MECHANISM OF THE MICROBIOLOGICAL OXIDATION OF AMMONIA. PART III.

BY G. GOPALA RAO AND W. V. SUNDARA RAO.

In this paper evidence is presented to show that the organisms of Winogradsky are mostly responsible for nitrification in the soil. It is emphasised here that autotrophic rganisms can flourish well in the presence of organic materials, if in the same medium heterotrophic organisms capable of acting on the particular organic substance are also present.

It was Winogradsky who first isolated in pure culture the nitrifying organisms, the *Nitrosomonas* and the *Nitrosococcus*, autotrophic organisms, which can oxidise ammonia to nitrite. Winogradsky (*Ann. Inst. Pasteur*, 1890, **4**, 213, 215; 1891, **5**, 92, 571) showed that soluble organic matter is very toxic to the growth and respiration of the bacteria. The bacteria are highly specific and act only on one substrate, namely ammonia, oxidising it to nitrite in the presence of atmospheric oxygen and fixing at the same time carbon dioxide into materials necessary for cell growth.

Winogradsky and Omeliansky (*Centrl. Bakt.*, 1899, **5**, 338, 377, 429) further showed that glucose, peptone, asparagine, glycerol and urea are toxic to the organism in concentrations of 0·2 to 1% in liquid cultures. Since this classical work of Winogradsky, the influence of organic substances on nitrifying bacteria has been the subject of investigation by numerous workers. If organic substances in low concentrations prove toxic to the nitrifying organisms, knowing that soil contains many types of organic materials, there arises the important question, how can the nitrification in soil be explained? Further we know that nitrification is also very intense in sewage filters and the so-called nitre beds. To explain this three possible avenues have been explored by previous investigators.

It is possible that the organisms isolated by Winogradsky are not the organisms responsible for bringing about nitrification in the soil There may be other species of nitrifying organisms in the soil which either tolerate organic matter, or which actually require organic matter for their growth. Fremlin (*J. Hygiene*, 1903, **3**, 364; 1930, **29**, 236) reported the isolation of a nitrite-forming organism which is capable of growing in media containing organic compounds. Mishustin (*Ber. Bact. Agron. Sta. Moskau*, 1926, **24**, 189) found two spore-forming bacteria capable of producing nitrites in media containing organic nitrogenous compounds but not in inorganic media containing ammonium salts. Runov (*Centrl. Bakt.*, 1928, **7**, 193) reported two species of bacteria, one of them produced nitrites from organic nitrogenous compounds and the other from ammonia in the presence of organic substances Neither organism grew on Winogradsky's medium and the author concluded that there are many bacteria in nature capable of forming nitrites in media containing various organic substances. Joshi (*Mem. Dept. Agrl. India, Bact. Series*, 1915, No. 3, p. 85) reported a new nitrite-forming organism. Recently Cutler and Mukherjee (*Proc. Roy. Soc.*; 1931, **B**, **108**, 384) reported four species of soil micro-organisms differing completely from the autotrophic organisms of Winogradsky, not only morphologically but also in that they need organic matter. Referring to this work Russell ("Soil Conditions and Plant Growth", London, 1932, p. 331) states, "There seems little doubt that these are the organisms operative in sewage filters, intensive nitre beds and soils containing much organic matter." Cutler and Mukherjee first found these bacteria in the affluent from a beet sugar factory but have since shown that they are widely distributed in soil. It must, however, be said that the amount of nitrites produced by these organisms are very small, the maximum amount of nitrite nitrogen ever formed being only 3·2 parts per million. Even this tends to disappear in the later stages of the experiment. The Winogradsky organisms can produce under favourable conditions, enormous quantities of nitrite, as much as 200 mg. per liter in a few weeks of incubation. Regarding the work of Runov and Cutler and Mukherjee, Boltjes (*Archiv Microbiol.*, 1935, **6**, 79) states, "It may be said that one cannot be absolutely certain whether the traces of nitrites obtained by them were really produced by the oxidation of ammonia and not from the absorption of traces of oxides of nitrogen present in the laboratory atmosphere, or more likely through reduction of nitrates." In any case the amount of nitrites obtained by these workers after several weeks of incubation are so little that these bacteria cannot be very important for soil nitrification. Nelson (*Centrl. Bakt.*, 1931, II, **83**, 280) comes to a similar conclusion. Regarding the work of Fremlin, Boltjes states that Fremlin was very likely the victim of an error in technique.

The other approach to the problem has been made by a school of investigators who believe that the organisms of Winogradsky may not be so sensitive to organic substances in the soil as they are in liquid cultures. Wimmer (*Z. Hyg.*, 1904, **48**, 135) made tests with cultures in sand and concluded that organic matter is not so toxic as in solution, but was nevertheless toxic in great concentration. Bazarewski (*Koch's Jahrb.*, 1906, **17**, 452) from similar experiments arrived at the conclusion that 1% dextrose stimulated nitrification, while larger amounts delayed the process but did not entirely prevent it. Coleman (*Centrl. Bakt.*, 1908, III, **20**, 401) came to a similar conclusion working with sand and the soil cultures. While work with pure cultures may be of great importance when studying the various characteristics of the organisms, it must be conceded that results obtained in such studies cannot be applied *in toto* to the nitrification process in the soil. The soil is a very complex system, being the habitat of numerous heterotrophic and autotrophic organisms. The interplay of these organisms must be taken into account. Many years ago Omeliansky (*Centrl. Bakt.*, 1899, II, **5**, 473) showed that in combination with *Bacterium ramosus*, the nitrite forming organisms can grow and oxidise ammonia even in dilute peptone broth (1 in 20); Boltjes (*loc. cit.*) showed that nitrification can proceed in the usual medium containing in addition peptone and urea, if the medium is inoculated with *Bacterium pseudomonas* and "*urobacillus*." Pandalai (*Science*, 1936, **84**, 440) found that glucose did not prove harmful and in some cases proved a stimulant to nitrifying organisms in the presence of various heterotrophic organisms like *Azotobacter*, *Bacterium mycoides*, etc.

Moreover, nobody seems to have worked on the influence of the different organic substances that are more likely to be present in normal soils. It will be conceded that glucose, peptone, cane sugar, etc., which have been investigated, would at no time be present in the soil at such high concentrations as to prove detrimental to the action of the bacteria that are responsible for nitrification. The organic matter of the soil results mostly from plant residues, green manure and stable manures. The most important substances added to the soil may be classified into (1) the starches and sugars, (2) pentosans, pectins and other hemicelluloses, (3) true celluloses, (4) lignins and tannins, (5) fats, waxes and fatty acids, (6) proteins and the degradation products of all these substances and lastly (7) the soil humus. A systematic investigation of the influence of these substances at concentrations that are likely to obtain in the soil is necessary. In this paper are reported the results of the study of the influence of humic acid, cellulose, glucose, cane sugar, starch and an amino-acid alanin and the sodium salt of organic acids like citric, oxalic and acetic acids.

EXPERIMENTAL.

The method employed is the liquid culture technique described in Parts I and II of this series.

Influence of Humic Acid.

This is the name given to the final degradation product of organic materials in the soil, a dark coloured material relatively very stable, soluble in alkalis and precipitated from such solutions on neutralisation with acid. In the following table are reported the results on the influence of humic acid isolated from a garden soil. Merck's humic acid gave similar results.

TABLE I.

		Mg. of nitrite nitrogen per litre.			
Incubated for	Control.	0·05%.	0·1%.	0·5%.	1%.
5 days	2·2	Nil	Nil	Nil	Nil
15	3·0	6·1	,,	,,	,,
27	18·1	38·1	7·1	,,	,,
37	50·7	74·3	35·4	4·3	5·4
50	124·4	154·5	96.6	41·5	41·8
60	—	185·0	154·5	112·6	98·1

Duplicates were kept in every case.

TABLE II.

Influence of cellulose.

Pure cellulose obtained from cotton fibre by the usual method was used.

3 days	2·5	2·3	2·3		2·2
19	4·2	5·6	8·6		—
26	20·0	31.1	43·1		7·7
34	28·0	45·9	47·7		22·4
45	80·0	124·4	124·4		81·8
57	101·9	131·8	133·3		97·4

TABLE III.

Influence of starch.

3 days	2·5	4·7	4·6	4·0	4·7
19	4·2	Nil	2·1	Nil	Nil
26	20·0	13·0	33·9	,,	,,
34	28·0	22·4	43·9	,,	,,
45	80·0	70·0	124·4	8·0	4·5
57	101·9	115·4	134·4	26·7	22·4

TABLE IV.

Influence of cane sugar.

Merck's pure cane sugar was used.

		Mg. of nitrite nitrogen per litre.			
Incubated for	Control.	0·05%.	0·1%.	0·5%.	1%.
3 days	2·5	4·2	4·1	4·4	8·2
9	2·8	Nil	Nil	Nil	Nil
19	4·2	4·0	,,	,,	,,
26	20·0	65·9	,,	,,	,,
34	28	82·6	2·9	,,	,,
45	80	140·0	29·5	,,	,,
57	101·9	142·7	56·0	,,	,,

TABLE V.

Influence of glucose.

Merck's pure dextrose was used.

3 days	2·4	1·30	4·5	Nil	Nil
9	7·7	Nil	Nil	,,	,,
19	3·8	,,	,,	,,	,,
26	20·0	16·4	14·0	,,	,,
45	78·3	31·1	78·3	,,	,,
57	112·0	44·8	104·3	,,	,,

TABLE VI.

Influence of organic acids.

The sodium salt of the acid was used.

(*a*) *Acetate.*

		Mg. of nitrite nitrogen per litre.		
Incubated for	Control	0·005 *M.*	0·05 *M.*	0·2 *M.*
0 days	2·3	2·3	2·3	2·3
13	3·7	5·6	Nil	Nil
21	5·9	36·6	,,	,,
28	20·7	43·2	,,	,,

TABLE VII.

(*b*) *Oxalate.*

Incubated for	Control.	Mg. of nitrite nitrogen per litre. 0·005 *M*.	0·05 *M*.	0·02 *M*.
0 days	2·3	3·2	3·4	3·8
13	3·7	20·9	4·9	4·0
21	5·9	32·3	3·0	3·7
28	20·7	63·6	4·9	4·7

TABLE VIII.

(*c*) *Citrate.*

0	2·3	2·3	2·2	2·3
13	3·7	Nil	Nil	Nil
21	5·9	,,	,,	,,
28	20·7	9·3	6·0	,,

DISCUSSION.

It will be seen from the foregoing tables that humic acid does not inhibit the action of nitrifying bacteria at concentrations in which it is likely to be present in the soil and may even exert a stimulating action on the bacteria. So is the case with cellulose; this substance does not inhibit even at a concentration of 1%. With starch up to 0·1 % there is no retardation; higher concentrations, 0·5 and 1%, however, have a pronounced inhibitory action. Cane sugar up to a concentration of 0·05% does not retard and may even act as a stimulant; higher concentrations will, however. completely stop the growth and respiration of the bacteria ; 0·5 and 1% glucose, even at a concentration of 0·05% markedly inhibits the reaction, which is completely brought to a standstill at 0·5%. Acetate and oxalate at 0·005 molar concentration have a beneficial action, while at 0·05 molar concentration they retard the reaction.

It would thus appear that insoluble organic matter like cellulose does not retard the reaction. It is unlikely that the soil contains appreciable amounts of soluble organic matter. Moreover, traces of soluble organic matter like cane sugar, acetate and oxalates may even act as stimulants. Humic acid, which is the end-product of the decomposition of different types of organic compounds in the soil and is relatively stable to further decomposition by bacteria and chemical agencies, has a beneficial action on the nitrifying bacteria in concentration in which it is present in most soils. Thus the contention of some workers that soil nitrification cannot be due to the autotrophic bacteria isolated by Winogradsky is untenable.

Even when the soluble organic matter is present in such high concentrations as to retard the action of the autotrophic bacteria in pure solution culture, we have to take into account the presence in the soil of numerous species of bacteria capable of decomposing organic matter which by their presence create favourable conditions for the action of the autotrophic bacteria. For the reason that organic matter is deleterious to the autotrophic nutrifying organisms in pure solution culture, we cannot rush to the conclusion that these organisms are of little significance for nitrification in the soil; if that be so, we have to assume a soil atmosphere free from oxygen for explaining the existence of anaerobic bacteria in the soil; and it can never be so. The fact is that the aerobic organisms present in the soil create conditions favourable for the growth of the anaerobes by an active utilisation of the oxygen. This can be imitated artificially in the laboratory; when anaerobes are grown readily under ordinary aerobic conditions in the presence of rapidly growing aerobic bacteria like *Bacterium subtilis;* although in the absence of the aerobic organisms, the anaerobes can be grown in pure culture only in the absence of oxygen. Another illustration of the phenomenon is the growth of the two nitrogen fixing organisms, the anaerobic *Clostridium pastorianum* and the aerobic *Azotobacter*. The soil harbours many types of organisms and in order to understand the complete picture of various soil processes in the soil, a knowledge of the mutual interrelations of the different organisms is very essential. Some micro-organims may produce a change in the reaction of the medium or in the oxygen tension, or in the concentration of nutrients or such other factors which may be favourable or otherwise for the growth and chemical activity of other micro-organisms. We have already referred to the fact that in the presence of heterotrophic bacteria like *Azotobacter* and *B. mycoides,* nitrifying autotrophic bacteria can oxidise ammonia even in the presence of glucose.

It will, therefore, be unnecessary to assume as has been done by Russell (*loc. cit.*) and others that nitrification in the soil cannot be due to the autotrophic organisms of Winogradsky but must be due to heterotrophic organisms which tolerate organic matter. As a matter of fact the heterotrophic organisms so far reported to be capable of nitrifying produce only very minute traces of nitrites compared with the very large amounts produced by the Winogradsky organisms. The authors believe that considerable advance in biochemical knowledge can be made by investigations on the mutual interrelations of bacteria.

CHEMICAL LABORATORY,
ANDHRA UNIVERSITY,
WALTAIR.

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