XXV. SOIL ORGANIC MATTER AS A CULTURE MEDIUM FOR *AZOTOBACTER*.

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(Received May 10th, 1915.)

Not the least important of the functions performed by soil organic matter is that of providing food material for the bacterial flora upon the activity of which the fertility of the soil so largely depends. The problem of maintaining soil fertility often resolves itself into a question of promoting bacterial activity, and it is safe to say that the most important of all the soil organisms are those concerned in the nitrogen cycle. The nitrifying organisms assimilate the carbon dioxide of the air, and are thus independent of organic carbon; the ammonifying organisms can do their work when provided with the humus, or any organic matter containing nitrogen, so long as potassium salts are present [Dumont, 1905]; but the nitrogen fixing organisms, and especially *Azotobacter*, are generally supposed to be somewhat sensitive in their requirements, flourishing only upon specialised non-nitrogenous organic compounds. A wide variety of such substances pass into the soil from the natural accumulations of humus by the ploughing in of green crops and in the application of rotted farm and stable manure. These compounds undergo decomposition in the soil through the agency of many micro-organisms, including the spores and mycelia of the higher fungi [C. van Iterson, 1904]. The various enzymes which are introduced with the organic matter remain active in the soil [Woods, 1899], and assist in the further decomposition of these compounds; while the intact roots of plants have the power of oxidising the organic matter and bringing about an appreciable change in its composition [Molisch, 1887; Schreiner and Reed, 1909]. The higher organisms

such as worms are active in splitting up the fats and other complex substances; while in addition to these organic agencies at work, there must be taken into account the purely chemical changes which are constantly taking place. The nett result of all these reactions is the production of a large number of organic compounds, both nitrogenous and non-nitrogenous; the latter ranging in complexity from carbohydrates down to the simplest alcohols. It is generally assumed that carbohydrate in some form is essential for the nutrition of *Azotobacter*, although cultures have been obtained upon certain other substances, notably some of the vegetable acids, and the object of the present investigation was to discover how far the organism is capable of utilising the wide range of non-nitrogenous organic compounds which occur in the soil.

Method of work.

A uniform method of procedure was adopted throughout the work. The culture medium employed, with variation of the source of organic carbon only, was that used by Bottomley and given in detail in a previous communication by the writer [Mockeridge, 1912]. For the determination of the nitrogen fixing power of the organism upon each substance, 50 cc. portions of the medium, containing 1 % of the nutrient, were placed in each of six 300 cc. Erlenmeyer flasks, and sterilised in the autoclave at a temperature of 140°. Where organic acids were employed, sufficient calcium carbonate to neutralise them was added to each flask, and in the case of volatile organic substances, these were not added to the medium until the flasks containing the inorganic salts in solution had cooled down after sterilisation. Each flask was provided with a sand slope consisting of 50 grams of pure sterile sand, and was then inoculated with 1 cc. of a uniform suspension in distilled water of *Azotobacter* from a mannitol-agar plate. Two flasks of each series were then again sterilised to serve as controls, and the whole series was incubated at a temperature of 26° until all trace of the organic substance originally supplied had disappeared from the medium, when the contents of each flask were analysed by the Kjeldahl process for its nitrogen content.

Humates.

It is doubtful whether soluble humates can serve as a source of food material for *Azotobacter*, although it has been proved [Krzemieniewski, 1908] that they stimulate the activity of the organism, when the latter is already

provided with another nutrient; and if this takes place in artificial media and under the abnormal conditions of the laboratory tests, the probability is that it occurs more markedly still in the soil. In the present work it was found possible to obtain a growth of *Azotobacter* upon a solution of ammonium humate containing the requisite mineral salts, although no increase in the nitrogen content of the medium took place; but such growth could not be obtained upon media containing potassium or calcium humate without any other source of organic carbon. The organism thus appears to be able to utilise the humates as a source of energy if provided with nitrogen already fixed, and ammonium humate is present in all fertile soils in small, though appreciable, quantities; the ammonia produced by the decomposition and ammonifying organisms uniting with the humic acid in the organic matter.

Polysaccharides.

The widespread occurrence of various gums and starches in vegetable tissues is well known, and these substances must be transferred to the soil in all accumulations of plant débris. The value of dextrans as a source of carbon for mixed cultures of nitrogen-fixing organisms has already been pointed out [Bottomley, 1912]; and since the enzyme (amylase) directly concerned in their production is practically always found in foliage leaves [Brown and Morris, 1893], the addition of these bodies to the soil in green manures, as well as their production in the soil itself and in rotting manure-heaps, by hydrolysis of cellulose and starch are matters of certainty. Inulin is also a common food reserve in plants. As therefore the various polysaccharides must naturally occur in soils, an examination was made of the availability of certain of them as nutrients for *Azotobacter*. Gum arabic, gum tragacanth, starch, dextrin and inulin were tested, and all proved to be readily available for the organism.

The fixations obtained were as follows:

TABLE I.

Substance	Nitrogen fixed on 1 gram (aver- age of 4 determin- ations). Mg.	Time taken completely to use 1 gram of the substance. Days
Gum arabic	6.13	60
Gum tragacanth .	9.13	20
Rice starch	6.40	18
Potato starch . . .	5.93	20
Dextrin	6.62	20
Inulin	9.76	16

Sugars.

Sugar in some form is the usual nutrient supplied to *Azotobacter* in artificial cultures, and the power of the organism to utilise most of the more readily obtainable sugars was tested. These included pentoses and hexoses among the monosaccharides, and some of the disaccharides.

The results obtained were as follows:

TABLE II.

Sugar	Nitrogen fixation on 1 gram (aver- age of 4 deter- minations). Mg.	Time taken completely to use 1 gram. Days
Arabinose	9.28	30
Xylose	9.00	28
Dextrose	6.57	20
Laevulose.....	10.32	18
Galactose	6.20	22
Sucrose	7.28	22
Maltose	7.55	17
Lactose	3.39	34

The fact that the organism is capable of utilising two of the pentoses, arabinose and xylose, renders it probable that other members of the group, which occur somewhat widely in nature, are also available; for pentose sugars are formed both in the soil and in the manure-heap by the hydrolysing action of certain bacteria and enzymes upon gums, pectin compounds, naturally occurring glucosides, nucleic acids, etc. Recently a pentosan of undetermined nature, and also rhamnose itself, have been isolated from samples of loam [Schreiner and Shorey, 1910; Shorey, 1913]. Similarly the wide distribution of the hexoses and disaccharides in nature, and the variety of the agencies by which they may be produced in all decomposing vegetable matter, indicate a wide range of available food for *Azotobacter* in the soil.

Alcohols.

Among the substances tested as a source of food for *Azotobacter* were some of the monohydric alcohols, besides representatives of some of the more complex alcoholic compounds, as ethylene glycol (a dihydric alcohol), glycerol (trihydric), erythritol (tetrahydric) and mannitol (hexahydric). The fact that substances of an alcoholic nature are readily assimilable by *Azotobacter* is shown in the following table:

TABLE III.

Alcohol	Nitrogen fixed on 1 gram (average of 4 determin- ations). Mg.	Time taken completely to use 1 gram. Days
Methyl alcohol ..	2.1	42
Ethyl alcohol ...	4.02	34
Propyl alcohol ..	9.2	22
Isobutyl alcohol .	4.69	72
Ethylene glycol .	16.74	34
Glycerol	5.0	56
Erythritol	4.88	60
Mannitol	11.62	15

Only one determination of the nitrogen fixed upon ethylene glycol was made, on account of the difficulty of obtaining larger quantities of the compound; but a very copious growth of the organism upon this nutrient was obtained.

Flasks containing amyl alcohol and inoculated with *Azotobacter* showed no growth of the organism at the end of a month, but upon a solid medium obtained by the use of agar-agar a fair growth appeared upon this nutrient. No determinations were made of the nitrogen fixed.

The alcohols are of very frequent occurrence in nature, and appear to be sufficiently abundant in soil organic matter to warrant their inclusion among the bacterial foodstuffs of the humus. The possible sources in the soil of the alcohols mentioned in the above table are indicated below. Methyl alcohol occurs in combination in various plants, and is produced in the free state in cow dung by the action of *B. boöcopricus*. Ethyl alcohol is a well-known product of yeast fermentations and of practically all bacterial action, while propyl alcohol usually accompanies it in small quantities as a by-product. Butyl alcohol is formed during butyric fermentation, while under certain conditions *B. amylozyma* will produce amyl alcohol. Ethylene glycol is very probably a by-product of bacterial action in the soil, for one of its higher homologues, butylene glycol, has been shown to be formed during the action of *B. lactis aerogenes* on glucose and mannitol [Harden and Walpole, 1906]. Glycerol is a well-known by-product in yeast fermentations and in many bacterial actions; erythritol is found in the free state in lichens, mosses and seaweeds, while mannitol occurs naturally in many plants. The last-named substance has been isolated in fairly large quantities from one soil [Shorey, 1913].

Hence it is probable that substances of an alcoholic nature occur fairly widely in the soil, and are sufficiently abundant to provide a convenient source of food for *Azotobacter*.

Vegetable acids (other than aliphatic).

The organic acids are constant products of decomposition of practically all organic matter, and so probably most of them occur in soils and manures. The nitrogen-fixing power of *Azotobacter* when grown upon a medium containing the calcium salts of the organic acids was tested, with the following results:

TABLE IV.

Acid	Nitrogen fixed upon 1 gram (average of 4 determinations)	Time taken to use completely 1 gram.
	Mg.	Days
Citric	6.44	48
Malic	5.19	16
Tartaric	4.54	28
Racemic ...	2.77	50
Succinic	8.60	20
Malonic	5.32	20
Mucic	6.79	40
Fumaric ...	2.00	50
Maleic	1.88	50
Glycollic ...	1.75	60
Lactic	12.01	17

The vegetable acids are widely distributed in nature, citric acid, malic acid and tartaric acid and its stereo-isomers being among the most commonly occurring; the first-named, besides its occurrence in the free state, arises also as a product of the action of various micro-organisms. Succinic acid is also found in many plants, besides which it is a product of putrefaction and occurs in the urine of goats, horses, rabbits and cows, so that it is probably transferred to the soil in rotting manure, in which is also to be found lactic acid—a product of the lactic fermentation of carbohydrates in the presence of nitrogenous animal matter. The remaining acids tested, mucic, glycollic, maleic, fumaric and malonic, though they do not occur in plant residues, so far as is known at present, may readily be produced in soil or manure from the other acids enumerated, or from other organic substances by the various agencies at work during the decomposition of soil organic matter. That mucic acid, for instance, probably exists in the soil is indicated by the fact that saccharic acid, an isomeric form, has actually been isolated [Shorey, 1913], although this is not known to occur in plants. From the same soil were isolated oxalic, succinic and acrylic acids, while from other samples paraffinic, lignoceric, α -hydroxystearic and some resin acids have been isolated [Schreiner and Shorey, 1910], while still other soils have been found to contain dihydroxystearic, picolinecarboxylic and agroceric acids [Schreiner and Shorey, 1909].

Meconic acid was found to be useless as food for *Azotobacter*, but the substance itself is not inhibitory to the organism, for good cultures were obtained upon mannitol-agar plates containing meconic acid. Probably some of its products of decomposition have a deleterious effect. The poisonous nature of oxalic acid accounts for the fact that it is not available as a source of food for the organism.

Organic acids of any kind are useless to *Azotobacter* until neutralised by lime or some other base, but this presents no difficulty, since there is abundance of lime for the purpose in all fertile soils.

Fatty acids.

In the series of aliphatic acids, those tested with regard to their availability for *Azotobacter* included formic, acetic, propionic, butyric, isovaleric, stearic and palmitic acids. On the last three no growth was obtained in liquid culture, but when the medium was solidified by the addition of 2 % agar-agar, and poured into Petri dishes, a fairly abundant growth was obtained upon the surface. The organism is thus capable of utilising these substances as a source of energy, although it requires special conditions, probably connected with air supply. No determination of the nitrogen fixed upon these substances was made. Formic, acetic, propionic and butyric acids formed readily available sources of food, and the figures obtained upon these media are given in greater detail than has hitherto been the case, since further reference will be made later to these results.

The fatty acids are very widely distributed in nature. The more complex fats and oils are of almost universal occurrence in the vegetable kingdom, being found as reserve stores in fruits and seeds. Oil cakes of various kinds form a large part of the diet of animals at fattening time, so that an appreciable quantity of undigested fat and oil is probably transferred to the manure heap. These complex bodies of a fatty nature undergo decomposition through bacterial action under certain conditions of light and aeration with production of the simpler aliphatic acids. Reserves of fat in plants are always accompanied by the fat splitting enzymes, so that in the soil the decomposition of the fat into glycerol and a fatty acid, most commonly oleic, palmitic and stearic, may proceed. The lower aliphatic acids are also distributed in fair quantity throughout rotting manures, being formed by the action of bacteria upon many substances, especially upon the degradation products of the proteins; so that members of this group, when

neutralised by lime or some other base, serve as a readily available source of food for *Azotobacter*.

TABLE V.

Nature of medium	Nitrogen content, mg.	Fixation on $\frac{1}{2}$ g., mg.	Fixation on 1 g., mg.	Mean Fixation, mg.	Time taken to use 1 g.
50 cc. 1 % formic acid control	0.29				
	0.29				
" " with <i>Azotobacter</i>	0.91	0.62	1.24	1.47	60 days
" " "	1.22	0.93	1.86		
" " "	1.06	0.77	1.54		
" " "	0.91	0.62	1.24		
50 cc. 1 % acetic acid control	0.16				
	0.16				
" " with <i>Azotobacter</i>	2.26	2.10	4.20	3.77	24 days
" " "	2.12	1.96	3.92		
" " "	1.98	1.82	3.64		
" " "	1.83	1.67	3.34		
50 cc. 1 % propionic acid control	0.41				
	0.41				
" " with <i>Azotobacter</i>	3.14	2.72	5.44	5.16	26 days
" " "	2.86	2.44	4.88		
" " "	3.00	2.58	5.16		
" " "	3.00	2.58	5.16		
50 cc. 1 % butyric acid control	0.14				
	0.14				
" " with <i>Azotobacter</i>	3.18	3.04	6.08	6.08	48 days
" " "	3.04	2.90	5.80		
" " "	3.32	3.18	6.36		
" " "	3.19	3.05	6.10		

Esters.

The ethereal salts, or esters, occur widely, although in small quantity, in the essences of flowers and fruits and certain leaves. They probably find their way into the soil from these sources, and there undergo decomposition, through the agency of the lipases or other hydrolysing agents, into their two primary constituents; but even in the free state they are able to serve as a source of energy for *Azotobacter*. Two only of the simple esters were tested: ethyl and butyl acetates, and upon neither of these in liquid culture could any growth be obtained. Upon agar plates containing these substances, however, a fairly abundant, though slow, growth took place.

Glucosides.

The glucosides are of very frequent occurrence in plant substances, but their availability as food for this nitrogen-fixing organism appears to be restricted somewhat by the products of their decomposition. An abundant

growth, but a very small fixation of nitrogen, was obtained upon a solution of amygdalin, a glucoside occurring in bitter almonds and the kernels of several stone fruits. This glucoside upon hydrolysis gives rise to glucose, benzaldehyde and hydrocyanic acid, and these are the products of the action of yeast upon it. The glucose, of course, is immediately available for *Azotobacter*, and neither the hydrocyanic acid nor the benzaldehyde appears to be inhibitory to its growth. Hydrocyanic acid is found extensively in the free state in plants, so that small quantities of this substance are evidently not deleterious to plant life.

Most of the naturally occurring glucosides, e.g. salicin, give an alcohol upon hydrolysis, and salicin was found to be useless for the growth of *Azotobacter*, either in liquid or solid media. This fact points to the conclusion either that the organism has no action on the glucoside or that its first action is a hydrolytic one; for salicin, upon hydrolysis, gives glucose and salicyl alcohol, which latter is a powerful antiseptic, and would accordingly inhibit the growth of the organism. The substance itself is not harmful to *Azotobacter*, for an abundant growth of the organism was obtained on mannitol-agar plates which contained salicin; a fact which indicates that it must be the decomposition products of the salicin which have a deleterious effect.

Benzene derivatives.

A similar state of affairs was found to obtain with all the benzene derivatives tested. These included phenylacetic acid, mandelic (phenylglycollic) acid, benzyl alcohol, pyrogallol, quinol and catechol. These substances will not serve as a food supply for the organism in either liquid or solid media, yet when mixed into mannitol-agar plates do not inhibit its growth. Such substances as benzyl alcohol, catechol, pyrogallol and quinol are not readily split up chemically, and the great stability of the benzene ring may account for the unsuitability of these substances as food for *Azotobacter*; moreover, from such substances as phenylacetic and phenylglycollic acids there is always the possibility of the formation of phenol, and this product might prevent any further bacterial growth. The inability of the organism to utilise substances of this nature thus apparently depends not upon any harmful effect produced by the compounds themselves, but either upon the injurious secondary products formed by their decomposition, or on the difficulty of breaking up the stable benzene ring.

General considerations.

The results obtained indicate the wide range of substances which are available as a source of energy for this nitrogen-fixing organism in the soil: substances which occur naturally and may be added to the soil in green manures or in accumulations of vegetable débris; compounds which may be produced by fermentation of rotting manures and added to the soil in that way; and products which arise by secondary and often purely chemical reactions in the soil itself. It is of interest to note also, that the large number of organic substances present in the soil, which the organism cannot utilise, do not necessarily inhibit its growth, so long as some available food material is present; for *Azotobacter* appears to be possessed of considerable selective power.

All classes of compounds do not give equally good returns of nitrogen fixed for the expenditure of food material, and, generally speaking, it seems to be the rule that the longer the time taken to use completely unit mass of the nutrient, the less the nitrogen fixed upon that medium. Though they serve as a general guide, too much reliance must not be placed upon the figures obtained in this respect, for, as the author has pointed out in previous work, the nitrogen-fixing power of the organism varies considerably with the length of time during which it has been grown upon artificial media.

In order to compare correctly the relative availability of a large number of nutrient substances, the same strain of *Azotobacter* should be used throughout for inoculating purposes, and this means that all the cultures required should be incubated at once, whereas in practice the work has to be carried out in sections.

Generally speaking, the carbohydrates give the greatest yield of nitrogen fixed per unit of material consumed, and it is only to be expected that in the breaking down of the more complex molecule more energy should be obtained by the organism, and thus that more growth and consequent fixation of nitrogen should take place. *Azotobacter* is essentially an aerobic organism, and Beijerinck and van Delden [1902] observed that it absorbed oxygen and eliminated carbon dioxide, their observations being confirmed by the careful work of Krzemieniewski [1908]. This points to a slow combustion of the organic material provided; and some idea of the energy thus obtained by the organism can be gained by a calculation of the heat of combustion of the carbon compound supplied. When these figures are calculated for the aliphatic acids and compared with the amounts of nitrogen fixed upon those substances as given in Table V, the following results are obtained:

TABLE VI.

Acid	Molecular weight	Heat of combust. of 1 gram-molecule	Heat of combust. of 1 gr.	N fixed on 1 gr.	Ratio N fixed: Heat of combust.
Formic	46	68300 cal.	1484 cal.	1.47 mg.	1 : 1009
Acetic	60	230900	3848	3.77	1 : 1020
Propionic ...	74	393500	5317	5.15	1 : 1032
Butyric	88	556100	6319	6.08	1 : 1039

Within the range of experimental error, these ratios may be taken to be approximately constant, a result which points to the conclusion that in these four media, the decomposition of the respective organic constituents follows the same course, a very definite proportion of the energy thus obtained being devoted to the fixation of nitrogen.

It is obvious that, on account of the varied nature of the possible products of decomposition, such comparisons can only be made between substances of similar constitution; but the figures obtained for some of the carbohydrates are of interest.

TABLE VII.

Carbohydrate	Heat of combustion of 1 gram	Fixation on 1 gram	Ratio N fixed: Heat of combustion
Starch ...	4145 cal.	6.40 mg.	1 : 647
Dextrin ..	4145	6.62	1 : 626
Gum arabic	4145	6.13	1 : 676
Inulin	4145	9.76	1 : 424

Allowing for error of experiment, which is considerably greater here than in the case of the simpler compounds, owing to the difficulty of ascertaining that all the secondary products of the carbohydrate have completely disappeared, the ratios for starch, dextrin and gum arabic may be taken to be approximately equal. Assuming that hydrolysis takes place, as it must do, the starch will be converted finally into dextrose, with dextrin and maltose as intermediate products. A comparison of the ratios obtained with these substances is found to be

The ascending order of these ratios bears out the assumption that a hydrolysis of the polyoses takes place to form the simple hexoses, which latter are then oxidised. The ratio obtained for inulin (1 : 424) is surprisingly high as compared with those for starch, dextrin and gum arabic. However, inulin upon hydrolysis gives laevulose, and the ratio between the nitrogen fixed on one gram of laevulose and the heat of combustion of one gram of the same body is found to be 1 : 372, which compares very well with the inulin ratio.

The close relation which seems to exist in the series of aliphatic acids between the nitrogen fixed and the nutrient consumed is not apparent in all classes of homologous compounds, for the series of monohydric alcohols shows no such relation; and some of the simpler compounds, as ethylene glycol, show a surprisingly high rate of fixation of nitrogen for the heat-energy obtainable. However, nothing is known as to the actual mechanism of fixation. It may be that the nitrogen is attached to very simple organic compounds produced by the splitting up of the nutrient, and that certain substances give decomposition products which are peculiarly adapted to the purpose, while the secondary products from others may be practically useless. On the other hand, a synthesis may take place between the simpler bodies to form substances more complex, before the nitrogen is attached, certain nutrients furnishing the necessary synthetic products with less waste of energy and material than others. That synthesis of non-nitrogenous substances takes place in the cells of *Azotobacter*, apart from nitrogen assimilation, is shown by the fact that appreciable quantities of fat have been found stored up in the cells [Lipman, 1904]. By no means the whole of the energy obtainable from the nutrient is utilised in nitrogen fixation, for the author above quoted states that according to thermo-chemical calculations, one gram of mannitol would allow of the fixation of 97.3 mg. of nitrogen. However, the few data obtained appear sufficient to warrant the conclusion that a detailed study of energy considerations may help to throw some light upon the manner in which the nitrogen is fixed; but no matter what course this may follow, it is evident that any ordinary fertile soil contains abundant food material for the growth of *Azotobacter*.

REFERENCES.

- Beijerinck and van Delden (1902). *Centr. Bakt. Par. Abt.* II, 9, 3.
 Bottomley, W. B. (1912). *Proc. Roy. Soc., B.*, 85, 466.
 Brown and Morris (1893). *J. Chem. Soc.*, 629.
 Dumont, J. (1905). *Les Sols Humifères*, 44.
 Harden and Walpole (1906). *Proc. Roy. Soc., B.*, 77, 399.
 Iterson, C. van (1904). *Centr. Bakt. Par. Abt.* II, 11, 689.
 Krzemieniewski, S. (1908). *Bull. Inter. Acad. Sci. Cracovie*. No. 9.
 Lipman (1904). *New Jersey State Agric. Expt. Stn. Report*, 268.
 Mockeridge, F. A. (1912). *Annals of Botany*, 26, 875.
 Molisch (1887). *Sitzungsber. Akad. Wiss., Wien, math. nat. Kl.*, 96, 84.
 Schreiner and Reed (1909). *U.S. Dept. of Agric. Bureau of Soils, Bull.* 56.
 Schreiner and Shorey (1909). *U.S. Dept. of Agric. Bureau of Soils, Bull.* 53, 28.
 — (1910). *U.S. Dept. of Agric. Bureau of Soils, Bull.* 74, 13.
 Shorey (1913). *U.S. Dept. of Agric. Bureau of Soils, Bull.* 88.
 Woods (1899). *Centr. Bakt. Par. Abt.* II, 5, 745.