Biochemistry of Nitrification in Soil

3. NITRIFICATION OF VARIOUS ORGANIC NITROGEN COMPOUNDS

By H. LEES and J. H. QUASTEL, *Agricultural Research Council Unit of Soil Metabolism,* at Rothamsted Experimental Station, Harpenden, Herts*

(*Received* 14 *February* 1946)

It has been established (Lees & Quastel, 1946*b*) that nitrification of ammonium salts in soil takes place at the soil surface, at the expense of NH_4^+ combined or adsorbed in the form of a base-exchange complex. It has also been shown (Lees & Quastel, 1946*b*) that the course of nitrate formation which is autocatalytic in freshly obtained air-dried soil becomes linear, and shows no 'lag', when such soils are enriched or saturated with nitrifying bacteria by preliminary perfusion of the soil with ammonium salts.

Obviously if a soil with nitrifying bacteria converts NH_4^+ at an immediate linear rate into NO_3^-, it will also convert any other nitrogen compound (say X) into NO_3^- at an immediate linear rate, if the nitrifying bacteria are themselves immediately capable of transforming X into NH_4^+ or NO_3^-. On the other hand, if these bacteria cannot accomplish the transformation of X into NH_4^+ (or directly into NO_3^-), conversion of X into NO_3^- must await the proliferation of new cells capable of changing X into NH_4^+ (which will then be attacked by the nitrifying bacteria) or directly into NO_3^-.

Thus to solve the problem whether X is transformed in soil into NH_4^+ or NO_3^- by nitrifying bacteria, the experimental procedure would be to perfuse a soil saturated with nitrifying bacteria. Such a soil should give an immediate linear rate of transformation of NH_4^+ into NO_3^-. If the conversion of X to NO_3^- is immediately linear, this will be good evidence that the nitrifying bacteria are themselves capable of transforming X to NO_3^-. If the rate, however, is autocatalytic exhibiting the typical lag phase of bacterial proliferation curves this may be taken as evidence that the nitrifying bacteria do not directly accomplish the conversion of X into NH_4^+ (or NO_3^-), and that a new organism must first develop which can bring about the initial preliminary breakdown of X. An alternative explanation is that the autocatalytic curve expresses the rate of formation in the nitrifying bacteria of adaptive enzymes capable of attacking the substrate X. Such an explanation would be, however, at present highly speculative; we propose not to consider it until direct evidence is forthcoming for the existence in nitrifying bacteria of such adaptive enzymes.

* Now at University College, Cathays Park, Cardiff.

By means of the perfusion technique (Lees & Quastel, 1946*a*) experiments on these lines have been carried out to determine whether nitrifying bacteria in soil can themselves attack substrates other than NH_4^+. The substrates examined have been aliphatic amines, hydroxylamine, hydrazine, guanidine, pyruvic-oxime, glycine and urea. Experiments have also been carried out to determine whether some of these substances interfere with the conversion of NH_4^+ into NO_3^-.

EXPERIMENTAL

Technique

The general experimental procedure has been first to perfuse at 70° F. 40 g. sieved (4·0–1·0 mm.) air-dried Romney Marsh soil with 200 ml. ammonium chloride or ammonium sulphate solution containing about 70 μg. ammonium-N/ml. until nitrification was just complete. This took between 10 and 15 days. The soil was then washed with water to remove all NO_3^- and reperfused at 70° F. with 200 ml. water containing either ammonium chloride (or sulphate) or the substance under investigation, each at a concentration of 50 or 70 μg. N/ml. All experiments were done at least in duplicate; those with urea were repeated six times. Nitrate estimations were made according to the colorimetric method already described (Lees & Quastel, 1946*a*), where all details of the perfusion technique will also be found.

Nitrification of aliphatic amines

The following amines have been investigated: methylamine hydrochloride, trimethylamine hydrochloride, and tetramethylammonium chloride. The experimental results are shown in Tables 1 and 5. It will be seen that in a bacteria-saturated soil which gives an immediate linear rate of transformation of NH_4^+ into NO_3^-, the rates of conversion of all the amines into NO_3^- exhibit lag periods (see Fig. 1). The ammonium chloride is fully nitrified in 2 days; but methylamine and trimethylamine each require over 5 days for complete nitrification, and tetramethylammonium chloride requires at least 8 days. The results show that although these amines are completely nitrified by the soil, they must undergo preliminary change by soil organisms other than nitrifiers before attack by the nitrifiers can take place.

Omeliansky (1899) has shown that methylamine and dimethylamine cannot serve as nutrient sources

of N for isolated nitrifying bacteria. Meyerhof (1916) later showed that methylamine, trimethylamine and tetramethylammonium chloride at concentrations of 0·005 M (i.e. 70 μg.N/ml.) inhibit the respiration of isolated nitrifying bacteria (*Nitrosomonas*) by 30, 50 and 12% respectively.

Table 1. *Nitrification of mono- and trimethylamine hydrochlorides by bacteria-saturated soil*

(Initial stimulating perfusate: 200 ml. NH_4Cl, containing 70 μg. ammonium-N/ml., perfused until nitrification was almost complete. Second perfusate: 200 ml. solution of amines or NH_4Cl at concentration of 70 μg. N/ml.)

	Rates of nitrate formed during second perfusion: Nitrate-N formed (μg./ml.; max. possible = 70)					
Days ...	1	2	3	5	6	7
NH_4Cl	36	68	69	69	69	68
Monomethylamine-HCl	0	0	9	55	65	60
Trimethylamine-HCl	0	0	22	59	59	61

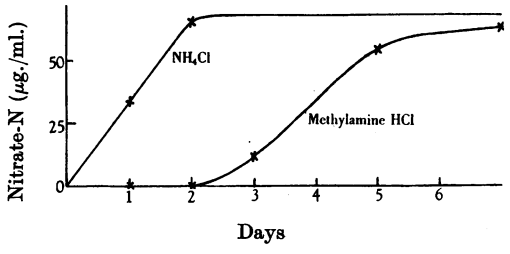

Fig. 1. Relative rates of nitrification of ammonium chloride (0·005 M) and of methylamine hydrochloride (0·005 M).

Nitrification of urea, glycine and guanidine

Urea. Urea is nitrified very rapidly by soil saturated with nitrifying bacteria. The combined results of six experiments are given in Tables 2 and 3. The rate of transformation of urea into NO_3^- exhibits, however, a definite time lag under conditions where the rate of transformation of NH_4^+ to NO_3^- follows an immediate linear course. Our conclusion is that urea is not converted in soil into NH_4^+ by the nitrifiers. The preliminary decomposition is presumably accomplished either by other soil organisms, or by non-biological means.

Glycine. Glycine is also nitrified very quickly by a soil saturated with nitrifying bacteria (see Table 2). The time lag in the curve of conversion of glycine into NO_3^- is, however, even more marked than that in the corresponding curve with urea. Glycine is therefore not directly attacked by the nitrifying bacteria. The preliminary decomposition necessary before nitrification is presumably accomplished either by other soil organisms or by non-biological means.

Guanidine. Guanidine carbonate is nitrified only very slowly by a soil saturated with nitrifying bacteria (see Tables 2 and 4). Under conditions where ammonium sulphate is completely nitrified in 2 days, and urea and glycine in 3 days, guanidine at equivalent concentrations of N is not fully nitrified in 16 days.

Table 2. *Nitrification of urea, glycine and guanidine by bacteria-saturated soils*

(Initial stimulating perfusate: 200 ml. ammonium sulphate solution, containing 70 μg. ammonium-N/ml., were perfused till nitrification was almost complete. Second perfusate: 200 ml. of ammonium sulphate, urea, glycine, or guanidine solution, each at a concentration of 50 μg. N/ml.)

	Rates of nitrate formed during second perfusion: Nitrate-N formed (μg./ml.; max. possible = 50)							
Days ...	1	2	3	5	8	10	14	16
Ammonium sulphate	25	50	52	57	—	—	—	—
Urea	15	40	52	53	—	—	—	—
Glycine	4	32	52	50	—	—	—	—
Guanidine carbonate	6	6	6	11	18	20	40	40

Table 3. *Nitrification of urea and sodium nitrite by bacteria-saturated soils*

(Initial stimulating perfusate: 200 ml. NH_4Cl containing 70 μg. ammonium-N/ml. were perfused until nitrification was almost complete. Second perfusate: 200 ml. of ammonium chloride, urea, or sodium nitrite solution at concentrations of 70 μg. N/ml.)

	Rates of nitrate formation during second perfusion: Nitrate-N formed (μg./ml.; max. possible = 70)		
Days ...	1	2	3
NH_4Cl	34	68	70
$NaNO_2$	45	70	70
Urea	21	50	68

An experiment was carried out to show whether guanidine inhibits nitrification of NH_4^+. A known quantity of NH_4^+ plus guanidine carbonate was perfused through a nitrifier-enriched soil and the rate of formation of NO_3^- measured. The results are given in Table 4. It will be seen that the normally high rate of conversion of NH_4^+ to NO_3^- was greatly diminished or suppressed by the presence of the guanidine. Even after an interval of 14 days ammonium chloride, which in the absence of guanidine is nitrified in 2 days, was not completely transformed into NO_3^-.

Guanidine is an even more active inhibitor of nitrification than is ethylurethane (Lees & Quastel, 1946*a*) for the results in Table 4 show that it is inhibitory at a concentration of 0·001 M. This is in accordance with the observation of Meyerhof (1916) that guanidine greatly inhibits the respiration of isolated nitrifying bacteria.

Table 4. *Nitrification of hydroxylamine, hydrazine and guanidine by bacteria-saturated soil*

(Initial stimulating perfusate: 200 ml. NH_4Cl containing 70 μg. ammonium-N/ml. were perfused until nitrification was almost complete. Second perfusate: 200 ml. of ammonium chloride, hydroxylamine, hydrazine or guanidine solution at 70 μg. N/ml. In duplicate tubes (marked *) after the second day of perfusion, NH_4Cl was added to the perfusate to give a concentration of 70 μg. ammonium-N/ml.)

	Rates of nitrate formation during second perfusion: Nitrate-N formed (μg./ml.)							
Days ...	1	2	3	4	5	7	12	17
Ammonium chloride	34	68	70	70	72	—	—	—
Hydroxylamine chloride	0	0	0	0	0	—	—	—
Hydroxylamine chloride + ammonium chloride	—	*	0	0	7	—	—	—
Hydrazine sulphate	0	0	10	12	—	16	—	—
Hydrazine sulphate + ammonium chloride	—	*	20	20	—	38	—	—
Guanidine carbonate	0	0	0	7	10	16	25	45
Guanidine carbonate + ammonium chloride	—	*	0	7	10	22	55	102

Sodium nitrite. As might have been expected from the results already described (Lees & Quastel, 1946*a*) the rate of conversion of NO_2^- to NO_3^- by a soil rich in nitrifying bacteria is very fast, faster than the conversion of NH_4^+ to NO_2^- by the same soil (see results in Table 3). The rate is too great to allow of sufficient accuracy, under our present experimental conditions, to determine if the course of transformation is linear.

Effects of hydroxylamine and hydrazine on soil nitrification

Both these substances at concentrations of 70 μg. N/ml. perfusate are nitrified with difficulty by a soil enriched with nitrifying organisms. Hydroxylamine shows no evidence of being nitrified but hydrazine is feebly but definitely attacked. When ammonium chloride (at a concentration of 70 μg. N/ml.) is added to a perfusate containing hydroxylamine, the added NH_4^+ is apparently either not nitrified or nitrified but slowly. Results illustrating these facts are shown in Table 4. It is clear, therefore, that hydroxylamine (0·005 M) is highly inhibitory of soil nitrification.

Hydrazine also suppresses, although it does not eliminate, the nitrification of added NH_4^+ (Table 4). At the concentration tested (0·0025 M) it is obviously an inhibitor of soil nitrification. Meyerhof (1916) has pointed out that both hydroxylamine and hydrazine at low concentration (0·001 M) inhibit the respiration of isolated nitrifying bacteria by about 40 %.

We should mention that in the experiments with hydroxylamine, the normal procedure was slightly modified. The soils were initially washed out with 1 % neutralized hydroxylamine hydrochloride in an attempt to remove the higher oxides of manganese which react with hydroxylamine. After this preliminary treatment, the soil was washed with water and then perfused with ammonium chloride to give a soil enriched with nitrifying organisms. This enriched soil was then perfused with hydroxylamine chloride or with a mixture of ammonium chloride and hydroxylamine chloride with the results quoted in Table 4. The fact that a soil treated first with 1 % hydroxylamine chloride, and then washed to remove all hydroxylamine, will allow nitrification of NH_4^+ to take place, shows that the inhibitory action of hydroxylamine is reversed by washing; this confirms our earlier results (Lees & Quastel, 1946*a*) on the reversibility of hydroxylamine toxicity.

Pyruvic oxime ($CH_3.C(:NOH).COOH$).

In view of the high toxicity of hydroxylamine to the nitrifying organisms and in view of the importance of discovering whether hydroxylamine can be attacked at sufficiently low concentrations by these organisms, an experiment was carried out to see whether pyruvic oxime can be nitrified by a bacteria-saturated soil. It is known that certain oximes liberate hydroxylamine in aqueous solution and the toxicity of such oximes to catalase (Sevag & Maiweg, 1934) has been ascribed to the highly inhibitory hydroxylamine yielded by dissociation (Keilin & Hartree, 1934). If a relatively small amount of hydroxylamine is formed by the dissociation of the 0·005 M-pyruvic oxime and if this can be nitrified, it follows that eventually all the pyruvic oxime will be nitrified, since the oxime will continuously supply hydroxylamine to restore equilibrium conditions as the hydroxylamine is removed by decomposition.

Experimental results given in Table 5 show that pyruvic oxime (0·005 M) is rapidly nitrified by a soil rich in nitrifying bacteria. The rate of NO_3^- formation is not quite as high as with an equivalent concentration of NH_4^+; the rate is in fact about the same as the rate with urea under the same experimental conditions. The course of nitrification of the pyruvic oxime appears to be almost linear; but further experiment is required to ascertain whether the process proceeds in an immediate linear manner or whether there is a small lag period.

The presence of pyruvic oxime in a perfusate containing NH_4^+ does not lead to an inhibition or suppression of the nitrification of NH_4^+ (Table 5). This

Table 5. *Nitrification of pyruvic oxime and tetramethylammonium chloride by bacteria-saturated soil*

(Initial stimulating perfusate: 200 ml. NH_4Cl containing 70 μg. ammonium-N/ml. perfused until nitrification was almost complete. Second perfusate: 200 ml. of ammonium chloride, pyruvic oxime, or tetramethylammonium chloride solution at concentrations of 70 μg. N/ml. In duplicate tubes (marked *) after the second day of perfusion, NH_4Cl was added to the perfusate to give a concentration of 70 μg. ammonium-N/ml.)

	Rates of nitrate formation during second perfusion: Nitrate-N formed (μg./ml.)						
Days ...	1	2	3	5	7	8	11
Ammonium chloride	22	40	60	75	75	75	76
Pyruvic oxime	14	31	45	70	68	70	69
Pyruvic oxime + ammonium chloride	—	*	75	—	121	140	145
Tetramethylammonium chloride	0	3	10	12	34	50	60

indicates either that the amount of hydroxylamine formed by dissociation of the oxime is insufficient to exert toxic effects on the nitrifying bacteria, or that the hydroxylamine is attacked almost as quickly as it is liberated by the breakdown of the oxime.

In an analogous experiment a mixture of hydroxylamine and sodium pyruvate in equimolecular proportion were perfused through soil in presence of ammonium chloride and the rate of nitrification compared with that of a mixture of hydroxylamine and ammonium chloride. The result of the experiment is shown in Table 6. It will be seen that the presence of the pyruvate entirely suppresses the toxic effect of hydroxylamine, which is completely nitrified.

This phenomenon is most easily explained in terms of the reversible reaction:

$$CH_3.CO.COOH + NH_2OH \rightleftharpoons CH_3.C(:NOH).COOH + H_2O.$$

The net effect of perfusing an equimolecular mixture of hydroxylamine and pyruvate is the same as that of perfusing an equivalent concentration of pyruvic oxime.

Table 6. *Nitrification of hydroxylamine in presence of sodium pyruvate*

(Initial stimulating perfusate: 200 ml. NH_4Cl containing 70 μg. ammonium-N/ml. perfusate until nitrification almost complete. Second perfusate: 200 ml. solution of (*a*) a mixture of hydroxylamine hydrochloride (70 μg. N/ml.) and ammonium chloride (70 μg. N/ml.) or (*b*) a mixture of hydroxylamine hydrochloride (70 μg. N/ml.), sodium pyruvate (0·005 M) and ammonium chloride (70 μg. N/ml.).)

	Rates of nitrate formation during second perfusion: Nitrate-N formed (μg./ml.)			
Days ...	1	2	3	5
Hydroxylamine + NH_4Cl	—	20	18	20
Hydroxylamine + Na pyruvate + NH_4Cl	22	60	88	125

DISCUSSION

The fact that pyruvic oxime undergoes rapid nitrification by a bacterially stimulated soil may be explained in one or more of the following ways:

(*a*) Hydroxylamine, produced by dissociation of the oxime, is capable of being directly attacked at low concentrations by the nitrifying organisms.

(*b*) Hydroxylamine, produced by dissociation of the oxime, is converted, at low concentrations, by organisms other than the nitrifiers, to a substance (e.g. NH_4^+) that is directly attacked by nitrifying organisms.

(*c*) Pyruvic oxime itself is directly attacked by nitrifying organisms to yield (eventually) NO_3^-.

(*d*) Pyruvic oxime itself is rapidly converted by organisms other than the nitrifiers to a substance (e.g. NH_4^+) that is directly attacked by the nitrifying organisms.

The fact (Meyerhof, 1916) that hydroxylamine cannot replace NH_4^+ as a nutrient source of nitrogen to the nitrifying bacteria (*Nitrosomonas*) makes the first interpretation unlikely. Moreover, it would have been expected that if either interpretation (*b*) or (*d*) were correct, the course of formation of nitrate would have exhibited a more definite time lag than appears to be the case. The time lags in the nitrification curves, shown by the amines (Table 1) or glycine (Table 2) when perfused through a soil enriched with nitrifying organisms are clearly defined. If interpretation (*c*), that pyruvic oxime is directly attacked by the nitrifying organisms, is correct, a most interesting question of the possible part played by this (or an analogous molecule) in the metabolism of nitrifying bacteria will arise. More work, however, is required to decide which of the above possible interpretations is correct.

SUMMARY

1. Soils which have been 'saturated' with nitrifying bacteria, by preliminary perfusion with an ammonium salt, convert NH_4^+ into NO_3^- at a linear rate. Such bacteria-saturated soils have been used for the perfusion of a variety of N-compounds in order to show whether these compounds are also converted to NO_3^- at a linear rate or whether their transformations to NO_3^- follow the sigmoid curves typical of bacterial proliferations. A sigmoid rate of NO_3^- production found in the perfusion of substance *X*

through a soil saturated with nitrifying bacteria is evidence that *X* must first be converted by other (non-stimulated) organisms into NH_4^+ which can then be attacked by the nitrifiers. On the other hand, if the substance under investigation is converted at an immediate linear rate into NO_3^- this is evidence that it may be attacked directly by the nitrifying organisms.

2. Although methylamine and trimethylamine are nitrified by a bacteria-saturated soil, their rates of transformation into NO_3^- show initial time lags which indicate that they are attacked by organisms other than the nitrifiers before nitrification. Tetramethylammonium chloride is nitrified with difficulty; its rate of conversion to NO_3^- shows a large time lag.

3. Urea and glycine are rapidly nitrified by bacteria-saturated soils but their courses of transformation to NO_3^- are not quite linear. Presumably they are attacked by organisms other than nitrifiers before nitrification takes place, or are converted chemically into substances which the nitrifiers can attack.

4. Guanidine carbonate (0·005 M) is nitrified with difficulty by a bacteria-saturated soil. It inhibits the conversion of NH_4^+ to NO_3^- in soil.

5. Hydroxylamine (0·005 M) is not nitrified by a bacteria-saturated soil. It suppresses the conversion of NH_4^+ to NO_3^- in soil.

6. Hydrazine (0·0025 M) undergoes feeble nitrification in a bacteria-saturated soil and greatly inhibits the conversion of NH_4^+ to NO_3^- in soil.

7. Pyruvic-oxime (0·005 M) is nitrified with great rapidity by a bacteria-saturated soil and does not inhibit the conversion of added NH_4^+ to NO_3^-.

8. The presence of sodium pyruvate (0·005 M) entirely eliminates the toxic effect of hydroxylamine (0·005 M) on soil nitrification and allows full nitrification of the hydroxylamine to take place. The significance of this fact and of the result with pyruvic oxime are briefly discussed.

REFERENCES

Keilin, D. & Hartree, E. F. (1934). *Nature, Lond.*, **134**, 933.
Lees, H. & Quastel, J. H. (1946*a*). *Biochem. J.* **40**, 803.
Lees, H. & Quastel, J. H. (1946*b*). *Biochem. J.* **40**, 815.
Meyerhof, C. (1916). *Pflüg. Arch. ges. Physiol.* **166**, 274.
Omeliansky, W. L. (1899). *Z. Bakt.* II, **5**, 485.
Sevag, M. G. & Maiweg, L. (1934). *Naturwissenschaften*, **22**, 561.

Colorimetric Determination of Magnesium in Plasma or Serum by Means of Titan Yellow

By R. J. GARNER, *British Postgraduate Medical School, London*

(*Received* 25 *June* 1946)

The principal methods employed for the estimation of blood magnesium require the precipitation of the magnesium either as the ammonium phosphate or as the 8-hydroxy-quinolinolate after preliminary removal of calcium by precipitation as oxalate. Of necessity these methods involve several steps, and are cumbersome and lengthy.

Several workers have described methods for the determination of magnesium based on the observation by Kolthoff (1927 *a*, *b*) that when magnesium is precipitated as the hydroxide by addition of sodium hydroxide in the presence of an acridine-sulpho-dye, Titan yellow, the colour of the dye is changed from yellow to red. Becka (1931) and Hirschfelder & Serles (1934) adapted the method for use with biological fluids, while the method has also found some favour for the determination of magnesium in water (Urbach & Baril, 1934; Müller-Neuglück, 1941; Ludwig & Johnson, 1942, and others).

The methods described for plasma (Becka; Hirschfelder & Serles) are open to some criticism in that these workers did not precipitate the plasma proteins. As the colour of normal plasma may vary from pale yellow to a frank red according to the degree of haemolysis, a considerable error may be introduced. There is also some controversy as to whether the presence of calcium interferes with the determination of magnesium.

In view of these points it was decided to investigate the method and, if possible, to develop a procedure suitable for the determination of magnesium in plasma and serum with the photoelectric colorimeter.