

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

## I. CHEMICAL REACTIONS.

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THAT the oxidation of ammonia to nitric acid is a transformation essential to the maintenance of life has long been realised, but our knowledge of the mechanism of the process is still far from complete. The change is of vital importance in the soil and it is from the viewpoint of the soil chemist that the subject is considered in the present paper. Although ammonium sulphate has for long ranked as a very popular fertiliser, the changes which it undergoes in the soil are still but imperfectly understood.

Since the discovery that nitrification was effected by bacteria [Schloesing and Müntz, 1877; 1878; 1879], it has been tacitly assumed that nitrification in the soil is entirely a biological process: it is true that nitrite formation from ammonium salts by ultra-violet radiation was reported over 20 years ago [Berthelot and Gaudechon, 1911] but it is only within the last year or two that evidence has been adduced to show that chemical forces may play an important rôle in nitrification.

The literature relevant to the biological aspects of the subject has been recently reviewed by Barritt [1933] and to this paper reference should be made.

Nitrite-forming organisms, other than the well-known autotrophic *Nitrosomonas*, have been described on several occasions, but their activities appear to be very limited compared with those of *Nitrosomonas*, and there seems every reason to believe that this organism is the most important factor in the biological production of nitrites (and hence of nitrates) from ammonia.

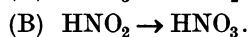
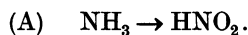
No compounds of composition intermediate between ammonia and nitrous acid have been detected hitherto during the oxidation processes, but it is clear that such compounds must have an existence, even if only a transient one, and there is evidence to show that their existence cannot be as evanescent as is usually assumed. Some work carried out by Beesley [1914] on the biological oxidation of ammonium salts showed that a considerable disappearance of nitrogen, amounting to nearly half the total nitrogen present, occurred in the oxidation of ammonia to nitrous acid; this disappearance was not attributable to the escape of gaseous nitrogen compounds, for most of the nitrogen was finally accounted for in the form of nitrate. That the missing nitrogen in Beesley's experiments was present in the form of some intermediate compound there can be little doubt.

The considerations mentioned above suggested that the whole subject of nitrification merited detailed investigation.

## SCHEME OF WORK.

The present investigation is concerned with the oxidation of ammonia to nitric acid by such processes as can take place in the soil. It soon became evident that the transformation of ammonia to nitric acid *in natura* is not solely a microbiological process, it being possible for the complete change to be effected by a chemical mechanism.

The nitrification of ammonia is most conveniently considered as comprising the two stages:



Both transformations can be effected by bacteria but both can take place in the absence of living organisms.

In temperate regions the change (A) is carried out in the soil primarily by the agency of *Nitrosomonas*, for the other nitrite-forming organisms appear to have little practical significance. This first stage can be effected also by the action of light and it is now evident that this photochemical change has more than a theoretical significance.

The second stage (B) may be carried out as the result of the activities of the nitrate-forming *Nitrobacter* but it is now clear that, in acid solution, the transformation is catalysed by the hydrogen ion. Evidently, therefore, in acid soils, the oxidation of nitrous acid to nitric acid must take place chemically so that no accumulation of nitrite is possible, and it is significant that many writers have reported the scarcity of *Nitrobacter* in soil compared with *Nitrosomonas*. In many of the biological oxidations carried out in this research, in which soil solution was used as the inoculum and the media were neutral or slightly alkaline, the oxidation did not proceed beyond the formation of nitrite.

It is interesting to note that none of the changes described here entails any loss of nitrogen in a gaseous form: this, however, does not preclude the possibility that the course of denitrification may be accompanied by losses of nitrogen as nitrous oxide or elementary nitrogen.

The present paper is concerned with the chemical changes occurring during nitrification, whilst the transformations effected by microbiological agency will be dealt with subsequently.

Special attention has been paid to the question of intermediate compounds, for the results obtained by Beesley show very clearly that at least one intermediate compound must be capable of a stable existence. It has been tentatively suggested by different writers that hydroxylamine and hyponitrous acid may occur as intermediate compounds during the process of nitrification but no evidence has been adduced in support of these views. It is true that these compounds are the most stable known of the possible intermediates, but it seems improbable that hydroxylamine plays a significant part, for its presence in solution with nitrite entails a loss of nitrogen in some gaseous form.

Recently hydroxylamine has been reported as an intermediate compound during the process of denitrification [Blom, 1928; Lindsay and Rhines, 1932] but this does not mean that it is probably present during the reverse change.

## DISCUSSION.

*Hyponitrous acid.*

One of the most interesting facts emerging from the present investigation is the identification of hyponitrous acid as an intermediate compound in the course of the oxidation of ammonia to nitrous acid, both by microbiological

agency and by the action of light. Little is known concerning the chemical nature of this compound but there appears to be no doubt that the calcium salt has a stable existence under the experimental conditions which obtained. The detection and estimation of hyponitrites is not easy: on standing, an aqueous solution of the calcium salt is decomposed in part, if not wholly, to nitrous oxide. This being so, any hyponitrite-nitrogen escapes detection and estimation during routine determinations of ammonia, nitrite and nitrate. Nevertheless, the stable existence of calcium hyponitrite in presence of certain concentrations of other salts is by no means precluded.

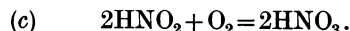
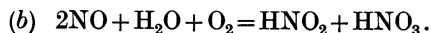
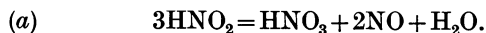
The formation of the greenish yellow, insoluble silver salt was the only qualitative test for hyponitrites found in the literature but this test is of little value in presence of large amounts of nitrites and other salts. An attempt to discover a colour reaction resulted in the elaboration of the following test for hyponitrites. Addition of resorcinol and potassium periodate to a neutral (or at least not strongly alkaline) solution of a hyponitrite produces an orange- or cherry-red colour which is quite intense after 5 minutes, if the substance is present in measurable quantity: after an hour or so, depending on the amount of hyponitrite present, the solution becomes strongly fluorescent<sup>1</sup>. Should the test solution be strongly alkaline a holly-green colour is produced which becomes red on acidification. This test appears to be sensitive for hyponitrite-nitrogen down to a concentration of only 10 parts per million: it gives reliable results in Beesley's medium and in presence of ammonia, nitrite and nitrate and has proved very valuable in the present investigation.

As far as has been ascertained, the only other substances which behave in a similar manner with resorcinol and potassium periodate are hydroxylamine, borates and bicarbonates. The first-named usually gives a much yellower colour and, moreover, it reduces Fehling's solution whereas hyponitrites do not. Borates and bicarbonates were not present during the current investigation so that no interference was experienced from this source.

It may be mentioned that Blom's test for hydroxylamine, depending on the Griess colour reaction for nitrites after oxidation with iodine, is not specific, being given also by hyponitrites in certain concentrations.

*The catalytic oxidation of nitrous acid to nitric acid.*

It has been established that nitrites in solution decompose according to equation (a). Barritt has shown, and the writer has confirmed, that in acid solution in presence of air practically the whole of the nitrite is converted into nitrate. The nitric oxide formed in solution is oxidised to nitrogen peroxide by oxygen from the air and is then converted into nitrous and nitric acids. Thus the reaction is continuous until the whole of the nitrous acid is oxidised according to the equations:



<sup>1</sup> Although satisfactory results were obtained with the culture media employed, it is not known how far the test is of general application. Experiments carried out by Dr W. R. Wooldridge have shown that, if the test solution is buffered at a  $p_{\text{H}}$  value between 5 and 6, an old gold colour is developed immediately resorcinol and potassium periodate are added in presence of hyponitrite, while fainter pink colours are produced after an interval of one minute with borates and bicarbonates. The colour developed with hydroxylamine is of a deep reddish hue and not yellow.

The results of a series of experiments showed that the rate of disappearance of nitrite from solution increased with increasing temperature and also with the degree of acidity of the solution. The data indicate that the removal of nitrite from acid soils by chemical means must be rapid, and it seems improbable that *Nitrobacter* occurs in such soils.

*The photochemical oxidation of ammonia to nitrous acid.*

The recent investigations of Dhar and his co-workers [Rao and Dhar, 1931; Dhar *et al.*, 1933] show clearly that nitrite formation can be effected in the absence of micro-organisms: it is pointed out that nitrification in soil is greatest in the top layer where photochemical activity is at a maximum. These workers found that the velocity of the reaction was increased in the presence of certain photosensitisers (of which titania proved to be the most active) but decreased with increasing acidity of the medium. It is submitted that, in tropical countries, the production of nitrite by photochemical agency may be of much greater significance than that resulting from microbiological activity.

In the present research experiments were carried out to determine the extent of the photochemical oxidation of ammonium sulphate, and the results obtained by the Indian investigators were confirmed. Although no attempt was made to follow the course of the reaction closely, it appears that the transformation is largely a surface phenomenon. It was not possible to make the irradiation process a continuous one and this fact must be taken into account in the interpretation of the results. It can be stated, however, that if the reverse change takes place in the dark it does so to an inappreciable extent.

The photochemical oxidation of ammonia to nitrous acid is accompanied by the formation of hyponitrous acid, according to the resorcinol-potassium periodate test, and this substance was detected in greatest amount during the early stages of the experiments but was never present in sufficient concentration to admit of quantitative estimation. In the studies of the biological oxidation of ammonia to nitrous acid, to be described in the next paper, however, the production of hyponitrous acid, as detected by the resorcinol-potassium periodate test, was confirmed by quantitative determinations.

*The photochemical reduction of nitric acid to nitrous acid.*

Although no systematic study of the process of denitrification was made at this stage, a few experiments were made to determine the extent of the reduction of nitrate to nitrite by the action of light. The procedure employed was similar to that used to investigate the photochemical oxidation of ammonia. It was found that the velocity of the photochemical reduction of nitrate was considerably greater than the rate of oxidation of ammonia to nitrite, under identical conditions. As in the case of the oxidation process, the transformation occurred at the surface but, unlike this former change, the reaction proceeded for a short time after removal of the light source. If the reverse action takes place in the dark it must be inappreciable.

EXPERIMENTAL.

*The catalytic oxidation of nitrous acid to nitric acid.*

In a series of experiments the relation between the rate of disappearance of nitrite from aqueous solutions of potassium nitrite and the  $p_H$  value and the temperature was investigated. The solutions were preserved in stoppered flasks

and incubated in the dark: the nitrite was estimated by means of standard potassium permanganate and sodium oxalate solutions, and the  $p_H$  was determined by the quinhydrone electrode.

It was found that, in the absence of air sufficient to supply oxygen according to the equations on p. 1577, the oxidation process was brought to a standstill and the nitrite remained in solution although the latter might be very acid.

In Tables I, II and III are given the losses of nitrite from aqueous solutions of potassium nitrite at different  $p_H$  values and different temperatures. The results given in Table III are expressed graphically in Fig. 1.

Table I. *Loss of nitrite from aqueous solution at different  $p_H$  values.*

Initial concentration, 0.222 g.  $KNO_2$  per 100 ml. solution. Temperature 21°.

$p_H$ value of solution	Percentage nitrite loss after 45 days
4.14	100.0
5.08	27.1
5.23	18.6
5.41	11.5
5.67	6.4
6.10	1.2

Table II. *Loss of nitrite from aqueous solution at different  $p_H$  values.*

Initial concentration, 0.221 g.  $KNO_2$  per 100 ml. solution. Temperature 32°.

$p_H$ value of solution	Percentage nitrite loss	
	After 1 day	After 8 days
4.35	49.4	100.0
4.42	34.7	89.5
4.68	15.4	54.3
5.32	1.0	9.6
6.93	0.0	1.0
8.38	0.0	0.0

Table III. *Loss of nitrite from aqueous solution at different temperatures.*

Initial concentration, 0.239 g.  $KNO_2$  per 100 ml. solution.  $p_H = 4.0$ .

Time in days	Percentage nitrite loss		
	4°	32°	37°
1	30.6	56.2	91.6
2	51.7	94.2	97.7
3	70.2	97.5	—
4.75	92.4	—	—
6.75	100.0	—	—

Initial concentration, 0.187 g.  $KNO_2$  per 100 ml. solution.  $p_H = 4.89$ .

1	10.8	17.8	—
2	10.8	18.7	—
3.25	14.2	28.9	—
7	18.7	34.5	—
16	24.6	38.1	—
24	31.2	42.7	—

In one experiment the nitrite *plus* nitrate-nitrogen remaining in solution was estimated, by reduction with Devarda's alloy, and the following figures were obtained.

$p_H$	Initial nitrous N content	Nitrous N content on 45th day	Total nitrous + nitric N on 45th day
5.23	0.0366	0.0297	0.0355
5.08	0.0366	0.0266	0.0348

*The photochemical oxidation of ammonia to nitrous acid.*

*Experiments with sunlight.* A quantity of Beesley's medium<sup>1</sup> was inoculated with a few drops of inoculum known to contain nitrite-forming organisms, and 10 ml. were measured into a number of test-tubes. Half of the tubes were

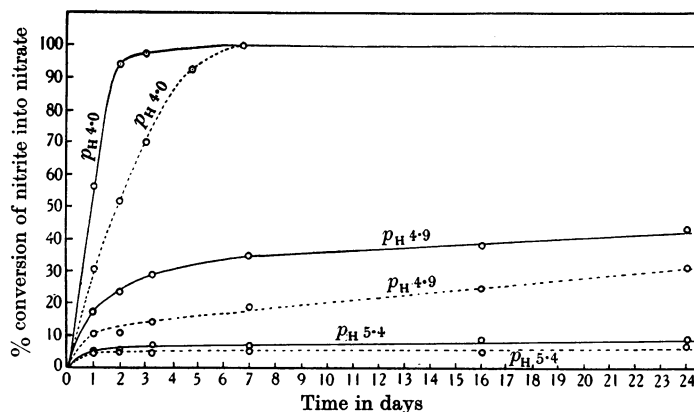


Fig. 1. Full lines, temperature 32°. Broken lines, temperature 4°.

kept in a dark cupboard, and the rest were exposed to sunlight in the window of the laboratory. Judging by the colour developed with the Griess reagent, at first the tubes in the dark cupboard contained more nitrite but after the 53rd day the tubes in the window were richer in nitrite.

The experiment was repeated with sterilised media, but nitrite formation was very slow: after 162 days the nitrite content of the tubes in the window was considerably greater than that of those kept in the cupboard. In neither experiment was nitrite formation accompanied by gas production.

In a further experiment, a 9.2% solution of ammonium sulphate was prepared and excess of calcium carbonate was added. The solution was preserved in stoppered flasks, one of which was placed in the window of the laboratory whilst the other was kept in a dark cupboard. Qualitative tests showed the presence of hyponitrite and nitrite to a much greater extent in the light than in the dark. The nitrite present was estimated colorimetrically by the Griess-Ilosvay method.

	mg. nitrous-N per 100 ml. solution	
	61 days	105 days
(a) Light	0.013	0.190
(b) Dark	0.000	0.008
Difference between (a) and (b)	0.013	0.182

*Irradiation by mercury vapour lamp.* In one experiment a 0.2% solution of ammonium sulphate, in presence of excess of calcium carbonate, was irradiated by means of a 100 volt D.C. mercury arc. The solution was placed under the arc in an open beaker and, at intervals, the nitrite content of the solution was determined colorimetrically. A correction was made for water lost from the solution by evaporation (Table IV).

<sup>1</sup> The composition of Beesley's medium is: 0.95 g. ammonium sulphate, 0.2 g. potassium dihydrogen phosphate, 0.2 g. sodium chloride, 0.066 g. MgSO<sub>4</sub>·7H<sub>2</sub>O, 20 g. calcium carbonate and 1000 ml. distilled water.

Table IV. *Nitrite formation from ammonium sulphate by irradiation.*

Hours of irradiation	mg. nitrous N per 100 ml. solution	Hours of irradiation	mg. nitrous N per 100 ml. solution
12	0.025	69	0.059
20	0.025	74.5	0.073
28.5	0.032	78	0.072
36.5	0.037	81.5	0.080
41.5	0.036	85.5	0.078
50	0.040	89.5	0.081
58	0.050	94.3	0.081
61.5	0.054		

The experiment was repeated and the nitrite content of the solution was determined at more frequent intervals (Table V).

 Table V. *Nitrite formation from ammonium sulphate by irradiation.*

Hours of irradiation	mg. nitrous N per 100 ml. solution	Hours of irradiation	mg. nitrous N per 100 ml. solution
1	0.004	5	0.026
2	0.009	6	0.034
3.5	0.011	6.5	0.040
4.5	0.022		

The solution was stored in a dark cupboard during the night and a determination of the nitrite content on the following morning showed that no change had taken place.

*The photochemical reduction of nitric acid to nitrous acid.*

*Experiments with sunlight.* A series of test-tube experiments with aqueous potassium nitrate showed that nitrite formation in tubes kept in the laboratory window was considerable compared with that in tubes in a cupboard. No gas evolution occurred during the reduction process.

A 0.25 % solution of potassium nitrate was prepared and placed in stoppered glass flasks, one of which was placed in the window, the other being kept in a cupboard. The nitrite present was estimated colorimetrically: no hyponitrite was detected in solution by the resorcinol-potassium periodate test.

	mg. nitrous N per 100 ml. solution	
	69 days	114 days
Light	0.003	0.024
Dark	0.000	0.000

*Irradiation by mercury vapour lamp.* A 0.25 % solution of potassium nitrate, in an open beaker, was irradiated by means of a 100 volt D.C. mercury arc and the nitrite content was determined at intervals by the Griess-Ilosvay colorimetric method (Table VI). A correction was made for the water lost by evaporation.

 Table VI. *Nitrite formation from potassium nitrate by irradiation.*

Hours of irradiation	mg. nitrous N per 100 ml. solution	Hours of irradiation	mg. nitrous N per 100 ml. solution
1	0.004	11	0.075
2	0.008	12	0.077
3	0.010	13	0.080
4	0.020	14	0.080
5	0.038	15	0.088
6	0.045	16	0.094
7	0.053	17	0.098
8	0.062	18	0.099
9	0.063	19	0.109
10	0.074		

## SUMMARY.

An investigation of the mechanism of the nitrification of ammonium sulphate has shown that certain of the stages may be effected by microbiological, chemical or photochemical agency.

The change  $\text{NH}_3 \rightarrow \text{HNO}_2$  may occur as a result of bacterial agency or by the action of light and in both cases hyponitrous acid has been detected as an intermediate product. The change  $\text{HNO}_2 \rightarrow \text{HNO}_3$  may be the result of bacterial activity or it may take place chemically, as the reaction is catalysed by the hydrogen ion. At  $p_{\text{H}}$  values above 5.0 the loss of nitrite in this way is not appreciable, but at lower values the loss may be considerable. This is of some practical importance and suggests that, under certain conditions, nitrification in tropical soils may be largely a chemical process.

It has also been established that the photochemical change  $\text{HNO}_3 \rightarrow \text{HNO}_2$  is by no means inappreciable.

With resorcinol and potassium periodate, hyponitrites give an orange- or cherry-red colour which is quite intense after 5–10 minutes and becomes strongly fluorescent after standing for an hour or so. Similar colours are given by hydroxylamine, borates and bicarbonates with these reagents but hydroxylamine is readily detected by its reducing action on Fehling's solution.

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