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The Nitrifying Process and Its Specific Ferment. Part I

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V. *The Nitrifying Process and its Specific Ferment.*—Part I.

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The spontaneous oxidation of nitrogen in nature is a process which has long attracted attention in consequence of the great practical importance of the products to which it gives rise. Indeed, so great is the demand for potassium nitrate, nitre, or saltpetre, the principal derivative of the oxidised nitrogen, that the process of nitrification is often artificially stimulated by placing nitrogen, in the form of refuse animal matter, under the most favourable conditions for undergoing this change. The process of nitrification has thus been carried on for ages as a regular industry in India, and even in some European countries, thus especially in France during the Great Blockade. For a number of years past, however, the principal source of nitric acid and its derivatives has been the enormous deposits of nitrate of soda occurring in South America, which deposits themselves may, however, possibly be the product of a vast nitrification-process in a former period of the Earth's history.

But although nitrification had thus been practically studied for centuries, it was only in 1878 that the process was shown by Schlœsing and Müntz ('Compt. Rend.,' vol. 84, p. 301; 85, p. 1018) to be a fermentation change, and entirely dependent upon the presence of certain minute forms of life or micro-organisms. But although this connection was thus first experimentally demonstrated in 1878, it had with characteristic foresight been already surmised by Pasteur in 1862.

In the communication referred to above, these investigators clearly show that the process only takes place under conditions compatible with the life and growth of micro-organisms, and is immediately arrested by conditions, such as the application of strong heat or antiseptics, which are fatal to these forms of life. They further showed, moreover, that the nitrification-process could be induced by the introduction into suitable materials of the minutest quantity of matter from a medium already undergoing nitrification.

In the same year these remarkable results were fully confirmed by Warington ('Chem. Soc. Journ.,' 1878, p. 44), who further greatly extended our knowledge of

the conditions affecting the process of nitrification, in a very elaborate paper communicated to the Chemical Society in the following year (1879, pp. 429–456).

In subsequent publications ('Compt. Rend.,' vol. 89, pp. 891 and 1074), SCHLŒSING and MÜNTZ claim to have separated the organism causing nitrification, and they briefly describe the general appearance which nitrifying media present when examined with high microscopic powers.

It is, however, almost needless to say that we have no guarantee that they actually accomplished the isolation of this important organism, as the methods of bacteriological research at that time in general use were of a very imperfect character. In any case, the subject demanded re-investigation with the aid of more modern methods.

The results of a number of other researches on the subject of nitrification have been published from time to time, amongst which we may specially mention an extremely interesting paper by MUNRO ('Chem. Soc. Journ.,' 1886, pp. 632–680), in which the nature of the nitrogenous substances capable of undergoing nitrification is very fully discussed. In none of these communications, however, is any material advance made towards the identification and isolation of the active organism of nitrification.

We append, however, the following list of the principal contributions which have been made to our knowledge of the subject :—

"Note on the Ferment-Theory of Nitrification." F. H. STORER ('Chem. Soc. Journ.,' Abst., 1878, p. 932); in this it is shown that ammoniacal salts undergo nitrification in contact with peat, if air, filtered through cotton wool, is drawn through the mixture, whilst if the same mixture is first sterilised by heat, then the current of filtered air fails to bring about any nitrification.

"Nitrification," E. W. DAVY ('Pharm. Journ. Trans.,' [3], vol. 10, pp. 1–3; 'Chem. Soc. Journ.,' Abstr., 1879, p. 1047), discusses the formation of nitrates and nitrites in natural waters, and contends that light is not inimical to the change, which takes place very readily between 70° and 80° Fahr. It is further pointed out that, in the presence of organic matter, nitrites are formed.

WARINGTON, in "Alterations in the Properties of the Nitric Ferment by Cultivation" ('Chemical News,' vol. 44, p. 217; 'Chem. Soc. Journ.,' Abst., 1882, p. 79), states that when ammoniacal solutions are seeded from old nitrifying solutions only nitrites and no nitrates are formed, and he attributes this phenomenon to diminished energy of the nitrifying organism.

In a subsequent paper entitled "Nitrification, Part III." ('Chem. Soc. Journ.,' 1884, p. 637), WARINGTON describes a very extensive series of experiments, throwing light upon the special conditions which further nitrification.

PICHARD, in "Comparative Nitrifying Effect of Various Salts" ('Compt. Rend., vol. 98, p. 1289; 'Chem. Soc. Journ.', Abst., 1884, p. 924) shows that the presence of calcium sulphate greatly promotes nitrification, and institutes a comparison between the relative effect produced by the following salts; thus—

Calcium sulphate	100·00
Sodium sulphate	47·91
Potassium sulphate	35·78
Calcium carbonate	13·32
Magnesium carbonate	12·52

Pichard's results as regards the beneficial effect of calcium sulphate are confirmed by Warington in a paper "On the Action of Gypsum in Promoting Nitrification" ('Chem. Soc. Journ.,' 1885, p. 758), and again, in a further communication "On the Distribution of the Nitrifying Organism in the Soil" ('Chem. Soc. Journ.,' 1887, p. 119), he shows that by adding calcium sulphate to the ammoniacal solutions the latter may be made to nitrify by seeding with soil taken from greater depths than had hitherto been found capable of causing nitrification.

Munro, in "Formation of Nitrites during Nitrification of Ammoniacal Solutions" ('Chemical News,' vol. 56, pp. 62–64; 'Chem. Soc. Journ.,' Abst., 1888, p. 82), contends, in opposition to Gayon and Dupetit, that the nitrite found during nitrification is actually the result of the direct oxidation of the ammoniacal nitrogen, and not due to a reduction of nitrate first formed.

In "Distribution of the Nitric Ferment and its Function in the Disintegration of Rocks" ('Annalen Chem. Phys.,' [6], vol. 11, pp. 136–144; 'Chem. Soc. Journ.,' Abst., 1887, p. 1135), Müntz states that the nitrifying ferment is to be found at the highest elevations of the Alps, and ascribes to it the power of causing the disintegration of rocks. He also observes that organisms which appear to be identical with the nitrifying ones are capable of reducing nitrates when air is excluded.

It is not a little surprising that after the overwhelming evidence of such numerous investigators, the vital nature of the nitrification process should have been again disputed. This has been done, however, by Frank ('Forschungen auf dem Gebiete der Agriculturphysik,' vol. 10, p. 56), whose contention has however, received no support, and has been rebutted by Plath ('Biedermann's Centralblatt,' 1888, pp. 6–8; 'Chem. Soc. Journ.,' Abst., 1888, p. 521), and by Landolt ('Biedermann's Centralblatt,' 1888, p. 577).

The modern methods of bacteriological study were first brought to bear upon the subject of nitrification by Heræus ('Zeitsch. f. Hygiene,' 1886, p. 193). This investigator, after inducing nitrification in an ammoniacal solution by means of a small quantity of garden soil, endeavoured to isolate the organisms present in the nitrifying solution by means of the method of gelatine-plate cultivation. In this manner he obtained four different organisms in a state of purity, and these he distinguished by the signs ρ, σ, ϕ and χ respectively. These pure cultures he inoculated into ammoniacal solutions of the following composition :—

	I.	II.
	grm.	grm.
Potassium phosphate	·05	·05
Magnesium sulphate	·01	·01
Calcium chloride	·05	·05
Ammonium carbonate	1·00	1·00
Grape sugar	1·00	none
	in 1000 c.c. of distilled water.	in 1000 c.c. of distilled water.

After remaining three days at 30° C. the solutions exhibited traces of nitrous acid as indicated by the iodide of starch reaction. After six days this reaction was decidedly stronger, but still the quantity of nitrous acid was not sufficient to admit of quantitative estimation. The experiments were repeated again with slight modifications, but again the amount of nitrous acid was insufficient for quantitative purposes. Upon this evidence, unsupported as it is by a single instance of nitrification on such a scale as to admit of the quantitative determination of the nitrous and nitric acids formed, Heræus claims for the organisms ρ, σ, ϕ, and χ the nitrifying power. This is the more remarkable, as nitrous and nitric acids are so eminently fitted for quantitative estimation, even when present in only the minutest traces. He even goes further and claims similar oxidizing properties for the well-known forms *Micrococcus prodigiosus*, *B. ramosus*, *the cheese Spirilla*, Finkler's *Spirilla*, *the typhoid bacillus of* Koch-Gaffky, *B. anthracis*, and *Staphylococcus citreus*, having obtained nitrous acid reactions when these organisms were respectively grown in sterilised and diluted urine. So positively, indeed, is it asserted in the above paper, that the organisms in question are endowed with nitrifying properties, that a casual reader will almost infallibly carry away the impression that the conclusions drawn are altogether beyond dispute. It is in this way, no doubt, that Heræus's statements have been generally incorporated without question in the ordinary text-book literature of the day.

Heræus's investigations were obviously calculated to be of the greatest importance to all interested in the question of nitrification, but his conclusions concerning the nitrifying power of the bacteria designated by ρ, σ, ϕ, and χ did not admit of immediate criticism, and could not be controverted or verified without a lengthy experimental inquiry. On the other hand, his assertions concerning the nitrifying properties of the well-known organisms mentioned above could be easily put to the test by any one possessing pure cultures of the same forms. Now, in the course of an investigation on "The Action of some Specific Micro-organisms on Nitric Acid" ('Chem. Soc. Journ.,' 1888, p. 373), one of us had occasion to experiment with two of the same organisms, viz., *M. prodigiosus* and *B. ramosus*, but in the case of neither could any evidence of nitrifying power be obtained, whilst, on the contrary, both organisms were found to be capable of reducing nitric to nitrous acid. This observation at once

indicated that a very different interpretation should be put upon HERÆUS's results to that which he had himself suggested.

The indications of nitrous acid which he had obtained on growing these organisms in diluted urine were obviously due to reduction of the small quantity of nitrates almost invariably present in normal urine, and not to any oxidation of the ammonia at all. This was pointed out by one of us in the paper in question, and, of course, casts a suspicion also upon the supposed nitrification obtained by HERÆUS with the pure cultures ρ, σ, ϕ, and χ, to which we had no access.

In further endeavours to obtain nitrification by means of pure cultures, one of us experimented with no less than 33 different forms obtained by us from air and water,* but in every case the results were negative. Such a number of negative results obtained when working under precisely similar conditions to those indicated by HERÆUS naturally tended to shake our belief in his having ever obtained more than traces of nitrous or nitric acids in any of his experiments with pure cultures.

NOTE.—Of other investigations professedly made with pure growths the following may be mentioned:—

"Nitrification," A. CELLI and F. MARINO-ZUCO ('Gazz. Chim. Ital.,' vol. 17, pp. 99–103; 'Chem. Soc. Journ.,' Abst., 1887, p. 858), in which the authors claim to have found a micrococcus (*M. cereus*) which behaved as a very efficient nitrifying agent. They further mention preliminary experiments purporting to show that certain organisms (*B. saprogenus*, *B. fluidificans*, and *M. luteus*) destroy nitrates when taken from gelatine, but produce nitrates when taken from potato-cultures.

"Changes induced in Water by the Development of Bacteria," T. LEONE ('Gazz. Chim. Ital.,' vol. 16, p. 505; 'Chem. Soc Journ.,' Abst., 1887, p. 615), in which the author asserts that the same organisms may successively produce nitrification and reduction of nitrates according to circumstances.

"The Chemical Action of some Micro-organisms," R. WARINGTON ('Chem. Soc. Journ.,' 1888, p. 751). In this paper the author records his failure to produce nitrification with pure growths obtained by gelatine-plate culture from nitrifying solutions; he reviews the present position of the question of nitrification, and concludes with the words "an organism which nitrifies as soil nitrifies has yet to be isolated."

Failing to obtain nitrification with any of the pure cultivations of organisms in our collection, we determined to approach the matter from a different side, viz., to induce nitrification by a mixture of organisms, and then from this nitrifying mixture to attempt to isolate the particular organism or organisms responsible for the process.

* "Studies on some New Micro-organisms obtained from Air," by GRACE C. FRANKLAND and PERCY F. FRANKLAND, 'Phil. Trans.,' 1887, p. 257; also "Ueber einige typische Mikro-organismen im Wasser und im Boden," by the same, 'Zeitsch. f. Hygiene,' vol. 6, 1889, p. 373.

The readiest means of inducing nitrification in suitable ammoniacal solutions is, as is well known, by the introduction of a minute trace of ordinary garden soil. The solution we employed for the purpose had the following composition :—

Salt solution*	100 c.c.	Diluted to 1000 c.c.
Ammonium chloride . .	·5 grm.	
Carbonate of lime (pure) .	5·0 grms.	

The solution was thus entirely free from organic matter, as the results of previous investigators had clearly indicated that nitrification can take place in purely mineral solutions, whilst we hoped, through the absence of organic matter, to eliminate such other forms as require the same for their growth and multiplication.

The solution was invariably placed in small 6 oz. medicine bottles, which were filled to a depth of 2–3 inches, the bottles being previously plugged with cotton-wool, and sterilised at 150° C., whilst after introducing the liquid, the whole was steam-sterilised for upwards of an hour on three successive days.

Five bottles of this description were inoculated with a minute quantity of garden soil on May 9th, 1887, and placed in an incubator at 30° C.

On examination after eleven days, the bottles were all of them turbid, exhibited a slight skin on the surface and yielded the characteristic reactions for nitrous and nitric acids. Three of these solutions, which had nitrified, were submitted to gelatine plate cultivation; the plates developed numerous colonies, many of which caused liquefaction of the gelatine. A number of different colonies were picked out with the sterile platinum needle, and individually inoculated into separate bottles containing the sterile ammoniacal solution, but not one of these yielded any nitrous or nitric acid on subsequent examination after suitable incubation.

From one of the original bottles which had nitrified after addition of garden soil, a second bottle of ammoniacal solution was inoculated with a platinum needle; when this bottle had undergone nitrification, a fresh bottle was similarly inoculated from it, and so on in a long series of generations. The dates of inoculation of these successive generations, with other particulars, are recorded in the following table :—

* This salt solution was prepared by dissolving 1 grm. of potassium phosphate, ·2 grm. of crystallized magnesium sulphate, and ·1 grm. fused calcium chloride in 1000 c.c. of water.

Generation.	Date of Inoculation.	Quantity taken for Inoculation.	Date when Nitrification first observed.	Remarks.
I.	9. 5.1887	Original garden soil . . .	20. 5.1887	* Incubator at 30° C.
II.	25. 6.1887	3 needle-loops from I.	30. 6.1887	* ,, ,,
III.	1. 7.1887	,, ,, II.	7. 7.1887	,, ,,
IV.	14. 7.1887	,, ,, III.	23. 7.1887	,, ,,
V.	25. 7.1887	,, ,, IV.	17. 8.1887	* ,, ,,
VI.	26. 8.1887	,, ,, V.	1.10.1887	,, ,,
VII.	3.10.1887	1 ,, ,, VI.	7.10.1887	* ,, ,,
VIII.	7.10.1887	1 needle-point from VII.	17.10.1887	,, ,,
IX.	17.10 1887	,, ,, VIII.	29.10.1887	,, ,,
X.	7.11.1887	,, ,, IX.	30.11.1887	At first in incubator then at 15–20° C.
XI.	1.12.1887	,, ,, X.	15.12.1887	15–20° C.
XII.	16.12.1887	,, ,, XI.	13. 1.1888	,,
XIII.	28. 1.1888	,, ,, XII.	20. 2.1888	* ,,
XIV.	29. 2.1888	,, ,, XIII.	5. 4.1888	,,
XV.	7. 4.1888	,, ,, XIV.	27. 4.1888	* ,,
XVI.	30. 4.1888	,, ,, XV.	10. 5.1888	,,
XVII.	12. 5.1888	,, ,, XVI.	26. 5.1888	,,
XVIII.	19. 7.1888	,, ,, XVII.	3. 9.1888	,,
XIX.	3. 9.1888	,, ,, XVIII.	1.10.1888	,,
XX.	11.10.1888	,, ,, XIX.	20.11.1888	,,
XXI.	24.11.1888	,, ,, XX.	26. 2.1889	,,
XXII.	26. 2.1889	,, ,, XXI.	4. 5.1889	,,
XXIII.	28. 6.1889	,, ,, XXII.	18.10.1889	,,
XXIV.	4.11.1889	,, ,, XXIII.	17.12.1889	,,

In the case of those generations marked with a * plates were poured and numerous bottles inoculated with isolated colonies,† but in no case could nitrification be induced. In the plates poured from the later generations the colonies had the appearance of being all of the same kind, and thus differed markedly from the plates obtained from the earlier generations in which there was a great variety of colonies, including liquefying ones which were entirely absent in the later plates.

Not only were bottles inoculated with single colonies, but, thinking that nitrification might be due to the combined action of two or more distinct organisms, bottles were sometimes inoculated with two or more different kinds of colonies from the same plate, and also in some cases with a large number of colonies taken from an overcrowded plate. In no single instance, however, have we succeeded in obtaining nitrification from a plate-cultivation, either from a single colony or from a number of colonies.

In addition to the series of experiments with the line of direct generations referred to above, several series of collateral experiments were being simultaneously carried on.

These collateral experiments were principally directed towards the separation and isolation of the nitrifying organism by the method of attenuation or dilution.

† These bottles were maintained in some cases at 30° C., in others at 15–20° C.

Dilution Experiments, Series I., commenced October 20, 1887.

50 c.c. of sterilised distilled water were placed in a sterile stoppered bottle and then inoculated with a few drops taken from the "sixth generation" (see p. 113), and the mixture was then violently shaken for some time to thoroughly disintegrate any coherent masses of organisms that might have been introduced. From the attenuation thus prepared a number of test-tubes containing sterile ammoniacal solution were respectively inoculated with one loop of a platinum needle, another series of test-tubes with two loops, and a single test-tube received as much as twelve drops from the above attenuation.

The several tubes thus inoculated on October 20, 1887, were examined on November 30, 1887, when the one which had received twelve drops yielded strong reactions both with diphenylamine and sulphanilic acid, whilst the tubes inoculated with one and two loops respectively gave no reactions, nor did they when subsequently examined on December 23, 1887. Thus only in the case of the tube which had received twelve drops had any nitrifying organisms been introduced, whilst the smaller quantities (one and two loops) employed for the other tubes must have been quite free from these organisms.

The solution which had been nitrified with the twelve drops added as above was reasonably to be regarded as purer than the "sixth generation" from which it had been obtained by large dilution. This "twelve-drop attenuation" as it may be called, therefore, was cultivated further in successive generations, each giving rise to well-marked nitrification. The third generation of this series was plate-cultivated, and inoculations made with a number of the colonies, but in no case did nitrification result.

The second generation of this twelve-drop attenuation was employed as the starting point for the

Dilution Experiments, Series II., commenced March 5, 1888.

The solution used for dilution in these experiments had been inoculated on December 1, 1887, it was found to have nitrified on December 23, 1887, but on re-examining on March 5, 1888, it not only gave reactions with diphenylamine and sulphanilic acid, but also with NESSLER'S solution, and it might, therefore, be taken that the nitrifying organism was still in full activity. Two drops of this solution were added to about 50 c.c. of sterilised water, well shaken, and then five bottles containing sterile ammoniacal solution were inoculated as follows :—

No. 1 bottle with 9 drops.
„ 2 „ „ 7 „
„ 3 „ „ 5 „
„ 4 „ „ 3 „
„ 5 „ „ 1 drop.

These bottles were kept in the dark at the ordinary temperature of the laboratory, and when examined on April 5, 1888, they were all found to give strong reactions both with diphenylamine and sulphanilic acid.

In this series the dilution employed had, therefore, been insufficient, but No. 5 bottle, which had received only one drop, would obviously be likely to be the purest of the series.

Plates were poured from this No. 5 bottle as the purest, and from the resulting colonies obtained a number of bottles containing sterile ammoniacal solution were inoculated, but in no case was nitrification induced.

Dilution Experiments, Series III., commenced April 7, 1888.

No. 5 bottle, as the purest of the last series of experiments, was taken as the starting-point for this series. 1 drop from this bottle was diluted with 50 c.c. of sterilised distilled water, and from this *first dilution* 3 bottles (Nos. 1, 2, and 3), containing sterile ammoniacal solution, were inoculated with 1 drop each. Each of these bottles thus received about $\frac{1}{1000}$ drop of the original solution (bottle No. 5 above).*

Further 5 c.c. of the above "first dilution" were diluted to 50 c.c. with sterile distilled water, and from this *second dilution* a bottle No. 4 was inoculated with ·5 c.c. (or $\frac{1}{1000}$ drop of original solution), a bottle No. 5 with 1 c.c. (or $\frac{1}{500}$ drop of original solution), a bottle No. 6 with 2 c.c. (or $\frac{1}{250}$ drop of original solution), and a bottle No. 7 with 3 c.c. (or $\frac{1}{167}$ drop of original solution).

The whole series thus consisted of—

No. 1	bottle containing about	$\frac{1}{1000}$	drop of	nitrifying	solution.
„ 2	„	„	„	„	„
„ 3	„	„	„	„	„
„ 4	„	„	„	„	„
„ 5	„	„	$\frac{1}{500}$	„	„
„ 6	„	„	$\frac{1}{250}$	„	„
„ 7	„	„	$\frac{1}{167}$	„	„

All these bottles were kept at 15–20° C. and were found to have nitrified when examined on May 10, 1888.

Thus, again, the dilution had been inadequate for assured isolation, but bottles Nos. 1–4 would obviously be likely to contain the nitrifying organism in a greater state of purity than the remainder Nos. 5–7. One of these purest bottles was, therefore, made the starting-point for the next series of experiments.

Dilution Experiments, Series IV., commenced May 12, 1888.

One of the presumably purest bottles of the previous series was made the point of departure for the renewed attempt at isolation.

* In this and subsequent calculations it is assumed that 1 c.c. consists of about 20 drops.

After a preliminary microscopic examination with a view to roughly estimating the number of micro-organisms in a given volume of the liquid, the following process of dilution was carried out :—

First Dilution (1 drop in 50 c.c. of sterile water).

No. 1 bottle inoculated with 1 drop (= $\frac{1}{1000}$ drop of original nitrifying solution).
No. 2 bottle ditto.

Second Dilution (3 drops of First Dilution in 50 c.c. of sterile water).

No. 3 bottle inoculated with 1 drop (= $\frac{3}{1,000,000}$ drop of original).
No. 4 bottle ditto.
No. 5 bottle ditto.

No. 6 bottle inoculated with 3 drops (= $\frac{9}{1,000,000}$ drop of original).
No. 7 bottle ditto.
No. 8 bottle ditto.

No. 9 bottle inoculated with 5 drops (= $\frac{15}{1,000,000}$ drop of original).
No. 10 bottle ditto.
No. 11 bottle ditto.

No. 12 bottle inoculated with 10 drops (= $\frac{30}{1,000,000}$ drop of original).
No. 13 bottle ditto.
No. 14 bottle ditto.

No. 15 bottle inoculated with 2 c.c. (= $\frac{120}{1,000,000}$ drop of original).
No. 16 bottle ditto.
No. 17 bottle ditto.

No. 18 bottle inoculated with 5 c.c. (= $\frac{300}{1,000,000}$ drop of original).

No. 19 bottle inoculated with 7 c.c. (= $\frac{420}{1,000,000}$ drop of original).

No. 20 bottle inoculated with 10 c.c. (= $\frac{600}{1,000,000}$ drop of original).

The object of this wide range of attenuation was to endeavour to secure nitrification in only a part of the series, in which case it would be probable that the most diluted bottle in which nitrification still took place had received only one or, at any rate, only few individuals of the nitrifying organism.

These bottles were examined on the 7th July, 1888, when it was found that of the three most highly diluted solutions, contained in bottles Nos. 3, 4, and 5, only bottle No. 4 had nitrified, whilst bottles Nos. 3 and 5 gave no reactions either with diphenylamine or sulphanilic acid. This, of course, suggested the possibility that bottle No. 4 had been nitrified by a pure growth. Two gelatine-tubes * were inoculated

* In all cases in which inoculations were made into gelatine-peptone, the needle was both stabbed into the material and also streaked along its surface, with a view to obtaining the depth and surface characters of any resulting growth.

from this bottle No. 4 on July 7, 1888, and on July 12 a very delicate growth in the depth and on the surface of the gelatine was observed. On microscopic examination, This was seen to be due to very characteristic long and slender bacilli. From one of these gelatine-tubes exhibiting this growth two bottles containing sterile ammoniacal solution were inoculated on July 12, 1888, and both of these bottles subsequently (examined on September 3 and October 1, 1888) nitrified.

This might at first sight be supposed to afford evidence that the nitrification had been caused by a pure growth taken from the gelatine-tubes, but on taking the following circumstances into consideration this was seen to bear another interpretation.

Thus, on July 7th, 1888, two bottles containing sterile ammoniacal solution were inoculated from the nitrified bottle No. 4 in the above series; already, on July 12th, these bottles were found to be distinctly opalescent, and one of them was tested for nitrous and nitric acids but with negative results; on microscopic examination, the bottle appeared to contain two different organisms, one a small fat bacillus, and the other a long bacillus, much resembling that observed in the gelatine-culture mentioned above. Bottles 3 and 5 of the above series, and which had not nitrified, were also microscopically examined and found to contain apparently pure growths of the short fat bacilli. Bottle 3 was inoculated into gelatine to compare the growth with that obtained from bottle 4, with the result that the same growth was formed, and this, on microscopical examination, was seen to consist of the same long bacillus. Somewhat later (July 15, 1888) the gelatine tube, inoculated from bottle 4, began developing a second and less conspicuous surface growth than that which first appeared, and this second growth, on microscopic examination, was found to consist of the short fat bacillus. Thus, both the long and short bacilli were obtained from bottle 4, which had nitrified, they were also both obtained from bottle 3, which had not nitrified; and, therefore, the only probable conclusion was that neither of these organisms were connected with the nitrifying process.

The nitrification in bottle 4 must, therefore, have been caused by some third organism which had not grown in the two gelatine-tubes inoculated therefrom, and the nitrification induced by inoculating these gelatine-tube growths, as described above, must have been due to the transference of some of this third organism along with the visible growth. This is the more likely as the inoculation from the gelatine-tubes into the bottles was made only five days after the inoculation (July 7, 1888) of the gelatine-tubes themselves from bottle No. 4; and, on subsequently (September 3, 1888) endeavouring to cause nitrification by inoculating from these gelatine-tubes, the attempt failed, probably because the growth had increased, and there was, therefore, less probability of carrying any of the original matter introduced into the tube with the inoculating needle.

Although the nitrification occasioned by inoculation from the gelatine-tubes referred to above, could certainly not have been caused by a pure growth, still there was every reason to believe that the bottles which had thus nitrified, would contain a less admix-

ture of other organisms than anything we had previously dealt with, and these were, therefore, made the basis for further attempts to isolate the nitrifying organisms.

One of these bottles, which had been inoculated from the gelatine-tube as above, and which had duly nitrified, was transmitted through four generations of culture in bottles containing sterile ammoniacal solution. Thus from the gelatine-tube inoculated on July 7, 1888 :—

I. Bottle containing ammoniacal solution was inoculated from above July 12, 1888 ; examined and found reactions with diphenylamine and sulphanilic acid, September 3, 1888.

II. Bottle inoculated from I. bottle, September 3, 1888 ; examined and found reactions for nitrification, October 1, 1888.

III. Bottle inoculated from II. bottle, November 11, 1888 ; examined and found reactions for nitrification, February 26, 1889.

This III. bottle was made the starting point for the following experiments :—

Dilution Experiments, Series V., commenced March 20, 1889.

One drop from the nitrifying solution in III. bottle referred to above, was diluted with 50 c.c. of sterile distilled water, giving the

First Dilution . 1 drop = $\frac{1}{1000}$ of original drop.

Second Dilution (1 drop of First Dilution mixed with 50 c.c. sterile water).

1 drop = $\frac{1}{1,000,000}$ of original drop.

No. 1 bottle inoculated with 1 drop (= $\frac{1}{1,000,000}$ of original drop).
No. 2 bottle ditto.
No. 3 bottle ditto.

No. 4 bottle inoculated with 2 drops (= $\frac{2}{1,000,000}$ of original drop).
No. 5 bottle ditto.
No. 6 bottle ditto.

No. 7 bottle inoculated with 3 drops (= $\frac{3}{1,000,000}$ of original drop).
No. 8 bottle ditto.
No. 9 bottle ditto.

No. 10 bottle inoculated with 10 drops (= $\frac{10}{1,000,000}$ of original drop).
No. 11 bottle ditto.
No. 12 bottle ditto.

Third Dilution (1 c.c. of Second Dilution mixed with 50 c.c. sterile water).

1 drop = $\frac{1}{50,000,000}$ of original drop.
No. 13 bottle inoculated with 1 drop (= $\frac{1}{50,000,000}$ of original drop).
No. 14 bottle ditto.
No. 15 bottle ditto.
No. 16 bottle ditto.

No. 17 bottle inoculated with 3 drops (= $\frac{3}{50,000,000}$ of original drop).
No. 18 bottle ditto.
No. 19 bottle ditto.
No. 20 bottle ditto.

No. 21 bottle inoculated with 5 drops (= $\frac{5}{50,000,000}$ of original drop).
No. 22 bottle ditto.
No. 23 bottle ditto.
No. 24 bottle ditto.

No. 25 bottle inoculated with 10 drops (= $\frac{10}{50,000,000}$ of original drop).
No. 26 bottle ditto.
No. 27 bottle ditto.
No. 28 bottle ditto.

No. 29 bottle inoculated with 2 c.c. (= $\frac{40}{50,000,000}$ of original drop).
No. 30 bottle ditto.
No. 31 bottle ditto.
No. 32 bottle ditto.

A few of the above bottles were examined for nitrous and nitric acids on May 4, 1889, or about six weeks after their inoculation, when the following results were obtained :—

Bottle No. 10 (dilution $\frac{10}{1,000,000}$) gave strong reactions with both diphenylamine and sulphanilic acid.
Bottle No. 1 (dilution $\frac{1}{1,000,000}$) gave distinct reactions.

Further bottles were similarly examined on May 7th, 1889 :—

Bottle No. 13 (dilution $\frac{1}{50,000,000}$) gave no reactions more than a blank bottle similarly preserved.
Bottle No. 14 (dilution $\frac{1}{50,000,000}$) ditto.
Bottle No. 15 (dilution $\frac{1}{50,000,000}$) ditto.
Bottle No. 2 (dilution $\frac{1}{1,000,000}$) ditto.
Bottle No. 11 (dilution $\frac{10}{1,000,000}$) gave slightly stronger reactions than the blank bottle.
Bottle No. 12 (dilution $\frac{10}{1,000,000}$) gave very strong reactions with both reagents.

Further examinations, both chemical and microscopical, were made on the following day, May 8th, 1889, with the following results :—

Bottles Nos. 1 and 3 (dilution $\frac{1}{1,000,000}$) yielded strong reactions both with diphenylamine and with sulphanilic acid, and both on microscopic examination were found to contain *a large number of very short almost micrococcus-like bacilli.*

Bottle No. 2 (dilution $\frac{1}{1,000,000}$), on the other hand, gave no reactions with either of the above reagents.

Bottle No. 4 (dilution $\frac{2}{1,000,000}$) gave no reactions.

Bottle No. 5 (dilution $\frac{2}{1,000,000}$) (examined on May 28, 1889) gave strong reactions with both reagents.

Bottle No. 6 (dilution $\frac{2}{1,000,000}$) (examined May 28, 1889) gave no reactions with the two reagents.

Bottles Nos. 7 *and* 9 (dilution $\frac{3}{1,000,000}$) gave strong reactions with both reagents.

Bottle No. 8 (dilution $\frac{3}{1,000,000}$), on the other hand, gave no reactions.

On May 28th, 1889, some of the bottles containing liquid still more dilute than $\frac{1}{1,000,000}$ were examined, but in every case with negative results. Thus :—

Bottles Nos. 29, 30, 31, *and* 32 (dilution $\frac{40}{50,000,000}$), all yielded negative results with both reagents.

Thus of a number of bottles inoculated with about $\frac{1}{1,000,000}$ of the original, some nitrified, whilst others did not, or, in other words, into some a nitrifying organism was introduced, whilst in others the inoculation failed to convey such an organism.

On the other hand, all the bottles containing less than $\frac{1}{1,000,000}$ of the original failed to nitrify, no nitrifying organisms having been conveyed in these more highly attenuated inoculations.

The most highly attenuated bottles which underwent nitrification being those which had received the dilution $\frac{1}{1,000,000}$, or bottles Nos. 1, 2, and 3, it was in these that the nitrifying organism in the greatest state of purity was to be expected.

It was upon these bottles, therefore, that our further attention was concentrated.

Of these bottles Nos. 1, 2, and 3, as already mentioned, Nos. 1 and 3 had nitrified strongly, and contained micro-organisms visible with the microscope, whilst No. 2 had not nitrified.

The contents of these three bottles were now submitted to investigation by means of cultivation-experiments. The results of these cultivation-experiments were highly remarkable.

Thus on inoculating from each of these bottles into tubes of gelatine-peptone—

(1) The tube from bottle No. 1, which had nitrified, never developed any growth whatever, although preserved for many months, indeed until the gelatine had become quite dried up.

(2) The tube from bottle No. 2, which had not nitrified, exhibited already on the third day a growth, consisting of a white surface expansion and beaded in the depth, and causing no liquefaction of the gelatine. Under the microscope the growth was seen to consist of chains of small bacilli, with bright shining spores.

(3) The tube from bottle No. 3, which had nitrified, also exhibited a growth.

These results clearly showed that in these three bottles we had two distinct

organisms to deal with; the one causing nitrification, but refusing to grow in the gelatine, the other growing in the gelatine, but incapable of producing nitrification. On this hypothesis bottle No. 1 contained the nitrifying organism in a state of purity, bottle No. 2 the non-nitrifying organism also in a state of purity, whilst bottle No. 3 contained a mixture of the two organisms. This supposition was the more probable as the less highly attenuated, and therefore presumably less pure, bottle No. 5 (dilution $\frac{2}{1,000,000}$), and bottles Nos. 10 and 12 (dilution $\frac{10}{1,000,000}$), all of which had nitrified, yielded growths on being inoculated into gelatine-tubes.

These observations were confirmed both by repetition and by the pouring of gelatine-plates from bottles Nos. 1 and 5, the plates from No. 1 yielded no colonies whatever, whilst those from No. 5 showed numerous small colonies.

It was thus established, beyond doubt, that the bottle No. 1 had nitrified, that it contained numerous micro-organisms of a very short bacillar form, but that this organism could not be cultivated on gelatine-peptone.

We next endeavoured to continue the cultivation of this organism in the ammoniacal solution, in order to ascertain whether it retained its nitrifying power, as well as its property of refusing to grow in gelatine.

Accordingly, on June 28, 1889, five bottles containing sterile ammoniacal solution were inoculated with a needle from bottle No. 1. These bottles, which may be described as No. 1 (2nd generation), were kept in the dark, at the temperature of the air, during the long vacation, and examined on October 18, 1889. *They were all five found to have strongly nitrified, but on inoculating them respectively into five different gelatine-tubes, in no single case was a growth developed.*

By way of control we had on the same day inoculated a gelatine-tube from the 23rd generation of the direct series experiments referred to on page 113, and this tube had already on the third day developed a visible growth.

On microscopically examining these five bottles (No. 1, 2nd generation), we again found in each case the characteristic small, almost micrococcus-like bacillus, to which we have had occasion to refer so frequently before, and which we may now fitly describe more in detail, having by the above experiments clearly established its causal connection with the process of nitrification.

Characterisation of the Bacillus of Nitrification.

(1) We have noticed that the solutions which had undergone nitrification by the organism in question remained perfectly clear, whilst the solutions which suffered nitrification by the mixture of organisms present in the direct series of experiments (see p. 113), generally exhibited a thin surface-film, and sometimes slight opalescence.

(2) One of the most remarkable features of the organism is its capacity of apparently indefinite growth in a medium practically destitute of organic matter,

although this property does not serve to distinguish it from the other organisms which accompanied it until the final separation by the process of dilution was accomplished.

(3) On microscopical examination, the solutions nitrified by the pure culture exhibited numerous small bacillar forms, which stain with somewhat more difficulty than most micro-organisms. The bacilli are about $0{\cdot}8\mu$ in length, and hardly longer than broad, in fact, their form is intermediate between that of a bacillus and micrococcus, so that the term "bacillococcus" may not inappropriately be used to designate them. They occur both isolated, in pairs, and in small irregular groups.

The accompanying figure exhibits their appearance as taken from bottle No. 1 itself.

Fig. 1.

Thus their appearance, although not so characteristic as to admit of their ready detection when present in small numbers along with other organisms, is still sufficiently definite to enable them to be distinguished from many other forms.

The organisms in the living state exhibit vibratory movement only.

Quantitative Determination of Nitrification.

We have already pointed out how necessary it is in speaking of nitrification, to indicate quantitatively the extent to which the process has taken place, to insure that the slight traces of nitrous and nitric acids so frequently exhibited by water which has been in contact with the air have not been mistaken for true nitrification.

We have, therefore, quantitatively determined the condition of the mineral nitrogen in several of the solutions in question. In these quantitative determinations we have employed the methods previously described by one of us ('Chem. Soc. Journ.,' vol. 53, p. 368).

Thus the ammonia was determined by means of NESSLER'S reagent, and comparison of the tint produced with that obtained with a standard solution of ammonium chloride. In determining the nitric acid, the nitrite was first destroyed by evaporating the solution with an excess of ammonium chloride, and then decomposing the residual nitrate with sulphuric acid and mercury, the nitric oxide evolved being carefully measured in a gas-analysis apparatus.

In the determination of the nitrous acid, on the other hand, the solution was evaporated to dryness, with a little pure caustic soda, to prevent the loss of nitrite by decomposition with ammonia present; the residue was then decomposed with urea and dilute sulphuric acid, the gas evolved (consisting of nitrogen and carbonic anhydride) was freed from carbonic anhydride by means of strong caustic soda, and the residual nitrogen was then carefully measured in the gas-analysis apparatus.

Bottle No. 1 (*2nd Generation*), inoculated on June 28, 1889, was thus examined on November 21, 1889, and found to contain :—

Ammoniacal nitrogen	=	5·76	parts per 100,000
Nitrous ,,	=	6·43	,,
Nitric ,,	=	0	,,

22*nd Generation of Direct Series from Garden Soil* (see p. 113), inoculated on February 2, 1889, was examined on December 20, 1889, and found to contain :—

Ammoniacal nitrogen	=	0	parts per 100,000
Nitrous ,,	=	10·71	,,
Nitric ,,	=	0	,,

23*rd Generation of Direct Series from Garden Soil*, inoculated on May 20, 1889, was examined on November 28, 1889, and found to contain :—

Ammoniacal nitrogen, not determined			
Nitrous ,,	=	8·92	parts per 100,000
Nitric ,,	=	0	,,

The Ammoniacal Solution used in these experiments was found to contain on November 21, 1889 :—

Ammoniacal nitrogen = 11·12 parts per 100,000.

From the above results it appears, therefore, that the pure growth had converted more than one-half of the ammoniacal nitrogen into nitrous nitrogen in less than five months, whilst no nitric nitrogen had been formed. The impure growths contained in the 22nd and 23rd generations had also only given rise to nitrous without any nitric nitrogen, even in the course of ten and a half months. In fact, in the case of the 22nd generation, the whole of the ammonia had been almost quantitatively converted into nitrous nitrogen.

That in the case of " Bottle No. 1 (2nd generation)," the sum of the ammoniacal and nitrous nitrogen is in slight excess of the ammoniacal nitrogen present in the original solution employed is probably due to the bottle in which this 2nd generation had undergone nitrification having been considerably larger than the others, and consequently more concentration by evaporation through the cotton-wool stopper must doubtless have taken place.

In order to put this point to the test, we, later on (February 15, 1890), again took a similar large bottle, in fact, a duplicate of No. 1 above, and found

N as NH_3	=	4·53	parts per 100,000
N as N_2O_3	=	8·00	,,
Total N	=	12·53	,,

thus clearly showing that the excess of total nitrogen in these large bottles was due to the greater amount of concentration by evaporation through the cotton-wool stoppers.

The exclusive production of nitrous nitrogen in these experiments is of special interest, as it raises the possibility of there being distinct organisms concerned in the formation of nitrites and nitrates respectively; whilst, on the other hand, it is by no means impossible that the formation of nitrate may yet take place in these solutions, or that the same organism may produce nitrates under other conditions. These are points which will require considerably more time to determine.*

The principal results of our investigation may be summarised as follows:—

1. The isolation, by the method of fractional dilution, of a micro-organism present in ammoniacal solutions undergoing nitrification originally induced by a minute quantity of garden soil.

2. The organism in question is possessed of a characteristic form, being a very short bacillus, about ·8μ long, hardly longer than broad, and exhibiting only vibratory motion.

3. The organism can be cultivated in suitable ammoniacal solutions to which no organic matter whatsoever has been added. In such solutions we have cultivated it for nearly three years.

4. In these solutions the growth of the micro-organism is accompanied by the gradual transformation of the ammoniacal into nitrous nitrogen, whilst hitherto we have not observed the formation of any nitric nitrogen in solutions inoculated with the pure growth.

5. The solutions thus nitrified remain perfectly transparent and pellucid.

6. The solutions nitrified by inoculating the organism in question, after its purification by the process of fractional dilution, have in every case yielded absolutely negative results when introduced into gelatine-peptone, the organism, as taken from such nitrifying solutions being apparently incapable of growth in this solid medium.

On the other hand, in the process of purification by dilution referred to above, the less diluted, and, therefore, presumably less pure portions, invariably yielded growths on being introduced into gelatine-peptone. The refusal to grow in gelatine thus serves as an invaluable guide in ascertaining the purity of the organism.

We are at present engaged in the further investigation of this interesting organism, but in consequence of the large amount of time which these observations involve, owing to the slowness of the process of nitrification, we have deemed it advisable no longer to delay the publication of this first part of our enquiry.

* In his study of the phenomena of nitrification, WARINGTON ('Chemical News,' vol. 44, p. 217) found that ammoniacal solutions seeded from old nitrified solutions generally only yielded nitrites and no nitrates.

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Appendix.

Although in the experiments recorded above we have uniformly found that the pure nitrifying solutions yielded no growth in gelatine-peptone, we have by no means abandoned the hope of cultivating the nitrifying organism on this and possibly on other solid media.

Thus, in our most recent experiments we find that on inoculating from the pure nitrifying solutions into broth, the latter develops after a considerable time (about twenty days at the ordinary temperature), a very characteristic growth, the liquid becomes turbid, a very thin whitish pellicle forms on the surface, and afterwards a considerable amount of glutinous deposit collects on the bottom, the whole liquid in fact becomes highly viscous and adheres to a needle in long strings.

On microscopic examination, this growth is seen to consist of small bacilli, about 1·5μ in length, and about ·5μ in breadth, sometimes, however, forming threads (5·7μ in length) in which the divisions are generally not apparent, but sometimes the divisions were sufficiently discernible to show that these threads were really made up of four or five individuals hanging together, end to end.

The accompanying drawing shows the appearance of the organism taken from a broth-cultivation.

Fig. 2.

On inoculating from such a broth-tube into a second, it is found that the growth makes its appearance more rapidly than in the first, thus whilst tubes inoculated from the nitrifying solutions required about ten to twenty days before any conspicuous growth was developed, the broth-tubes inoculated from these exhibited the characteristic growth in about six to ten days.

Although the microscopic appearance of the organisms in the broth-cultures thus somewhat departs from their appearance in the nitrifying solutions (compare figs. 1 and 2), such slight divergence in form is by no means uncommon in the case of one and the same organism when growing in different media, and in previous communications on the morphological characters of micro-organisms we have frequently had to call attention to such differences of shape, which are in fact well recognised by all who are acquainted with the nature of these low forms of life. We are, however, able to place beyond all dispute that the organisms growing in the nitrifying solutions (fig. 1), and those growing in the broth (fig. 2) are one and the same, for on inoculating the broth-growth into an ammoniacal solution, the latter (which, as we shall point out below, actually nitrified), again yielded the same characteristic forms as were represented in fig. 1. Thus the accompanying drawing shows the microscopic appearance

of the organisms taken from the ammoniacal and nitrifying solution inoculated from a broth-tube, which had itself been inoculated from a previous nitrifying solution :—

Fig. 3.

Not only, however, is the growth in broth much more rapid when the inoculation is made from a previous broth-tube, instead of from the nitrifying solution itself, but on inoculating from the broth into gelatine-peptone, a very slow growth actually makes its appearance in the latter.

This growth in the gelatine appeared after about three weeks in the first inoculation from broth, but on subsequently inoculating from this gelatine-culture in gelatine again, the growth was more rapid, appearing in from ten to twelve days.

This gelatine-growth appears as a transparent, smooth, shining, thin greyish expansion on the surface, which slowly increases in thickness, and causes a depression with gradual liquefaction. On microscopic examination, the growth was found to consist of small bacilli, often hanging together in pairs, in fact their appearance was intermediate between that of the bacilli from broth on the one hand (fig. 2) and from the nitrifying solutions (figs. 1 and 3) on the other. Fig. 4 represents the appearance of the organism taken from a gelatine cultivation.

Fig. 4.

Our nitrification experiments with these broth and gelatine cultures have not yet proceeded far; we have, however, already obtained undoubted conversion of ammonia into nitrous acid by means of two of the broth-cultures. Thus a broth-tube was inoculated from one of the pure nitrifying solutions on October 21, 1889; on November 2, 1889 (*i.e.*, after twelve days), the broth had become slightly turbid; on November 4, 1889, two bottles containing ammoniacal solution were inoculated from the broth-tube; one of these bottles was placed in the incubator at 25° to 27° C., whilst the other was left at the ordinary temperature of the air. On December 17, 1889 (or after forty-three days), the incubator-bottle gave undoubted reactions with both diphenylamine and sulphanilic acid. On January 10, 1890, these reactions had become very strong, and on February 12, 1890, the mineral nitrogen was quantitatively determined according to the methods described on page 122, with the following results :—

Ammoniacal nitrogen	=	10·70	parts per 100,000.
Nitrous ,,	=	1·10	,, ,,
Total . . .		11·80	,, ,,

Nitric nitrogen was presumably absent; it could not, however, be tested for, owing to the insufficiency of the liquid available.

As the ammoniacal solution originally contained about 11 parts of ammoniacal nitrogen (see analysis, page 123), and as some concentration of the liquid had taken place whilst in the incubator, it is obvious that the above results indicate marked conversion of ammonia into nitrous acid.

In the same way a bottle containing ammoniacal solution was inoculated from a similar broth cultivation on November 19, 1889, and placed in the incubator at 24° C. On January 10, 1890, this yielded strong reactions with diphenylamine, and also with sulphanilic acid.

The duplicate bottle inoculated on November 4, 1889, but kept at the ordinary temperature (15–20° C.) has not yet nitrified. Nor has any nitrification yet taken place in a bottle inoculated from a gelatine culture on December 10, 1889.

There is, however, nothing surprising in the retarded nitrification, which appears thus, at best, to take place from broth or gelatine cultures, for there are many other instances of the physiological properties of organisms being modified by change of soil. It has, indeed, been frequently observed by one of us in the case of other fermentations, that the fermentative power of a particular organism towards a particular substance may be greatly modified by growing it in different media. This appears to have been already recognised by Fitz in his later work ('Berlin, Chem. Ges. Berichte,' 1882, p. 878) in which the following very suggestive, but hitherto little recognised passage occurs :—

"Die Fähigkeit, Gährung zu erregen, wird ausser durch hohe Temperatur auch aufgehoben, wenn dem Spaltpilz sehr reichlich und andauernd Sauerstoff dargeboten wird.

"Wenn man, z. B., eine einzige Zelle in eine verhältnissmässig grosse Menge Kulturflüssigkeit aussät, so wird die Gährfähigkeit beträchtlich herabgestimmt und oft auch ganz aufgehoben.

"Ebenso verhält es sich wenn man den Spaltpilz bei sehr reichlichem Luftzutritt viele Generationen hindurch in einer Kulturflüssigkeit, in welcher er keine Gährung verursachen kann, kultivirt."

Brieger ('Zeitsch. f. Physiol. Chemie', vol. 8, pp. 306–311) points out that the "pneumococcus" of Friedländer loses its pathogenic properties when cultivated in sugar solutions, and only regains them when again cultivated in the ordinary solid media.

Another very remarkable instance of the chemical properties of a micro-organism being altered by the medium in which it is cultivated, is exhibited by the well-known *B. prodigiosus,* which on long-continued culture on gelatine or agar-agar loses its pigment-producing power, which can, however, be restored by cultivation on potatoes.

In the present instance the nitrifying organism had been cultivated, as already mentioned, for nearly three years, in the dilute ammoniacal solutions practically

destitute of organic matter, to which we have referred, so that its sudden transference to rich, nutritive media, like broth and gelatine-peptone, might not unnaturally be expected to produce a profound change in its organisation.

We have already shown how the capacity of growing in gelatine was entirely lost through this long residence in the dilute ammoniacal medium, which capacity was only restored by a preliminary cultivation in broth, similarly, we presume that cultivation in broth and gelatine may greatly diminish, and in some cases possibly extinguish, the nitrifying power of the organism.

It is particularly noteworthy that the ammoniacal solutions inoculated from the broth cultures, *on inoculation into gelatine, yielded no growth whatever, but gave the characteristic tardy growth in broth*, in this respect, as in the microscopic form of the organism they contained, precisely resembling the pure nitrifying solutions from which these broth-cultures were originally obtained.

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