CXXXVII. THE FORMATION OF HYPONITROUS ACID AS AN INTERMEDIATE COMPOUND IN THE BIOLOGICAL OR PHOTOCHEMICAL OXIDATION OF AMMONIA TO NITROUS ACID.

II. MICROBIOLOGICAL OXIDATION[1].

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THE present investigation is concerned with the oxidation of ammonium salts to nitric acid in soil. In Part I [Corbet, 1934] the chemical aspects of the subject were dealt with, and in this paper the reactions effected by bacterial agency are considered.

For work on the biological oxidation of ammonia, aqueous solutions of ammonium sulphate containing small amounts of nutrient salts were inoculated with nitrifying organisms from soil. No particular attention was paid to the species of organisms present, but difficulty was experienced frequently in obtaining a culture capable of effecting complete and rapid nitrification under experimental conditions. On many occasions inoculation of Beesley's medium with small quantities of soil from arable land failed to produce more than traces of nitrite, even after prolonged incubation. With a few soil samples obtained at Jealotts Hill, nitrite production in Beesley's medium was strong and rapid but usually less than 5 % of the ammonium salt was decomposed after several weeks' incubation at 32°.

In one instance only did nitrification proceed beyond the stage of nitrite formation, and no attempt was made to ensure nitrate production, as the present investigation was concerned primarily with the intermediate compounds formed during the oxidation of ammonia to nitrous acid.

The formation of intermediate compounds.

It has been found in this investigation that the microbiological oxidation of ammonium (as sulphate) can proceed by the following stages:

$$NH_3 \rightarrow NH_2OH \rightarrow H_2N_2O_2 \rightarrow HNO_2 \rightarrow HNO_3.$$

There appears to be no doubt that hydroxylamine occurs as an intermediate compound during the early stages of nitrification, although on account of the reaction of the medium it is present in quantity too small to admit of accurate estimation. In some series of experiments, when nitrification had begun, this compound was detected by its reducing action on Fehling's solution and on iron alum, but it was never present when appreciable quantities of nitrite had accumulated.

[1] Some aspects of the work described in this paper were considered in a paper read at the Annual General Meeting of the Association of Applied Biologists held in London on 15th February 1935.

It was shown experimentally that cultures of soil micro-organisms able to convert ammonium (as sulphate) into nitrite transformed hydroxylamine into nitrite under the same conditions. In one such experiment, 95 % of the nitrogen originally present as hydroxylamine disappeared, leaving the residue in the form of nitrite. It was evident that the hydroxylamine had undergone oxidation to nitrite, and obviously the resulting hydroxylamine nitrite had broken down to nitrous oxide and water.

Although hydroxylamine was detected as an intermediate product on occasions, it could not accumulate in the culture flasks since it becomes progressively less stable with increasing alkalinity of the medium. The data given in Table V show that some decomposition of hydroxylamine takes place at p_H 4·6, while at higher values the breakdown is rapid. In presence of nitrite, hydroxylamine is unstable whatever be the reaction of the medium (Table VI). Addition of nitrite is an easy and effective means of removing hydroxylamine from solution, and advantage was taken of this fact in the analytical procedure.

Hyponitrous acid was frequently detected as an intermediate compound during the early stages of nitrification and, with some cultures of soil micro-organisms, it was present in quantity.

In one series of experiments with ammonium sulphate, a considerable portion of the nitrogen known to be present could not be accounted for as ammonia, hydroxylamine, nitrous or nitric acid, and this deficit was greatest between the disappearance of ammonia and the formation of nitrite. It was concluded that the nitrogen was present in the form of hyponitrite.

This experiment was carried out in the early stages of the investigation, before a satisfactory method of estimating hyponitrous acid had been elaborated. Later work however fully confirmed the tentative conclusion. This substance was often detected during the early stages of nitrification, but in one series of experiments it constituted an important intermediate substance in a biological reaction, whereby the nitrogen in ammonium sulphate was finally lost, presumably in a gaseous form.

Calcium hyponitrite is stable in aqueous solution and in presence of nitrite, but on warming it decomposes and the nitrogen is lost in gaseous form. Although no chemical mechanism is known whereby hyponitrites are oxidised to nitrite, when an aqueous solution of calcium hyponitrite was inoculated with soil micro-organisms, there was an accumulation of nitrite and no gas evolution took place.

Attention has already been drawn [Corbet, 1934] to the autoxidation of nitrous acid which takes place rapidly in media of $p_H < 5$.

Discussion.

In Tables I–IV and Figs. 1–3 are given some of the results obtained during the present investigation. It was found preferable to work with a number of separate culture flasks, since nitrification was neither vigorous nor rapid when a large bulk of liquid medium was inoculated and aliquot portions were withdrawn at intervals, and so in some instances the experimental points do not lie on a smooth curve.

It will be seen that while hydroxylamine was hardly ever present in measurable amount, hyponitrous acid constituted an important intermediate compound in the first and fourth series of experiments. Since the mutual presence of hydroxylamine and nitrite results in loss of nitrogen, it is not surprising that these two compounds do not co-exist at any stage during the biological nitri-

fication of Beesley's medium. The fact that hydroxylamine and hyponitrous acid occur during the initial stages of the decomposition and are absent later, when appreciable amounts of ammonia still remain to be oxidised, is discussed on p. 1089.

Table I. *First series of nitrification experiments.*

	Nitrogen present in mg. per 100 ml. solution							
Days after inoculation	0	8	14	18	24	30	42	76
Nitrogen present as:								
Ammonia	19·6	15·0	4·7	10·7	9·0	7·8	8·8	0·1
Nitrous acid	.	1·7	3·0	7·1	9·8	11·0	10·3	19·6
Total accounted for	19·6	16·7	7·7	17·8	18·8	18·8	19·1	19·7
Deficit	.	2·9	11·9	1·8	0·8	0·8	0·5	.
Hyponitrous acid-N present according to titration with $KMnO_4$	.	3·0	11·4	3·6	0·8	0·8	0·3	.

No nitrate was found in any of the culture flasks. These results are expressed graphically in Fig. 1. There was, of course, no actual regeneration of ammonia: the apparent increase in ammonia-nitrogen on the 18th day is attributable to the fact that the reaction did not proceed at the same speed in all the culture flasks.

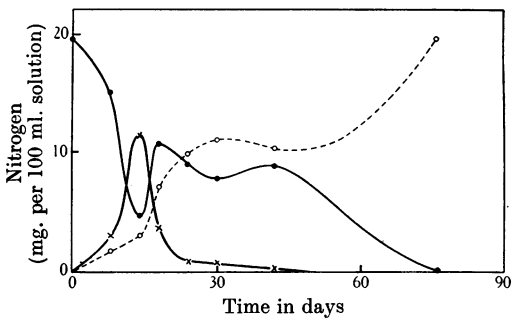

Fig. 1. First series of nitrification experiments.
•—• Ammonia-N. o- - -o Nitrous-N. ×—× Hyponitrous-N.

Table II. *Second series of nitrification experiments.*

	Nitrogen present in mg. per 100 ml. solution							
Days after inoculation:	0	11	15	21	27	33	40	48
Nitrogen present as:								
Ammonia	19·2	18·8	18·5	18·0	18·5	18·9	18·3	18·6
Hydroxylamine	.	.	.	0·1	.	.	.	.
Nitrous acid	.	0·4	0·2	0·7	0·3	0·2	0·4	0·6
Total accounted for	19·2	19·2	18·7	18·8	18·8	19·1	18·7	19·2
Deficit	.	0·0	0·5	0·4	0·4	0·1	0·5	0·0

No nitrate was detected in this series of experiments.

Subcultures from this experiment and many other cultures of soil micro-organisms gave similar results. In one experiment, in which the course of nitrification was followed by measurement of the diminution in pressure, it was found that biological activity ceased when a small proportion of the ammonia had been oxidised.

The culture flasks were incubated at 32°, and qualitative experiments showed that the course of nitrification was similar when incubation took place at 4°.

Table III. *Third series of nitrification experiments.*

	Nitrogen present in mg. per 100 ml. solution										
Days after inoculation	0	3	5	7	10	13	17	19	24	28	42
Nitrogen present as:											
Ammonia	19·2	18·5	18·2	18·7	17·8	15·9	11·0	11·3	7·2	2·1	3·5
Nitrous acid	.	0·0	0·4	0·3	1·0	2·7	7·1	6·8	9·1	16·7	15·3
Total accounted for	19·2	18·5	18·6	19·0	18·8	18·6	18·1	18·1	16·3*	18·8	18·8
Deficit	.	0·7	0·6	0·2	0·4	0·6	1·1	1·1	2·9	0·4	0·4
Qualitative tests:											
Hydroxylamine	−	+	−	+	−	+	−	−	−	−	−
Hyponitrous acid	−	+	+	+	+	+	−	−	−	−	−
Nitric acid	−	−	−	−	−	−	−	−	−	−	−

* No satisfactory explanation of this low value was discovered.
A subculture from the above experiment gave similar results.

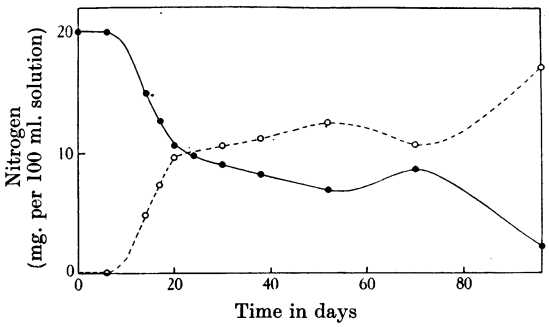

Fig. 2. Third series of nitrification experiments.
•—• Ammonia-N. o- - -o Nitrous-N.

In Part I attention was drawn to work carried out by Beesley [1914] on the biological oxidation of ammonium salts, in which, at one stage during an experiment, a considerable proportion of the nitrogen known to be present was unaccounted for, although finally it appeared in the form of nitrate. That the missing nitrogen was present partly, if not entirely, as calcium hyponitrite there can now be little doubt. The results obtained by Beesley were closely paralleled in the first series of nitrification experiments performed by the present writer (Table I and Fig. 1). Although in this case the hyponitrite was not estimated gasometrically, the qualitative tests leave no room for doubt that the missing nitrogen existed at least partly, and probably entirely, in the form of calcium hyponitrite. Chemical evidence has been adduced to show that there could have been no accumulation of hydroxylamine under the circumstances.

The evidence available strongly suggests that nitrification may be effected by a number of different species or strains of bacteria. It is unlikely that cultivation in the laboratory has produced drastic changes in the enzymic activities of the micro-organisms, for subcultures invariably gave the same type of reaction as the original cultures. The only way in which artificial conditions appeared responsible for any change was that in the type detailed in the third series of experiments production of hydroxylamine and hyponitrous acid was definitely associated with the logarithmic increase phase of growth, although repeated subculturing reduced these intermediates to vanishing point.

Table IV. *Fourth series of nitrification experiments.*

	Nitrogen present in mg. per 100 ml. solution									
Days after inoculation	0	2	3	6	8	10	13	17	35*	55
Nitrogen present as:										
Ammonia	19·8	18·0	18·0	16·5	15·8	15·0	14·0	14·0	10·2	5·9
Hyponitrous acid	.	1·4	n.d.	2·4	n.d.	1·7	2·1	n.d.	n.d.	0·7
Nitrous acid	.	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Total accounted for	19·8	19·4	.	18·9	.	16·7	16·1	.	.	6·6
Deficit	.	0·4	.	0·9	.	3·1	3·7	.	.	13·2
Qualitative tests:										
Hydroxylamine	−	−	−	−	−	−	−	−	−	−
Hyponitrous acid	−	+	+	+	+	+	+	+	+	+
Nitric acid	−	−	−	−	−	−	−	−	−	−

n.d. means not determined.

* Determination of the total nitrogen by the Kjeldahl method (using selenium as a catalyst) in a parallel flask on the 35th day gave a value of 10·0 mg. nitrogen per 100 ml. solution.

The results are plotted in Fig. 3. Subcultures gave precisely similar results. In one series, a solution which contained initially 20·3 mg. NH_3-N per 100 ml. solution contained 4·4 mg. NH_3-N and 1·0 mg. $H_2N_2O_2$-N and no hydroxylamine or nitrate and only a trace of nitrite after 81 days.

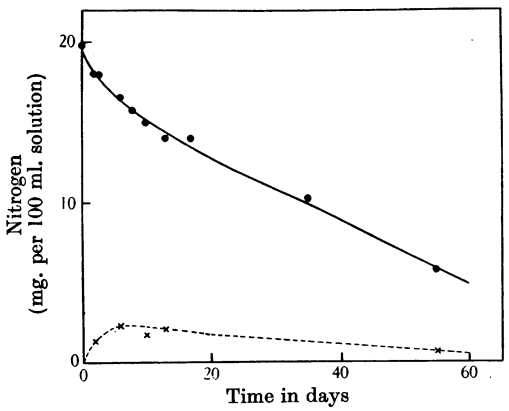

Fig. 3. Fourth series of nitrification experiments.
•—• Ammonia-N. ×---× Hyponitrous-N.

The most interesting type of biological nitrification encountered is that detailed in the fourth series of experiments (Table IV and Fig. 3) with a culture obtained from soil under grass which had been treated with "nitrochalk" some 6 months previously. This particular soil was selected since it repeatedly gave a strong positive reaction for hyponitrous acid with the resorcinol and potassium periodate test. With this culture, disappearance of ammonia was rapid, and after about 10 % of the nitrogen had accumulated in the form of hyponitrite, the proportion of the latter remained constant throughout the experiment. Presumably the missing nitrogen escaped in gaseous form.

Nitrification in soil.

It appears that the biological production of nitrite from ammonia in soil takes place in a variety of ways. The repeated failures to isolate nitrate-forming bacteria from soil suggest that *Nitrobacter* is by no means so widely distributed

as the nitrite-forming organisms, and that the further oxidation of nitrite to nitrate may be attributable to autoxidation in acid media.

Evidence obtained from laboratory experiments indicates that hyponitrites may occur in soils, at least under some conditions, and tests at Jealotts Hill have shown that small amounts of hyponitrite are in fact present in certain soils. On the other hand, evidence has been advanced by Gopala Rao and Dhar [1931; Gopala Rao, 1934] and by the writer, showing that in exposed soils in the tropics, nitrification is, at least partly, a chemical process. Nitrite formation may be effected by a photochemical reaction and, to a lesser extent, by alternate wetting and drying-out of soils.

The depth to which ultraviolet light can penetrate the soil is an important matter in this connection in the tropics. It is believed that photonitrification of ammonium salts is effected by light of the wave-length 3100–2900 Å. The mercury arc emits radiations down to 1800 Å., while the solar spectrum does not penetrate into ultraviolet regions beyond 2900 Å. It was found in the laboratory that whilst light from a mercury vapour lamp effected nitrite-formation in a silica gel impregnated with ammonium sulphate to a depth of several inches, no liberation of iodine from potassium iodide occurred through 0·25 inch of moist or dry soil. Nevertheless, the question of penetration of soil by chemically active radiations from sunlight cannot be readily dismissed: Hoerlin [1934] states that the ultraviolet radiation in equatorial latitudes is of different intensity and composition from that in temperate regions. Many of the chemical reactions caused by ultraviolet light are effected also by the action of hydrogen peroxide (*e.g.* liberation of iodine from potassium iodide, nitrite production from ammonia [Weith and Weber, 1874] and the formation of a pink substance from ammonium thiocyanate), and this may have some bearing on the question of the depth of photonitrification in exposed tropical soils which are wetted daily. It must also be borne in mind that ammonium salts often occur "free" in such soils, not being adsorbed by the soil colloids, and in equatorial latitudes the top soil layer is much more shallow than in temperate regions.

Once nitrite is formed in the acid soils of equatorial countries, the further oxidation to nitrate must be largely effected by chemical means.

The criticisms advanced by Fraps and Sterges [1935] against the work of Dhar and Gopala Rao are without substance, since in their experiments soil was exposed to the sun in pyrex beakers covered with glass. The writer has confirmed that no ultraviolet radiations of wave-length capable of oxidising ammonium salts to nitrite can penetrate these materials.

Denitrification in sunlight.

Recently Dhar [1934] has sought to explain the losses of nitrogen from soil, known to take place under certain conditions in presence of readily oxidisable organic matter, as a result of the decomposition of ammonium nitrite in sunlight. Dhar has found that when mixed with sterilised or unsterilised soil, or in presence of a photosensitiser such as titania, zinc oxide or ferric oxide, ammonium nitrite undergoes decomposition with liberation of free nitrogen: since nitrite is an intermediate in nitrification, it is supposed that ammonium nitrite is formed and then disrupted under the action of light.

The present writer found that a solution of ammonium chloride and sodium nitrite in stoichiometrical proportions (p_H 6·8) showed no decomposition in the dark during a period of 3 months, and only slight losses of ammonia occurred, the nitrite content remaining unchanged, after 87 hours' irradiation by the mercury arc (Table VII). In presence of freshly-ignited titania however, both

in sunlight and under the mercury arc, small quantities of gas were liberated. In view of the fact that nitrite is so readily transformed into nitrate in acid soils, it seems doubtful if any considerable losses from soil can occur in this way except, possibly, in arid regions where nitrite accumulation takes place.

While discussing nitrogen losses from the soil, attention may be drawn to the importance of the results of the fourth series of nitrification experiments.

Jenny [1929] has shown that, in general, the nitrogen content of the soil is a function of the mean annual temperature and humidity, and thus for any given mean annual temperature and humidity, the potential nitrogen content of the soil has a maximum value. Apart from this it is evident that the nitrogen content of the soil cannot be raised indefinitely, and that some means must exist of ridding the soil of surplus nitrogen when the potential nitrogen content is exceeded as, for instance, in heavy applications of nitrogenous fertilisers. The nature of this mechanism is obscure, and it may be biological or chemical in character.

In the fourth series of nitrification experiments described in this paper, a loss of nitrogen followed the conversion of ammonium sulphate into hyponitrite, and the only plausible explanation is that the missing nitrogen has been released in gaseous form. This may be an example of a biological mechanism effecting the escape of excessive amounts of nitrogen from the soil. In the present case however the detection and estimation of gaseous forms of nitrogen released by bacterial agency is difficult, since the reaction can proceed only in presence of adequate supplies of oxygen.

Experimental.

The decomposition of hydroxylamine.

Solutions of hydroxylamine hydrochloride were prepared, and the reaction was adjusted by addition of sulphuric acid or sodium hydroxide; the p_H values were determined colorimetrically. The solutions were preserved in the dark in stoppered flasks, and the hydroxylamine content was determined by boiling with ferric alum and sulphuric acid, followed by titration of the reduced iron with potassium permanganate.

Table V. *Loss of hydroxylamine from aqueous solution at different* p_H *values.*

Initial conc. 0·0073 g. NH_2OH per 100 ml. Temperature 32°.

	% Loss				
p_H of solution	After 1 day	After 7 days	After 14 days	After 23 days	After 38 days
2·2	0·0	0·0	0·0	0·0	0·0
4·6	1·0	1·7	9·8	—	—
5·9	46·1	46·9	59·4	61·1	60·2
8·4	46·1*	66·3	75·2	81·7	86·9

* Determination of other forms of nitrogen in this solution gave NH_3-N nil; nitrite-N, trace; nitrate-N, 0·001 %.

It is evident that the decomposition of hydroxylamine which occurs in solutions not strongly acid is an oxidation, for vigorous boiling of hydroxylamine hydrochloride with $N/10$ NaOH in an inert atmosphere resulted in but slight decomposition.

Losses of hydroxylamine from solution occurred in the presence of nitrite, whatever the reaction of the medium. For example, a solution was prepared

containing 0·039 g. NH_2OH-N and 0·005 g. nitrite-N per 100 ml. solution. Immediately after preparation, the hydroxylamine content dropped to 0·036 g. NH_2OH-N per 100 ml. solution, and 23 hours later the solution contained 0·034 g. NH_2OH-N and no nitrite-N per 100 ml. solution. Thus, the whole of the nitrite *plus* a stoichiometrically equivalent amount of hydroxylamine had disappeared within a day.

In a further experiment, a solution containing initially 0·030 g. NH_2OH-N and 0·057 g. nitrite-N per 100 ml. solution had the composition 0 g. NH_2OH-N and 0·043 g. nitrite-N per 100 ml. solution on the following day. Both experiments were carried out at 32°.

It is evident that the course of the reaction between hydroxylamine and nitrous acid must be affected by the reaction of the medium, and accordingly experiments were carried out, in stoppered flasks, at different p_H values (Table VI).

Table VI. *Loss of nitrogen from solutions of hydroxylamine and nitrous acid at different* p_H *values.*

Initial conc. 0·036 g. NH_2OH-N and 0·034 g. nitrite-N per 100 ml. solution. Temperature 32°.

p_H of solution	NH_2OH-N after 1 day	Nitrite-N after 1 day
2·4	0·001	0·000
4·8	0·000	0·000
5·9	0·000	0·016
7·6	0·000	0·028

The photochemical decomposition of ammonium nitrite.

Irradiation of an aqueous solution of ammonium nitrite by means of a 100 D.C. mercury arc gave the results shown in Table VII.

Table VII. *Irradiation of ammonium nitrite by ultraviolet light.*

Initial conc. 0·028 mg. NH_3-N and 0·027 mg. nitrite-N per 100 ml. solution (prepared from ammonium chloride and sodium nitrite).

Treatment	NH_3-N	Nitrite-N
3 months in dark	0·027	0·028
87 hours' irradiation by mercury arc	0·023	0·028

The biological oxidation of ammonium sulphate. Beesley's medium.

The medium employed for the culture of the nitrifying organisms was that recommended by Beesley [1914] and prepared according to the formula:

Nitrogen (in combination as ammonium sulphate) 0·20 g.; KH_2PO_4 0·20 g.; NaCl 0·20 g.; $MgSO_4, 7H_2O$ 0·066 g.; $CaCO_3$ 20·0 g.; distilled water 1000 ml.

The p_H of the medium was 6·9 (quinhydrone electrode) $1\frac{1}{2}$ hours after preparation.

Usually about 2 litres of Beesley's medium were prepared and inoculated with about 1 ml. of a vigorous culture of nitrifying organisms obtained from soil: after standing overnight, quantities of 100 or 200 ml. were placed in a number of 500 ml. conical flasks which were closed with rubber bungs or cotton-wool: when rubber bungs were employed, an adequate supply of air was present for complete nitrification. The flasks were incubated at 32° and the contents analysed at convenient intervals.

Analysis of the nitrifying solutions.

Routine analysis of Beesley's medium, after inoculation with nitrifying organisms, was carried out as follows. The calcium carbonate was removed by filtration and the filtrate made up to a standard volume with water. After qualitative tests for hydroxylamine (Fehling's solution), hyponitrous acid (resorcinol and potassium periodate), nitrous acid (Griess) and nitric acid (reduction to nitrite), aliquot portions of the liquid were analysed as follows.

(*a*) Ammonia was estimated by distillation with magnesia into standard sulphuric acid solution.

(*b*) Nitrite and nitrate together were determined by addition of Devarda's alloy to the solution, after the ammonia estimation had been completed, followed by distillation of the ammonia formed by reduction into standard acid as before.

(*c*) Nitrite was determined by the Griess-Ilosva colorimetric method.

(*d*) The solution was titrated with potassium permanganate in presence of sulphuric acid before and after boiling: in this way the nitrite *plus* hyponitrite, and nitrite alone were estimated, but see the remarks on p. 1095. Permanganate titrations were carried out only in the first series of experiments, a gasometric method being subsequently employed for the estimation of hyponitrite.

(*e*) When present in sufficient quantity, hydroxylamine was estimated by boiling with ferric alum and sulphuric acid, followed by titration with potassium permanganate.

(*f*) *The estimation of hyponitrous acid.* The method of estimating hyponitrous acid entailed collection and examination of the gases evolved on boiling. 100 ml. of the solution were placed in a Claisen distillation flask, and after displacement of the air by a current of pure carbon dioxide from a Farmer's apparatus [1920], the contents of the flask were maintained at boiling-point for about 1 hour. The liberated gases (nitrous and nitric oxides and nitrogen) were swept into a Schiff's nitrometer, over strong potassium hydroxide (saturated with nitrous and nitric oxides), by a further current of carbon dioxide. Nitric oxide was estimated by measurement of the diminution in volume after shaking with ferrous sulphate, and nitrous oxide by determination of the volume decrease after sparking in presence of excess of hydrogen. As air was often present, particularly during the initial stages of nitrification experiments, the amount of hydrogen used was found by noting the diminution in volume after further sparking with oxygen. Attempts to measure the proportions of the three gases present by measuring the volume changes, first after sparking with hydrogen and then after exploding with oxygen, often gave results explicable only on the supposition that any nitric oxide present had been largely reduced to ammonia. Nevertheless, the absorption of nitric oxide by ferrous sulphate solution is not an entirely satisfactory procedure, for the reaction is somewhat slow, and it is necessary first to saturate the absorbent with nitrous oxide; moreover, the precipitate formed as a result of potash from the nitrometer coming into contact with ferrous sulphate is often troublesome in the gas burette.

In some experiments, the hyponitrite in the culture solution was actually separated as the silver salt by precipitation with excess of silver nitrate; after standing overnight, the precipitate was washed free from silver nitrate and decomposed by boiling with sulphuric acid. Analysis of the mixed nitrogen gases evolved was carried out in the manner described. Vigorous boiling of a solution of ammonium nitrite with a small amount of calcium carbonate, the first-named being present in the maximum concentration in which it could occur in the culture liquid, resulted in the liberation of so small a quantity of gas that it was evident that no appreciable error was introduced into the hyponitrite estimation as a result of nitrogen gas liberated by the decomposition of any ammonium nitrite present.

The estimation of hyponitrous acid is not easy, and to this fact, doubtless, must be attributed the former lack of convincing evidence that this acid constitutes an intermediate in the oxidation of ammonia to nitrous acid.

Experiments were first conducted with silver hyponitrite, which was prepared as a pale greenish yellow, flocculent precipitate by addition of excess of silver nitrate to a solution of sodium hyponitrite. The salt dried to a green powder, which rapidly darkened when exposed to light. The silver was estimated as chloride and the hyponitrite determined by oxidation of the salt by $N/10$ $KMnO_4$, acidified with sulphuric acid, and collection of the nitrogen gases evolved on heating in a Schiff's nitrometer over potash, as described above. In this procedure a portion of

the nitrogen present in the hyponitrite remained in the flask as nitrate (see equations on p. 1096), and was subsequently estimated in the form of ammonia, after reduction with Devarda's alloy. The nitrogenous gases collected in the nitrometer were analysed in the manner already described.

Analysis of the silver salt gave the following results (%):

	Found				Calculated for $Ag_2N_2O_2$
	Preparation (*a*)		Preparation (*b*)		
Ag		75·7 %		74·8	78·25
N as NO	1·51		1·09		
N as N_2O	6·40		8·79		
N as N_2	0·00				
N as NO_3'	2·55		0·08		
*N_2O_2''		22·4		21·6	21·75
		98·1		96·4	100·00

Calcium hyponitrite was prepared by addition of excess of calcium nitrate to a solution of sodium hyponitrite: the resulting precipitate was washed with water, then with alcohol and finally with ether and dried on filter-paper.

Analysis of calcium hyponitrite:

	Found		Calculated for $CaN_2O_2,4H_2O$
Ca		23·35 (23·34) %	23·29
N as NO	1·85		
N as N_2O	9·57		
N as N_2	0·00		
N as NO_3'	4·17		
Total N	15·59		
*N_2O_2''		32·76	34·80
CO_3''		1·43	—
N_2O (by difference)		42·47	41·86

On heating to constant weight at 105°, the loss in weight was 35·41 %.

* This figure was arrived at by addition of the nitrogen found as NO, N_2O, N_2 and NO_3'' and multiplication by the appropriate factor.

In routine analysis of soil, ammonia is estimated by distillation with magnesia and collection in standard acid, whilst nitrite and nitrate together are determined by measurement of the further ammonia liberated by distillation with magnesia after reduction by Devarda's alloy. With pure hydroxylamine hydrochloride practically no "ammonia"-nitrogen is found by distillation with magnesia, but the value for the "nitrate"-nitrogen is between 70 and 90 % of the hydroxylamine-nitrogen known to be present. Hyponitrite is not detected during such soil analysis, since the nitrogen is lost in gaseous form on warming.

Fortunately, hydroxylamine was never present in more than very small amount during the present investigation, so that no error was introduced from this source. Hyponitrite and nitrite do not react but aqueous solutions of calcium hyponitrite slowly decompose on standing. In presence of hyponitrite, nitrite can be accurately estimated by permanganate titration, provided that the hyponitrite is first decomposed by boiling the solution for 10 minutes.

The action of potassium permanganate on hyponitrites. The action of permanganate on hyponitrites was investigated, but the results obtained are somewhat inconclusive.

Kirschner [1898] stated that oxidation of hyponitrous acid by potassium permanganate entailed the consumption of only one atom of oxygen per mol. of acid, but subsequent investigators have disagreed with this view. The present research showed that considerable variation occurs in the ratio $O/H_2N_2O_2$ during the oxidation of hyponitrites by addition of the latter to a cold, acidified solution of potassium permanganate. In general, the equation

$$4H_2N_2O_2+4O=2HNO_3+3H_2O+3N_2O$$

is satisfied, giving a value of unity to the ratio $O/H_2N_2O_2$, as claimed by Kirschner.

Eighteen determinations of the hyponitrite in silver hyponitrite by this method gave a mean value of 20·94 ± 1·31 % as against the theoretical value of 21·75 for pure $Ag_2N_2O_2$, but the figures varied between 11·02 and 31·94 %.

It appears that the equation given above may represent the sum of two reactions:

$$(a) \quad H_2N_2O_2 = H_2O + N_2O,$$

$$(b) \quad H_2N_2O_2 + 4O = 2HNO_3.$$

Under laboratory conditions with cold permanganate and sulphuric acid, the reaction shows a definite tendency to proceed so that three-fourths of the hyponitrous acid decomposes according to (*a*) and one-fourth is oxidised according to (*b*), although this relationship appears to be fortuitous.

SUMMARY.

The oxidation of ammonia (as ammonium sulphate) by cultures of soil micro-organisms proceeds in a variety of ways. The type most frequently encountered was that in which oxidation ceased after about 5 % of the ammonium salt had been converted into nitrite: with other cultures nitrite formation was vigorous and rapid.

The question of the presence of intermediate compounds during the course of nitrification has been investigated in detail and it has been established that, while hydroxylamine has only an ephemeral existence at p_H values of 6 and above, calcium hyponitrite constitutes an important intermediate compound during nitrification by certain strains of soil bacteria. The presence of hyponitrite was detected by the colour given with resorcinol and potassium periodate, and its estimation was effected by collection and examination of the nitrogenous gases liberated by boiling the culture liquids, or by separation of the silver salt, followed by measurement of the nitrogenous gases evolved by heating with sulphuric acid.

In one series of nitrification experiments, ammonia was oxidised to hyponitrite and this was followed by loss of nitrogen; in this particular series neither nitrite nor nitrate was detected at any time. In all cases, subcultures gave precisely similar results.

Nitrification in soils, by biological and chemical agencies, is discussed in the light of the results obtained during the present investigation.

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Note added April 26th, 1935. It has been found that the losses of nitrogen in gaseous form, which occurred in the fourth series of experiments, are attributable, at least in part, to the release of nitrous oxide. Berthelot and Gaudechon [*Compt. Rend. Acad. Sci.* (1910) **150**, 1517] found that nitrous oxide was decomposed into its elements under the action of ultraviolet light and this would explain why no accumulation of this gas takes places in the atmosphere.