A NEW ASPECT OF THE MECHANISM OF NITRIFICATION IN SOIL.

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One of the characteristic features about the classical nitrifying bacteria is their repugnance to organic substances. Indeed, the presence of more than traces of substances such as sugar or peptone entirely inhibits their growth and respiration. In apparent contrast to this, nitrification proceeds unimpaired in soil and sewage where there is a wealth of organic matter of animal and vegetable origin.

The apparent inconsistency in the behaviour of these organisms has been explained as being due to one or more of the following :—

(1) Omeliansky [1] (1899) has shown that, in combination with *Bacterium ramosus*, the nitrite-forming organism can grow, respire and cause the oxidation of ammonia even in dilute peptone broth (1 in 20). Ammonia is first produced and, after several days, nitrite appears. When in addition to these two bacteria, the nitrate former is inoculated in the dilute broth, nitrate is produced, but only after a month. It appears, therefore, that under conditions such as occur in nature, the growth and activity of the nitrite-forming bacteria are not interfered with by the presence of organic material.

Cutler and his associates (*cf.* Russell) [2,3,4] have recently described a number of species of bacteria which can produce nitrite from ammonia. These organisms were first found in the effluent from a beet sugar factory, and have since been shown to be widely distributed in the soil. They function in presence of organic matter and can withstand high acidity (pH 4·8). The quantities of ammonia nitrified by these organisms are however 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. Under similar conditions, *Nitrosomonas* can produce much more nitrite (nearly a hundred times as much), so that the potential possibilities of the classical organisms under the soil conditions cannot be entirely ignored.

Barritt [5] (1933) has recently suggested that there is a possibility of the nitrifying bacteria being a phase in the life-cycle of heterotrophic organisms. This view of course vaguely questions the strict autotrophic character of the organisms tacitly assumed by Winogradsky and somewhat rigidly demonstrated by other workers. Readily assimilating carbonic acid, the organisms of nitrification appear as perfect autotrophs, the various attempts in proving them rather to be facultative heterotrophs having failed entirely.

No conclusive evidence regarding any of the foregoing suggestions is so far available. The question of the internal metabolism of the nitrifying bacteria has not yet been discussed. The synthesis of sugar and protein implies the existence of an enzyme system similar to that occurring in the protoplasm of plants. The writer has, however, shown[6] that the process of nitroso-fermentation is essentially a surface catalytic reaction and that there is no evidence to show that any one particular enzyme system is active in the process of nitrification.

Bonazzi[7] has suggested the possibility of carbon dioxide respiration of the autotrophic bacteria to account for nitrification in the absence of free carbon dioxide in nitrifying cultures, but since free carbon dioxide is not essential for the process, this is not evidence of katabolism, but in mixed cultures (as for example, cultures obtained in soil and sewage) the necessary carbon dioxide is obtainable from the normal respiration of the heterotrophic bacteria and it is possible therefore that there is a process of 'give and take' between the organisms of the soil—in other words, some sort of 'Chemomixotrophic' metabolism or 'symbiosis' occurring in nature.

Starkey[8] observed that the nitrifying bacteria showed increased ability to oxidise ammonia in regions of maximum root development. He also found that there was close correlation between carbon dioxide production and abundance of microbial population. The profuse use of oxygen by the nitrifying bacteria is very well known and this, taken with the fact that adsorbed or condensed oxygen in soil has an unfavourable effect on the ordinary heterotrophic microflora of soil, points to a far reaching possibility that organisms in soil derive mutual benefit from one another in various ways.

With a view to throwing some light on this aspect of the problem, the present enquiry was undertaken. The procedure was to follow the oxidation of ammonia in presence of organic matter by the nitroso bacteria when acting alone or in conjunction with other organisms involved in the transformation of nitrogen in soil, e.g., *Bacterium mycoides*, *Bacterium megatherium*, two very important organisms of the ammonifying group, and *Azotobacter chroococcum*, a very important nitrogen fixing organism. In the case of *Azotobacter*, Barthel[9], and Kellermann and Smith[10] have definitely shown that the bacterial cells do not nitrify at all, and, as such, cannot produce nitrate in synthetic media. The experiments were made in purely liquid or soil cultures.

Experimental.

The bacterial strains used in this work* were freshly cultured in the respective media and incubated until vigorous growth was obtained. At this stage the colonies were picked by a sterile platinum loop and inoculated into

* I am indebted to Messrs. S. C. Pillai and T. R. Bhaskaran of these laboratories through whose courtesy I got the strains of *Bacterium mycoides* and *Azotobacter* respectively and to whom my sincere thanks are due.

sterile water until a very strong suspension was obtained. This was carefully aerated with the necessary precautions and portions of this active suspension used for the experiments.

The Omeliansky medium was used as the source of ammonia for the organisms. Magnesium carbonate (2 per cent) was added to the medium except in cases when ammonium carbonate was substituted for the sulphate. An actively nitrifying culture of the nitroso organism was employed for these experiments. The study of nitrification consisted in following the course of the reaction by the Griess-Ilosvay colorimetric method and in certain cases by the method evolved by the writer[11]. The cultures were incubated at 30° and the nitrite formed was estimated from time to time.

I. *Nitrification of Omeliansky solution using mixed flora of soil as inoculum and the influence of glucose on the oxidation of ammonia.*

Fresh garden soil was used as the source of the mixed flora. Aliquots (50 c.c.) of Omeliansky medium were placed in several 250 c.c. Erlenmeyer flasks plugged with cotton wool and magnesium carbonate (separately sterilised ; lg.) was added to each flask. Various concentrations of sterile glucose solution were added to several of the flasks followed by 5 c.c. portions of a suspension of fresh soil in distilled water. The volume of liquid in each flask was finally brought up to 100 c.c. by addition of requisite quantities of sterile water. Duplicates were run in all the cases and control cultures without treatment were also kept. Table I gives the results of these experiments, and Table II those of a study of the action of glucose on nitrification by a pure culture of the nitroso organism.

A comparison of the two sets of results will show that while glucose is toxic to the organisms oxidising ammonia to nitrite (*vide* Table II) when mixed flora of the soil are used instead of the pure culture, nitrification can go on even in presence of glucose so long as the concentrations are fairly low.

TABLE I.

Influence of glucose on nitrification by the mixed flora of the soil.

Days of incubation.	Nitrite nitrogen (in mg.) formed per litre.					
	Concentration of glucose (per cent).					
	0	0·025	0·05	0·075	0·1	0·2
0	Trace	Trace	Trace	Trace	Trace	Trace
7	,,	4·0	4·0	,,	,,	Nil
14	13·7	15·7	11·9	,,	,,	,,
21	29·4	36·0	33·4	7·9	5·3	,,
28	43·0	63·7	48·7	28·0	15·8	,,
40	65·9	88·9	70·0	36·0	20·0	,,

TABLE II.

Influence of glucose on nitrification by the nitroso bacteria.

Days of incubation.	Nitrite nitrogen (in mg.) formed per litre.					
	Concentration of glucose (per cent).					
	0	0·025	0·05	0·075	0·1	0·2
0	2·0	2·0	2·0	2·0	2·0	2·0
7	12·8	2·0	2·0	Trace	Nil	Nil
14	24·9	1·9	2·0	,,	,,	,,
21	49·0	5·5	,,	,,	,,	,,
28	66·0	7·6	,,	,,	,,	,,
40	87·0	13·0	Trace	,,	,,	,,

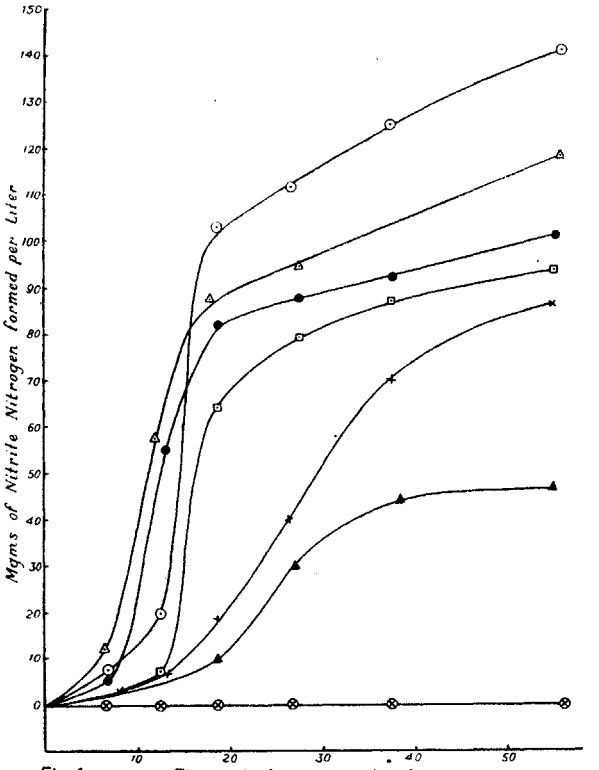

Fig. 1.

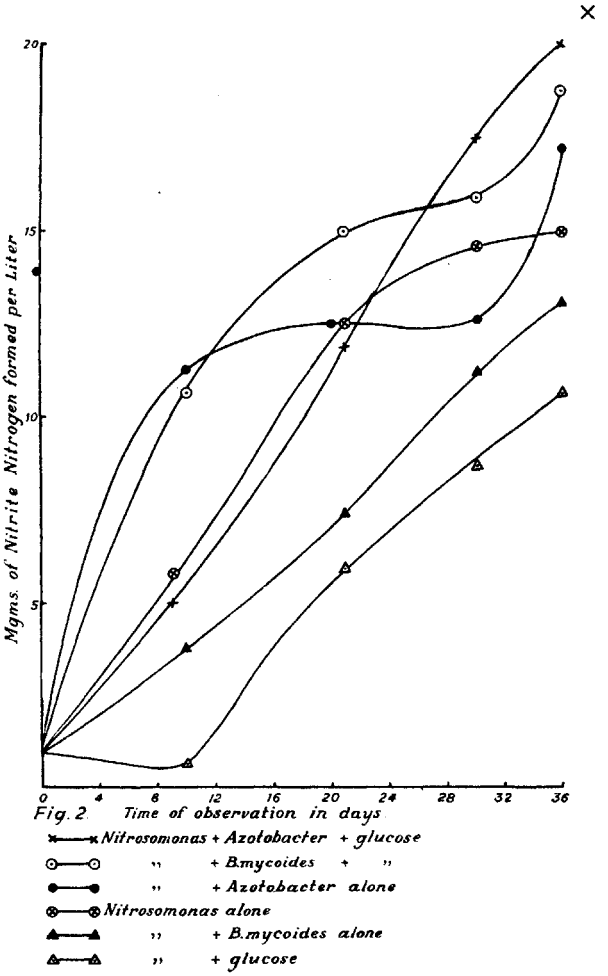

Fig. 2.

II. *Nitrification of Omeliansky medium by nitroso bacteria in presence of organic matter and the effect of mixed inoculations of the nitroso ferment with* Bacterium mycoides, Bacterium megatherium, *and* Azotobacter chroococcum *respectively.*

(1) *Glucose.*—The methods of culturing and study were exactly as detailed above. Thick bacterial suspension (2 c.c.) was used for inoculation in each case. The results are represented in Fig. I. The concentration of glucose used was 0·1 per cent. It is known that nitrification is inhibited by a glucose concentration of 0·025 to 0·05 per cent and completely checked by glucose concentration of 0·2 per cent in pure cultures.

These results show that whenever *Nitrosomonas* was accompanied by other strains of organisms, glucose did not depress nitrification ; on the other hand, in several cases, nitrification was enhanced. Fig. II represents the results of a similar study in soil cultures. 100 gram portions of a local specimen of soil (powdered to pass through a 30 mesh sieve) were employed for each culture together with 1 to 2 gms. of calcium carbonate. The samples were sterlized and 20 c.c. of sterile Omeliansky medium added to each culture. They were then inoculated with the different organisms, the details of procedure being the same as outlined above. The results show that there is very striking parallelism between nitrification as obtained in liquid cultures and that in soil cultures.

There was no nitrification when *Bacterium mycoides* or *Azotobacter* was inoculated into soil cultures in the absence of nitrite formers.

(II) *Humic acid.*—Since humic acid is one of the most important soil constituents, it was considered desirable to study nitrification by mixed cultures in presence of this substance. Humic acid used in this study was prepared according to Burk *et al* [12]. The requisite quantity was weighed into each culture flask and sterilised and then sterile media added. The results are given in Table III.

The nitrifiers used in these experiments were very active. It may be added that natural humic acid (freshly prepared) and a specimen of humic acid from Messrs. E. Merck (Dramstadt) exhibited no fundamental difference in their influence on nitrification. The natural product showed a higher nitrification in all the cases.

It may be seen that at very low concentration (0·025 per cent) humic acid stimulates nitrification. As the concentration increases, there is a steady fall in efficiency until, at about 0·5 per cent, there is practically no nitrification.

When *Azotobacter chroococcum* or *B. mycoides* is combined with the nitrifying organism, a different type of result is obtained. There is no stimulation at low concentrations of humic acid, but the adverse effect of the higher concentrations is not so pronounced as when the nitrifying organism functions alone.

(III) *Peptone.*—Increasing concentrations of peptone retard nitrification. There is marked depression at 0·25 per cent while at 1 per cent there is no nitrification for over 6 weeks. Results are given in Table IV. The adverse effect is not appreciably removed by combining the nitrifying organism with *Bacterium mycoides*, but there is slight improvement in presence of *Azotobacter chroococcum*.

No nitrification was observed in cultures inoculated with *B. mycoides* or *Azotobacter* alone into sterile medium with and without peptone.

Preliminary experiments have shown that in the presence of very dilute solutions of nutrient broth nitrification takes place when inoculated with a combination of the nitrifying bacteria with *B. mycoides* or *Azotobacter*.

TABLE III.

Influence of Humic acid on Nitrite forming bacteria when present alone and when in mixed cultures.

Days of observation.	NITRITE NITROGEN (in mg.) FORMED PER LITRE.															
	Nitroso Bacteria alone.						Nitroso Bacteria + *Azotobacter chroococcum*					Nitroso Bacteria + *Bacterium mycoides*				
	Concentration of Humic acid (per cent)						Concentration of Humic acid (per cent)					Concentration of Humic acid (per cent)				
	0	0·025	0·05	0·1	0·2	0·5	0·025	0·05	0·1	0·2	0·5	0·025	0·05	0·1	0·2	0·5
0	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6	2·6
10	62	120	40·4	25·5	14·6	3·9	45·0	70	17·0	2·8	5·7	4·7	16·8	24·7	18·6	14
20	104·7	128	107	98·3	14·6	3·9	76·2	115	29·0	3·8	30	4·7	22·4	87·5	65	53
32	125·8	132·5	114·2	100	14·6	3·9	108·7	119	53·3	43·1	35	4·7	83·6	97·4	70	53
60	170·0	199	126	112	14·6	3·9	121·8	140	88	45·0	49·0	5·2	93·3	131·8	74	55
90	207·4	213	172	133	14·6	3·9	160	180	104	48·0	55·0	37·3	142	132	74	56
114	229	239	182	118	22·4	4·0	187	182	110	50·0	60·0	94·5	146	132	70	47

TABLE IV.

Nitrification of Omeliansky medium in presence of peptone by the nitrite forming bacteria when acting alone and when in presence of mixed cultures.

Days of incubation.	NITRITE NITROGEN (in mg.) FORMED PER LITRE.											
	Nitroso bacteria alone				Nitroso bacteria + *Bacterium mycoides*				Nitroso bacteria + *Azotobacter chroococcum*			
	Concentration of Peptone (per cent).											
	0·0 (control)	0·25	0·5	1·0	0·0 (control)	0·25	0·5	1·0	0·0 (control)	0·25	0·5	1·0
0	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
14	4·5	7·0	1·12	Nil	31·0	13	3·2	Nil	30	28	20	Nil
21	15·6	11·0	1·12	,,	80·0	48	5·5	,,	83	69	39	,,
36	88·0	30·0	1·12	,,	98·0	69	8·4	,,	100	74	48	,,
48	112·0	55·0	1·12	,,	120·0	86	10·4	,,	123	100	60	,,
60	162·3	90·7	1·12	,,	154·0	91·4	53	,,	149	112	60	,,
72	172·4	114	..	..	159·0	100	75	5·9	155	112	70	14
90	186·7	118	..	..	162·0	112	76	21·6	180	127	87	56
114	188	114	..	..	160·0	112	79	32	188	130	86	68

Discussion.

The most striking observation that arises from the present enquiry is that the nitrifying organisms, which, by themselves, are strict autotrophs can, under certain conditions, not only tolerate organic matter but also function actively in its presence. This apparently inconsistent behaviour is due to the presence of the other organisms of the soil.

The manner in which the individual organisms that were experimented with, or the combined flora of the soil, facilitate nitrification cannot yet be adequately explained. It may be pointed out, however, that the commoner saphrophytes destroy sugars and other forms of fermentable organic matter, so that, by removing such undesirable constituents, they would be indirectly assisting the nitrifying organisms.

It has been observed that nitrification is stimulated by similar organisms even in presence of sugar. Although the available data are insufficient to explain this phenomenon, it may yet be observed that the classical nitrifying organisms are more potent in the soil than their behaviour in pure cultures would suggest.

Peptone inhibits nitrification, but its adverse effect may not persist long in the soil. It undergoes rapid ammonification by the other organisms of the soil and thus indirectly provides material for further nitrification.

Further work is required to elucidate the precise manner in which the interfering organic substances are destroyed by the mixed flora of the soil; whether the immediate products of decomposition are so inimical to nitrification as the original substances. Information is also needed regarding the nature of the relation between nitrifiers and the other organisms of the soil; as to how the latter stimulate nitrification, especially in presence of organic substances. These and allied problems are under investigation and will form the subjects of later communications.

Summary.

(1) Nitrification by *Nitrosomonas* is inhibited by even very low concentrations (0·025 per cent) of glucose. On the other hand, in presence of the mixed flora of the soil, nitrification proceeds even at much higher concentrations (0·1 per cent) of sugar.

(2) When combined with *B. mycoides*, *B. megatherium* or *Azotobacter chroococcum*, *Nitrosomonas* functions actively even in presence of sugar. In some cases, the nitrification proceeded even to a greater extent than in presence of *Nitrosomonas* alone.

(3) When present in more than minute quantities, humic acid does not interfere with nitrification. Peptone has an adverse effect at concentrations of 0·25 per cent and above. In both the cases, the inhibitory action is mitigated by the presence of other organisms in association with *Nitrosomonas*.

(4) The mechanism of the related processes has been discussed. Evidence has been adduced to show that *Nitrosomonas* and the other organisms of the soil function in close association. Fermentable organic matter is rapidly destroyed by the saphrophytes of the soil thus leaving the conditions favourable to nitrification. The presence of the other organisms also helps the nitrifiers to tolerate certain forms of organic matter commonly present in the soil.

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