20–29 % barium content have been obtained, of which the latter is the more reliable. The preparation of a barium salt from acid solution, after the manner of Carré & Haynes [1922] for the calcium salts of pectic acid, yielded a product containing approximately 5 % barium. The use of barium as an agent in the separation and characterization of the mucilage has therefore been discarded. Such analytical data as we possess have been obtained from the free mucilage. Qualitative tests have revealed nothing but the presence of C, H and O; and empirical analysis gave C, 33·3 %, and H, 6 %, O (by difference), 60·7 %; equivalent, 1430. A more highly purified "fraction" (see below) gave C, 40·1 %, H, 6·7 % and O (by difference) 53·2 % and an equivalent of 1107. Too great a

stress must not be laid upon the above figures whilst any uncertainty exists—as it does—as to whether a single substance is under examination or a mixture. Hutchinson & Clayton refer to the possibility that it may represent "a whole range of degradation products"; whilst we ourselves have been able to fractionate the mucilage (i.e. the apparently homogeneous substance precipitated by acid alcohol) and are at present engaged in examining these fractions. It is interesting that one of them should appear to exhibit in an exaggerated degree the frothing tendency characteristic of the whole mucilage fraction. To return to the consideration of the latter as a single substance, there is no doubt that it has many features in which it resembles the pectin group of substances; but quite early we were able to exclude pectin itself by demonstrating that the mucilage contained no methoxyl groups. On the other hand, there is much to be said for the view that it is akin to oxycellulose.

With a view therefore to characterizing the substance, we have carried out estimations of its furfuraldehyde value and its uronic acid content. The former was determined by the method described by Dorée [1933], based on the original Kröber [1900] procedure; the uronic acid by the method of Nanji *et al.* [1925]. The results of these estimations have varied somewhat according to the treatment meted out to the mucilage during purification. The most reliable results are given below in Table III, inset between typical values for pectic acid and oxycellulose culled from the literature and inserted for comparison purposes.

Table III

	Furfural %	Uronic acid %
Pectic acid	20	70
Mucilage	6	14
Oxycellulose (acidic)	1–3	3–12

It would seem that the mucilage is rather more oxidized than oxycellulose. The figures for chemically prepared oxycellulose, however, relate to what is admittedly a mixed product, for it is known that acidic oxycellulose when extracted with alkali reveals a certain proportion of unchanged cellulose. If allowance be made for this fraction, it would bring the figures nearer to those of the mucilage. It may be of interest to note that the uronic acid value for the mucilage would indicate a minimum molecular weight of 1257; whilst the equivalent (p. 36) was found to be 1107–1430.

Hydrolysis of the mucilage has been effected by boiling under a reflux with $N/2$ sulphuric acid, in the first place for 4 hr. and in a second experiment for 12 hr. The solution goes black and there is much humin-like material left even at the end of 12 hr. At the beginning of hydrolysis there is an appreciable evolution of carbon dioxide, indicative of the uronic acid content. The removal of the sulphuric acid quantitatively with baryta reveals that there has been no increase in acidity; there are thus no acids other than uronic in the mucilage molecule. The neutralized hydrolysate was found to reduce Benedict's solution and to give positive reactions for pentose. It was concentrated and submitted to fractionation with alcohol of various strengths. Insufficient material being obtained for characterization of any individual compound, the final fraction was used in making an osazone in the usual way. Recrystallized from water, this osazone had M.P. of 160–161° (uncorr.). This, together with its appearance under the microscope, indicated it to be the osazone of xylose; a finding confirmed by its rotation. No other product was detected. The yield

however is unsatisfactory and it will be necessary to obtain quantitative information concerning the products of hydrolysis before it can be claimed that xylose is the only significant product. When sufficient starting material has been accumulated, other methods of hydrolysis will be attempted. Other workers have failed to obtain reducing substances on hydrolysis and it is clear that this reaction, as with cellulose itself, is not straightforward. We conclude therefore that the mucilage is an oxycellulose, in the sense that this is taken to mean fragmentation of the cellulose chain coupled with oxidation of a certain proportion of primary alcohol to carboxyl groups (in this case 1 in 8).

Pigment. The characterization of the yellow pigment has proved elusive. The following procedure has been adopted for its separation. The alcoholic extract of crude pigment is concentrated by distillation under reduced pressure to approximately 1/10 of its volume. The concentrate is made alkaline with soda, filtered, acidified with hydrochloric acid and extracted with ether. Invariably a small proportion of the pigment remains in the aqueous phase and cannot be extracted by further treatment with ether. The ether extract is dried with anhydrous sodium sulphate, filtered and the ether evaporated. The dark brown viscid material so obtained has a somewhat pungent odour and is acidic in reaction. It is insoluble in water and soluble in all the usual organic solvents. It will dissolve in alkalis giving brown solutions which are colloidal, owing to the presence at this stage of a small amount of higher fatty acid. This latter may be separated from the pigment by solution in methyl alcohol or in acetone, from which there is deposited on cooling a small amount of white material, soluble in light petroleum, and of M.P. approximately 32°; this product forms a colloidal solution in alkalis and gives a lead salts soluble in ether. It is presumed to be a fatty acid, but insufficient material has been available for further investigation. Its removal leaves the properties of the pigment unchanged, except that the latter now gives clear brown solutions in alkalis. A great variety of procedures has been tried with a view to obtaining the pigment (or a derivative of it), in crystalline form, all without success. Similarly, persistent qualitative investigation has yielded nothing tangible. Such evidence as we have suggests that the pigment is an unsaturated aliphatic acid of relatively small molecular dimensions. There is certainly no support for the suggestion of Hutchinson & Clayton that it is a carotenoid pigment. Chemically, the outstanding points to note are the great ease with which it is oxidized by permanganate (acid, alkaline or neutral) and the formation of iodoform on treatment with alkali and iodine.

Miscellaneous products

The most important of these quantitatively is carbon dioxide. As will be seen in the next section, approximately two-thirds of the cellulose which is decomposed appears as carbon dioxide. Apart from a slight initial amount, apparently due to the inoculum, the evolution of carbon dioxide synchronizes with the visible signs of decomposition.

The presence of material judged to be higher fatty acid has already been recorded. The amount is so small as to suggest the possibility that it is a structural constituent rather than metabolic product of the organism. Of volatile fatty acids, as reported in traces by Hutchinson & Clayton, we could find no evidence.

A small amount of some carbohydrate accompanies the pigment in the early stages of its separation; it must therefore be soluble in 75% alcohol. Preliminary evidence indicates that it is non-reducing, but gives rise to pentose on hydrolysis.

Somewhat similar products can be obtained by fractionation of the crude mucilage. Doubtless they represent late stages in the degradation of cellulose.

Finally, mention should be made of the odour arising from large-scale cultures, such as 2 l. On the small scale, the odour is too slight to detect. This odour is quite distinctive and is an invariable feature in massive cultures. It is difficult to describe; it is not unpleasant to most people, although rather sickly; it recalls the aroma of a stagnant woodland pool. We have not succeeded in finding a suitable vehicle for trapping it and it is produced in extremely small amount in all probability.

It will thus be seen that there are several by-products of metabolism, produced only in small amounts, which it seems desirable to investigate further. In consequence, attention has lately been directed towards increasing the rate of decomposition by the organism, with a view to collecting larger amounts of metabolic material.

Improved method of cultivation

It was obvious that a plentiful supply of oxygen was the most important requirement and various methods of cultivation have been tried with this in mind. Conditions which ensure a plentiful supply of oxygen often minimize the amount of fluid medium, whereas the second desideratum for vigorous growth of *Cytophaga* is that the mucilage formed should be washed away from the unattacked cellulose fibres. We have now succeeded in achieving both these objectives by the use of flasks similar to those devised by Kluyver [1925] but of 2-l. capacity. In these flasks, the air, entering through the medium, is broken up into fine bubbles by means of a sintered glass filter; this ensures saturation of the medium with oxygen and efficient agitation at one and the same time. Using this form of cultivation, we have been able to increase the total decomposition of cellulose in a culture and to shorten its duration (see Table IV). By this means we hope to collect sufficient material for further investigation.

Quantitative results

The following data are supplied as a guide to the fate of the cellulose which disappears under the attack of *Cytophaga*. They do not pretend to give an exact carbon balance sheet, since they were largely accumulated in experiments concerned primarily with the collection of metabolic products. For instance, it was our regular practice, whatever the purpose of the experiment, to determine the amount of cellulose not attacked. The procedure for carrying this out has already been described.

Some idea of the extent to which decomposition takes place may be gained from Table IV, which records the residual cellulose in most of our experiments of a comparable nature.

Experiments omitted from Table IV are concerned with variations in the medium or other conditions.

It will be seen that a general increase in the amount of decomposition was effected through the substitution of cellulose wadding for filter paper. The introduction of cigarette paper resulted in still greater decomposition, but this type of cellulose has drawbacks, which have been mentioned earlier. The table brings out the marked improvement arising from the use of Kluyver type flasks, especially when regard is paid to the duration of such experiments. The lesser degree of decomposition in the case of cellophane (Ex. 69) is due to its texture and greater tendency to sink, with consequent anaerobiosis.

Table IV

Exp. no.	Duration in days	Type of cellulose	Initial amount of cellulose g.	Residual cellulose g.	Cellulose lost g.	Decomposition %
26	23	C.F.P.	20	13·11	6·89	34·45
29	22	C.F.P.	20	13·77	6·23	31·15
32	31	C.F.P.	20	16·24	3·76	18·80
33	28	C.F.P.	20	16·84	3·16	15·80
34	26	C.F.P.	20	17·18	2·82	14·10
35	28	C.F.P.	20	15·68	4·32	21·60
36	30	C.W.	12	7·17	4·83	40·24
38	24	C.W.	20	14·45	5·55	27·75
39	18	C.W.	10	6·88	3·12	31·20
41	30	C.W.	20	15·77	4·23	21·15
42	23	C.W.	20	13·59	6·41	32·05
43	20	C.W.	20	14·58	5·42	27·10
44	20	C.W.	20	14·27	5·73	28·65
45	20	C.W.	20	14·64	5·36	26·80
46	20	C.W.	20	15·33	4·67	23·35
47	49	C.W.	20	12·32	7·68	38·40
48	38	C.W.	20	14·71	5·29	26·45
50	28	C.W.	40	25·48	14·52	36·30
52	37	C.W.	40	28·88	11·12	27·80
54	21	C.W.	40	24·08	15·92	39·80
56	27	C.W.	40	28·00	12·00	30·00
58	28	C.W.	40	21·47	18·53	46·32
60	28	C.W.	40	27·85	12·15	30·40
62	47	C.W.	40	25·87	14·13	35·32
66	27	C.W.	40	25·84	14·16	35·37
67	36	C.P.	20	8·71	11·29	56·45
68	40	C.P.	20	10·46	9·54	47·70
69	40	Ce.	20	13·88	6·12	30·60
72*	10	C.W.	15	7·65	7·35	49·00
73*	21	C.W.	7·5	2·4	5·1	68·00
75*	8	C.P.	5·29	2·62	2·67	50·47
77*	7	C.P.	5·4	2·84	2·56	47·41
					Mean	33·40

C.F.P. = Chopped filter paper. C.W. = Cellulose wadding.
C.P. = Cigarette paper. Ce. = cellophane.
* Kluyver flask.

It is thus apparent that about one-third of the cellulose was decomposed in most of our experiments. With a view to tracing the fate of the cellulose which disappeared, two series of experiments were carried out. In the first of these, the carbon dioxide evolved was measured and is related in Table V to the carbon removed. The carbon dioxide was absorbed in a series of baryta bottles, the contents of which were subsequently titrated; in some experiments aeration was by means of oxygen from a cylinder and in others by suction applied to the Kluyver flask.

Table V

Exp. no.	Duration in days	Initial cellulose g.	Residual cellulose g.	Carbon disappeared g. (A)	Carbon evolved as CO_2 g. (B)	Ratio B/A
1	—	1·0	0·799	0·0893	0·0493	0·552
2	—	1·0	0·661	0·1507	0·0988	0·6557
3	—	1·0	0·6295	0·1646	0·0916	0·5564
4	—	1·0	0·7975	0·09	0·04815	0·535
5	10	15·0	7·653	3·265	2·147	0·6575
6	8	5·407	2·844	1·139	0·775	0·6789

It would seem therefore that the ratio of carbon appearing as carbon dioxide to carbon disappearing is fairly constant and that the carbon dioxide accounts approximately for two-thirds of the cellulose which is decomposed. The remaining third of the carbon lost is accounted for by the mucilage principally and to a lesser extent by the other metabolic products already mentioned. That this is so, we have established by estimating in another series of experiments the carbon in solution, after the manner of Birkinshaw & Raistrick [1931]. The results are given in Table VI.

Table VI

Exp. no.	Duration in days	Initial cellulose g.	Residual cellulose g.	Carbon disappeared g. (A)	Carbon in solution g. (B)	Ratio B/A
35	28	20	15·68	1·92	0·638	0·332
37	28	32	24·679	3·254	1·212	0·372
38/39	22	30	21·328	3·854	1·419	0·3681
41/42	26	40	29·36	4·507	1·589	0·3606
44	20	20	14·27	2·55	0·91	0·3569

It is obvious that these figures, although from a different series of experiments, form the complement to those in Table V. It will also be noticed that there is no significant loss of carbon due to any volatile product; the substance responsible for the aroma can thus arise only in traces. To sum up: two-thirds of the carbon disappearing is in the form of carbon dioxide and one-third remains in solution. This latter is made up of mucilage and other products, and with a view to assessing the relative amounts of each we have attempted the direct measurement of the mucilage. In Table VII below are recorded residual cellulose, carbon dioxide evolved and mucilage formed in each of two experiments. The mucilage was separated as described on p. 36, washed with a little alcohol, dried and weighed. The figures for it given in Table VII are the actual weight of mucilage multiplied by the factor 0·7425, derived from the relative carbon contents of mucilage and cellulose.

Table VII

	Exp. 72 g.	Exp. 78 g.
Initial cellulose	15·0	10·0
Residual cellulose	7·653	6·054
CO_2 as cellulose	4·831	2·618
Mucilage as cellulose	1·782	0·726
Unaccounted for	0·734	0·602

The cellulose not accounted for must be allocated to the formation of the pigment and other metabolic products mentioned in the text as being present only in small amounts.

Discussion and Conclusions

The metabolism of *Cytophaga* presents many interesting points, some of which cannot be discussed here; such are its specific requirement of the most complicated carbohydrate and its simple requirements in all else; its mode of attack on cellulose, particularly the initial reaction; the relation of its activities to those of other micro-organisms in nature. Confining ourselves to the knowledge so far gleaned, we may say that the principal product of the decomposition of cellulose by *Cytophaga* is carbon dioxide, accounting as it does for some two-thirds of the cellulose disappearing. This must be a significant factor in bringing

back into the carbon cycle the immense amounts of cellulose returned to the soil annually. The extent of the decomposition, too, is important, as much as 68% in 21 days and 50% in 8 days having been achieved. It may be contended that this is under laboratory, and therefore optimal, conditions, but the two are not synonymous. Admittedly everything possible is done to facilitate the decomposition, but this may not be as effective as natural processes. Then again the duration of the laboratory experiment is but a fraction of the time available in natural decomposition.

If carbon dioxide is the principal product, it is not the most interesting. It is a big jump chemically from this, the simplest product, to the mucilage. There can be little doubt that this substance should be classed as oxycellulose. This is what Winogradsky suggested, although his evidence was qualitative and he was led to remark that the absence of reducing power constituted an essential difference between biological and chemical oxycelluloses. This distinction does not seem to us to be called for, since oxycellulose is admittedly an indefinite substance. There are, furthermore, two types of oxycellulose generally recognized; an acidic type, having no reducing value and an aldehydic type, having a relatively high copper number. The mucilage has no reducing value and should therefore belong to the acidic type of oxycellulose; this type has a high methylene blue number and does not give a yellow colour with alkalis, features with which the mucilage is in harmony. Nevertheless, it would be safer perhaps to describe it as *an* oxycellulose and more particularly as an oxycellulose formed in alkaline solution. It would seem that the primary alcoholic groups of the cellulose molecule are oxidized to carboxyl throughout the length of the chain; that the consequent instability causes (or renders easier) fragmentation of the large molecule and that the partially oxidized fragments are further attacked by the organism, but that the attack is not carried to completion. To account in this manner for the occurrence of the mucilage implies that any given molecule of cellulose is only partially consumed, which we find easier to believe than that a certain proportion (2/3) of the oxidized cellulose is completely consumed and the remainder not further attacked. It may be remarked here that although the organism seems to require solid cellulose to act upon, micellar structure is not important, as evidenced by the fact that it can utilize cellophane and parchment paper. In discussing here the mucilage as a single entity we are not unmindful that in reality it is probably a mixture, as has been pointed out earlier (p. 37); but in our fractionation the complex acidic polyuronide or oxycellulose predominates and it is this substance we call the mucilage. The second component, and possibly others, are much smaller in amount and may be considered to be later stages in a series of degradations, of which the yield is progressively less and which culminate in carbon dioxide. It is possible that the carbohydrate or polyuronide noted amongst the traces of metabolic products represents some such later stage; whilst the pigment as well may be a mere by-product of the penultimate stage of degradation. The composition of the mucilage is such that the formation of furfuryl derivatives is by no means a remote contingency; the formation of yellow pigment and the slight aroma are also not incompatible with some connexion with furfuraldehyde. We have often searched for evidence for the existence of furfuryl derivatives in the various metabolic products, but such evidence, if occasionally suggestive, has been on the whole negative.

There remains one further point to which attention should be directed. It is our general experience that the mucilage is not readily attacked by bacteria, although it does succumb to moulds. This resistance to bacterial decomposition, together with its great capacity for holding water, suggests that it might well be

one of the "organic colloids" of the soil. Most workers who have handled the material are impressed with its resemblance to humic substances and its location, mode of formation and general properties fit in well with such a role.

Summary

1. The metabolism of the aerobic, cellulose-decomposing organism, *Cytophaga Hutchinsoni* has been studied and the cultivation of the organism described.

2. Of the cellulose decomposed by the organism, it is shown that two-thirds appear as carbon dioxide and one-third principally as mucilage, together with small amounts of other products.

3. Evidence is presented which supports the view that the mucilage is to be considered as an oxycellulose of the acidic, non-reducing type. Xylose has been identified amongst its hydrolysis products.

4. The yellow pigment formed is considered to be an unsaturated aliphatic acid, of relatively small molecular dimensions, and is not, as stated in the literature, a carotenoid pigment.

5. Other metabolic products noted, but in traces only, are: a higher fatty acid, a carbohydrate soluble in 75% alcohol and an unidentified constituent responsible for a slight aroma. No evidence was obtained for the existence of volatile fatty acids.

6. By means of a new technique, ensuring luxuriant oxygenation, it is possible to achieve a 50% decomposition of cellulose in about eight days.

7. Attention is drawn to the biological significance of this type of decomposition.

The authors are grateful to Prof. R. A. Peters for his interest in this work.

REFERENCES

Birkinshaw & Raistrick (1931). *Philos. Trans.* B, **220**, 11.
Bokor (1929). *Arch. Mikrobiol.* **1**, 1.
Carré & Haynes (1922). *Biochem. J.* **16**, 60.
Dorée (1933). The Methods of Cellulose Chemistry. (London.)
Hutchinson & Clayton (1919). *J. agric. Sci.* **9**, 143.
Khouvine (1934). Exposés de chimie biologique, No. 164.
Kluyver (1925). *Biochem. Z.* **161**, 361.
Kröber (1900). *J. Landw.* **48**, 357.
Krzemieniewska (1933). *Arch. Mikrobiol.* **4**, 394.
Nanji, Paton & Ling (1925). *J. Soc. chem. Ind., Lond.*, **44**, 253 T.
Pringsheim (1932). Chemistry of the Saccharides. (New York.)
Rippel & Stoess (1932). *Arch. Mikrobiol.* **3**, 492.
Thaysen & Bunker (1927). The Microbiology of Cellulose, Hemicelluloses, Pectin and Gums. (Oxford.)
van Iterson (1904). *Zbl. Bakt.* **11**, 689.
Waksman (1931). Principles of Soil Microbiology. (London.)
Walker & Warren (1934). *J. Soc. chem. Ind., Lond.*, **53**, 452.
Winogradsky (1929). *Ann. Inst. Pasteur*, **43**, 549.