ON THE CULTIVATION OF THE NITROSO-BACTERIUM.

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Since the time that Schloesing and Muntz[1] first discovered that ammonia could be converted into nitric acid through the agency of micro-organisms much attention has been paid to these bacteria. Many experiments were made by Warington[2] and P. and G. C. Frankland[3] to obtain pure cultures; but since they were only able to grow their micro-organisms in fluid media their results were not perfected. Winogradsky[4] has obtained more definite results by cultivating the micro-organisms both in fluid media and on plates. In growing the nitroso-bacteria Winogradsky employed a solution containing ammonium sulphate, potassium phosphate, and a basic carbonate, chiefly magnesium. Solutions made in this way were inoculated with soil, and when the ammonia showed evidence of oxidation sub-cultures were taken. He further isolated the bacterium from the sub-cultures by cultivation. As a result of his experiments he came to the conclusion that the nitroso-bacterium could not grow in the presence of organic matter, and that there are two distinct species of bacteria, morphologically similar, that can exist side by side in inorganic solutions. One of these, the nitroso-bacterium, can oxidise ammonia in an inorganic solution, but is unable to grow on agar, gelatine, or other ordinary medium, and dies in beef broth. The other species, although similar in form, does not oxidise ammonia, but can grow in inorganic solutions and thrives

[1] *Comptes Rendus*, vols. 84, 85, 86, and 89.

[2] *S. Chem. Soc.* Aug. 1888.

[3] *Proc. Roy. Soc. London*, vol. 47, S. 296, 1890.

[4] *Annales de l'Institut Pasteur*, nos. 4, 5, and 12, 1890; and *Archiv des Sciences Biologique*, St Petersburg, 1892.

on agar, gelatine, etc. In order therefore to isolate the nitroso-bacterium he prepared plates from silica and certain inorganic salts in solution, and by these means he was able to obtain good growth of the bacterium in question in plates composed entirely of inorganic constituents. In order to isolate the nitroso-bacterium by means of organic media Winogradsky advised that particles of magnesia taken from an oxidised ammonia tube be scattered on a gelatine plate; any particles showing no growth would have a pure culture of this species adhering to them.

In view of Winogradsky's work I commenced these investigations in 1895. During the progress of my work there have been important papers by Burri and Stutzer[1] criticising Winogradsky's results. In a more recent paper Winogradsky, in conjunction with Omeliansky[2], showed that the nitroso-bacterium is able to grow in the presence of large amounts of organic matter.

The solution used by Winogradsky for growing the nitroso-bacterium consists of water containing 1 per 1,000 ammonium sulphate, 1 per 1,000 potassium phosphate and 1 per 100 magnesium carbonate. The carbonate solution is sterilised separately and added to the solution of salts after sterilising to prevent chemical decomposition.

This solution I have continued to use all through the work, and in this paper it will be referred to as the *ammonia solution*. It was kept in test tubes, 10 c.c. being in each tube. It has always been tested for the presence of oxides of nitrogen, and a control kept when batches of the solution were inoculated.

The presence of nitrites is judged by a solution of diphenylamine in sulphuric acid, Ilosvay's solution being used as a control when necessary.

When I commenced the work I obtained cultures of the nitroso-bacterium by inoculating ammonia solutions with small quantities, 0·2 g. or less, of various kinds of soil; rich garden soil, humus, sand, etc. Tubes of the solution so inoculated were kept at room temperature, and placed in a dark cupboard in order to avoid exposure to light. The evidence of the growth of the nitroso-bacteria is found in the conversion of the ammonia in the solution into nitrous acid, and is the only one which I relied on in my work on these organisms, although there is much evidence to show that this change does not always take place even when good growth occurs. The conversion of

[1] *Centralblatt für Bakteriologie*, Abt. II. Band 11, no. 4, 1896.
[2] *Centralblatt für Bakteriologie*, Abt. II. Band 5, no. 12, 1899.

ammonia into nitrous acid is at first a slow process, and the chemical tests show that it does not as a rule commence for some 3 weeks, in tubes inoculated with soil; and having commenced, a week or two more are usually required before the whole of the contained ammonia is oxidised. Although this is a rule, many tubes have to be kept for months, and at times a year, before oxidation of the ammonia occurs.

I. *Original Cultures.*

I have inoculated ammonia solutions with various soils 43 times. Of these 43 solutions, 30, or 70%, oxidised, the majority within one month. One tube required four months before this occurred. The remaining 13 tubes did not oxidise during the time they were under observation, which extended to over a year in most instances. From tubes that showed oxidation of the contained ammonia, gelatine plates were poured; these showed moulds, yeasts, liquefying and non-liquefying bacilli; some species occurring on one culture and some on another. The only species constantly present on all the plates was an oval bacillus, morphologically like the nitroso-bacterium.

Dilutions from the original tubes.

In making sub-cultures I took 1/20 of a c.c. from the bottom of a culture tube. This had the two-fold advantage of excluding some extraneous organisms, and giving the smaller number of nitroso-bacteria a large amount of ammonia to oxidise. The comparatively few nitroso-bacteria present in a solution would result in some time elapsing before the ammonia was oxidised, and this length of time would tend to kill out most of the extraneous bacteria since there would be no organic matter present for them to feed on.

First Dilutions: 18 tubes were inoculated, and of these, 14 or 77% oxidised in 8 weeks. The remaining 4 tubes did not show any evidence of oxidation of the contained ammonia whilst under observation. Microscopic specimens made from the first dilution tubes that showed oxidation, showed chiefly oval bacilli corresponding in form to the nitroso-bacterium, rod-shaped bacilli being also present.

Second Dilutions: From the first dilution tubes seven sub-cultures were made. Six of these, or 85%, showed oxidation of the ammonia in 2 or 3 months. One tube failed to show oxidation whilst under observation. Microscopic specimens showed chiefly the oval bacteria as before, a few rod-shaped forms being also present.

Third Dilutions: Sub-cultures from the second dilutions were made in 10 instances. All of these oxidised the contained ammonia in 1 month. Microscopic specimens corresponded to those prepared from the second dilutions.

Fourth Dilutions: 14 sub-cultures were made from the third dilutions; of these 13, or 93%, showed oxidation. 11 oxidised the ammonia in 1 month: 2 oxidised

the ammonia in 3 months; 1 showed no evidence of oxidation. Microscopic specimens showed the oval bacteria in almost pure culture.

Fifth Dilutions: 9 sub-cultures were made from the fourth dilution tubes. Of these 7, or 77%, showed oxidation. 6 oxidised the ammonia in 2 months. 1 oxidised the ammonia in some months; 2 showed no change. Microscopic specimens from these solutions showed the oval bacteria in almost pure culture.

Cultures of the Nitroso-Bacterium from Thames Water.

15 tubes which contained the ammonia solution were inoculated with small quantities of unfiltered Thames river water. Of these, 10 showed oxidation of the ammonia in from 1 to 2 months. The other 5 tubes exhibited no evidence of oxidation.

From the oxidised tubes 6 sub-cultures were made; but these gave no evidence of oxidation after being kept for periods of from 2 to 15 months.

No further experiments were made.

Ammonia solution, containing no carbonate, used as a medium.

In order to observe the effect of the absence of carbonate I prepared a solution containing 1 per 1,000 ammonium sulphate and 1 per 1,000 potassium phosphate but containing no carbonate. Four tubes of this solution were inoculated with strongly nitrifying cultures. These four tubes all showed oxidation of the contained ammonia in 10 weeks.

From these cultures sub-cultures in the same medium were made; these showed no evidence of oxidation during the 5 months that they were under observation.

These experiments are interesting as showing that a certain amount of nitrification can occur without a basic carbonate being present, but this change cannot continue indefinitely. After a time the action ceases, and for continuous nitrification the carbonate is required.

A solution containing 1 *in* 10,000 *ammonium sulphate and potassium phosphate used as a medium.*

Experiments were made with an ammonia solution containing only 1 in 10,000 parts of ammonium sulphate and potassium phosphate, a fair quantity of magnesium carbonate being present. Several tubes of this solution were inoculated with strongly nitrifying cultures, but in no instance did oxidation occur during the several months in which the tubes were under observation.

Absence of nitrification was probably due to the small quantity of phosphates present.

II. *Liquid Media containing organic matter.*

I made a series of experiments with ammonia solutions containing various quantities of peptone beef broth, Witte's powdered peptone, and urea. Each of these substances was added to solutions containing the usual amount of ammonia, and potash phosphate and magnesium

carbonate, and the solutions were tested from time to time for the presence of nitrites.

Peptone Beef Broth.

The beef broth was added to the ammonia solutions in quantities varying from 1 in 11,000 to 10 per cent.; controls being kept in all cases.

1 *in* 11,000 *beef broth:* Six tubes were inoculated, one with earth, the other 5 with strongly nitrifying solutions. The tube inoculated with earth showed oxidation in 6 weeks. The 5 tubes inoculated with strongly nitrifying cultures oxidised in 2 months. Three sub-cultures all oxidised after some months. Control tubes showed no oxidation.

1 *in* 5,000 *beef broth:* Three tubes of this solution were inoculated. No. 1 with soil, No. 2 with a strongly nitrifying culture, No. 3 with a 1 in 11,000 beef broth tube that had oxidised. No. 1 oxidised in 12 months, No. 2 oxidised in 9 months, No. 3 oxidised in $2\frac{1}{4}$ months. The control tube showed no change.

1 *in* 2,000 *beef broth:* Two tubes were inoculated from the solutions oxidising in 1 in 5,000 beef broth. These both showed oxidation of the contained ammonia in 9 months. A control tube showed no oxidation.

1 *in* 1,000 *beef broth:* Two tubes of this solution were inoculated from 1 in 5,000 beef broth oxidising solution. These both showed oxidation of the contained ammonia in $2\frac{1}{2}$ months. Control tube showed no oxidation.

1 *in* 500 *beef broth:* Two tubes of this solution were inoculated from 1 in 1,000 beef broth oxidising solution. These showed oxidation of the contained ammonia in 6 weeks. Control tube showed no oxidation.

1 *in* 100 *beef broth:* Two tubes of this solution were inoculated from 1 in 1,000 beef broth oxidising solution. These showed oxidation of the contained ammonia in $5\frac{1}{2}$ months.

1 *in* 10 *beef broth:* Two tubes of this solution were inoculated from 1 in 1,000 beef broth oxidising solution. Directly after inoculation one of the tubes showed a very faint nitrite reaction, the other none. Both solutions showed good oxidation of the contained ammonia in $5\frac{1}{2}$ months. Control tubes showed no change.

Ammonia Solution containing Witte's peptone used as a medium.

1 *in* 11,000 *peptone:* Five tubes containing this solution were inoculated from strongly nitrifying cultures.

Results: 1 tube showed oxidation of contained ammonia in 1 month; 1 tube showed oxidation of contained ammonia in 2 months; 2 tubes showed oxidation of contained ammonia in 5 months; 1 tube showed oxidation of contained ammonia in 12 months. 2 Sub-cultures obtained from robust nitrifying cultures oxidised in 1 month.

1 *in* 5,000 *peptone:* 9 tubes inoculated; 5 with soil and 4 from 1 in 11,000 peptone solution that showed oxidation.

Results from soil: 2 tubes showed oxidation in $1\frac{1}{2}$ months; 1 tube showed oxidation in 2 months; 1 tube showed oxidation in 3 months; 1 tube no change.

Results from 1 *in* 11,000 *peptone:* The 4 tubes inoculated showed oxidation in

2 months. Sub-culture showed oxidation of the ammonia in 9 months. Control tube showed no change.

Ammonia Solution containing Urea used as a medium.

1 *in* 11,000 *urea:* 7 tubes of this solution were inoculated, one with soil and 6 from strongly nitrifying solutions. The tube inoculated with soil showed oxidation in $1\frac{1}{2}$ months. The 6 tubes inoculated from the nitrifying solutions showed oxidation in periods varying from 1 month upward.

1 *in* 5,000 *urea:* 8 tubes inoculated. 4 with soil and 4 from the 1 in 11,000 urea solution that showed oxidation of the ammonia.

Results from soil: 3 tubes showed oxidation in 2 months; 1 tube showed oxidation some time later.

Results from 1 *in* 11,000 *urea solution:* 1 tube showed oxidation in $1\frac{1}{4}$ months; 2 tubes showed oxidation in 2 months; 1 tube showed oxidation some months later.

3 sub-cultures from tubes actively nitriting oxidised in 9 months. Control tube showed no oxidation.

1 *in* 2,000 *urea:* 3 tubes of this solution were inoculated with 1 in 5,000 urea solution showing oxidation. Of these three 1 showed oxidation in $2\frac{3}{4}$ months. The others showed no change in 10 months.

1 *in* 1,000 *urea:* 2 tubes containing this solution were inoculated with 1 in 5,000 urea solution that showed oxidation. No change was observed at the end of 10 months.

III. *Experiments with Solid Media.*

Plate Cultures.

In carrying out the work of isolation of the nitroso-bacterium by plate culture I have worked with silica, gelatine, and agar plates.

Although for clearness of description I shall describe the silica, agar, and gelatine plates separately I was working with all three at one and the same time.

Silica Plates.

The following is the method adopted by Winogradsky in preparing silica plates:

Sodium silicate, or waterglass, is diluted with water until its specific gravity is 1·05. To 100 c.c. of this solution, 50 c.c. of hydrochloric acid specific gravity 1·1 are added, and this mixture is then poured into a dialyser. The dialyser containing the solution is kept in running tap-water for one day, it is then removed from the tap-water and placed in distilled water for two days, this distilled water being frequently renewed. The presence or absence of chlorides is noted by the reaction with silver nitrate solution. After the chlorides have almost entirely disappeared the solution is removed from the dialyser and concentrated by heat to about half its volume, and its setting power tested. This dialysed silica solution is then mixed

with a solution of various inorganic salts which is used as a culture medium. The coagulation of the silica in this solution forms the silica plate.

The solution of salts used consists of:

Ammonium Sulphate	...	0·4 gramme
Magnesium Sulphate	...	0·05 ,,
Potassium Phosphate	...	0·1 ,,
Calcium Chloride ...	...	a trace
Sodium Carbonate	...	0·6 to 0·9 gramme
Distilled Water ...	...	100 c.c.

A solution of the sulphates and chlorides, and another of the phosphates and carbonate are made and sterilised separately, and mixed when cool. The solutions are kept separate in order to avoid chemical change.

The solution of salts that is mixed with the silica solution serves two purposes. In the first place it aids in producing coagulation of the silica in solution. It also serves as a food for the nitroso-bacterium.

In endeavouring to prepare silica plates by this method many difficulties were encountered and it was months before I obtained plates that would grow the nitroso-bacterium. The chief difficulties that I met with were due to one or other of the following causes:

Using crude silicate of soda solution (commercial); preparing a pure silicate of soda in the laboratory; leaky dialyser; using too strong solutions, either of silicate of soda or of hydrochloric acid; mixing the acid and the silicate of soda solution in the wrong order; exposing the dialysed silica solution in a large mouthed vessel to the atmosphere of the room.

The first of these difficulties can easily be obviated by using pure silicate of soda solution. The second I endeavoured to overcome by making pure sodium silicate in the laboratory. This I found to be a difficult and tedious process and liable to the error of introducing too much sodium carbonate in its manufacture. I therefore came to the conclusion that it was better and simpler to buy pure sodium silicate solution from a good firm, in spite of the drawback of having to wait a month or more for it (it cannot be obtained from English firms apparently). The difficulty with a leaky dialyser is usually avoided if great care is taken in testing the dialysing paper in the first place. Yet in spite of every care a leak will at times occur; this is found out when testing the water for chlorides, silica giving a marked cloudiness in the presence of nitrate of silver, which clears up on addition of nitric acid. Using too strong a solution of either silicate of soda or hydrochloric acid causes premature coagulation. If the solutions be very much too strong immediate coagulation occurs, if slightly too strong coagulation occurs in the dialyser. This can be avoided by using solutions of proper strength. If the silicate of soda solution be added to the acid, premature coagulation occurs; the acid must be added to the silicate of soda solution. If the dialysed solution be poured into a beaker and left, merely covered over for a few hours, before the salts have been added, it will coagulate spontaneously, through the CO_2 present in the air of the room acting on the large surface exposed, I believe. It is therefore necessary to keep the dialysed fluid in a narrow-necked vessel, and to use it as soon as possible.

In my earlier experiments I had to contend against each and all of these

difficulties, and the outcome of my experience was that I found it necessary to slightly alter the method advocated by Winogradsky.

The method eventually adopted was as follows: I took pure silicate of soda of a specific gravity somewhat less than that advised by Winogradsky; its strength varied from 3·3 to 4%; to this I added an equal quantity of hydrochloric acid solution, specific gravity 1·1. It is, as before mentioned, necessary to add the acid to the silica, otherwise premature coagulation occurs. The silicate of soda solution and acid thus mixed were then dialysed. I dialysed the solution for 4 days in running tap-water in order to remove the chlorides, present as chloride of sodium, in the solution. It is not absolutely essential to remove all the sodium chloride, since this salt is not in any way prejudicial to the growth of the nitroso-bacterium if only present in small quantity, but I have found it preferable to remove it from the silica solution at this stage since any quantity above a trace would tend to produce spontaneous coagulation of the silica. After the greater portion of the chloride had been eliminated by dialysing in tap-water, the dialysis was continued with distilled water until all traces of chlorides had disappeared. After this process had been completed the dialysed fluid was poured into a narrow-necked flask and evaporated down to about half its bulk. When it had been concentrated to this extent it was ready to receive the salts, which were added to afford an inorganic means of sustenance to the micro-organisms. The solution of salts used for this purpose was the same as that advised by Winogradsky.

To prepare a silica plate the evaporated silica solution is pipetted off into a fairly deep Petri dish, and about half the quantity of the solution of salts added. The whole is then evaporated slowly over hot water until the silica coagulates and forms a clear whitish jelly-like mass with a smooth surface. The plate is then ready for inoculation. To inoculate such a plate the culture is first mixed with sterile water and then pipetted over the surface of the silica plate and any excess of the inoculating fluid poured off.

Growth of Organisms on Silica Plates.

The plates were inoculated with the following cultures:—

1. Ammonia solution which oxidised directly from soil (impure cultures).

2. Cultures containing few if any species of extraneous organisms, these having been excluded by dilutions and plate cultivation.

3. Cultures obtained from silica plate colonies (pure cultures).

Plate from impure culture.

From an impure culture one silica plate was inoculated. After eleven days colonies of various species were seen, these included two kinds of bacilli; also yeasts and moulds. From this result I found at once that a medium containing only inorganic materials did not exclude or prevent the growth of all organisms except those which I sought, namely, the nitroso-bacterium; and this fact at first created some doubts as to the possibility of my being able to obtain a pure culture of such

species by this means. Seeing that I must work from purer cultures I continued to inoculate silica plates from solutions in which most of the extraneous bacteria had died out.

Plates from 2nd Group of Cultures.

Several plates were inoculated. After 7 days at room temperature numerous tiny colonies were seen which looked like points and were almost invisible to the naked eye. Under the A. A. Zeiss these colonies were seen to be yellow or brown in colour, homogeneous in appearance, variable in size, with serrate or dentate margins. A "Klatsch," or contact specimen, showed that these colonies were made up of oval organisms.

All the plates inoculated showed great numbers of these micro-organisms. In half the plates they were in pure culture. In two a few bacilli were also seen surrounding the oval forms and forming with these latter a colony. A large oval organism was also noted in some one or two plates associated with the small oval bacillus. All the silica plates inoculated from the second group of cultures showed oxidation of the contained ammonia. In two instances this occurred in 12 days.

Plates from 3rd Group of Cultures.

Silica plates were inoculated with ammonia solutions that oxidised from colonies taken from earlier plates. After some days the colonies developed, and corresponded to those already described. A contact specimen showed a pure culture of oval bacilli corresponding to those found on the earlier plates.

From the silica plates that showed oxidation of the contained ammonia pieces containing colonies were on 9 occasions inoculated into ammonia solutions. Of these solutions 5 oxidised, one in 1 month, two in 2 months, one in $2\frac{1}{2}$ months, and one in 3 months. Several ammonia solutions were also inoculated with single colonies from silica plates which showed oxidation of the ammonia. One such ammonia solution showed oxidation. From this oxidised solution bouillon agar plates were poured and these plates showed good growth of colonies. On the original plate they were tiny, and under the A. A. Zeiss they were seen to be yellowish-brown in the centre with ground-glass-like margins. Their outline was regular. The dilution plates showed cream-coloured colonies varying in size up to 4 mm. in diameter. A microscopical specimen showed oval micro-organisms morphologically like the nitroso-bacterium.

Gelatine Plates.

The gelatine that was chiefly used was prepared from beef broth in the usual way. Besides this a gelatine medium was also prepared from extracts of various soils, these soil extracts being filtered and sterilised, and then gelatine to 10 % added. Neither peptone nor sodium chloride was added, and the reaction was not altered unless it was acid to litmus. At first gelatine plates were poured and allowed to set; then particles of magnesia were taken from ammonia solutions that had oxidised, and scattered on them, as advised by Winogradsky, in the hope that one or other of these particles would show no growth and so yield a pure culture of the nitroso-bacterium. In no case however did I meet with this result, invariably numerous colonies developed round the particles of magnesia. The colonies that developed round the particles were often in nearly pure culture, and I noticed that they were made up of oval organisms, morphologically resembling the nitroso-bacterium. Secondly, I poured several plates from oxidised ammonia solutions. Those inoculated from the ammonia solutions oxidising directly, those from earth usually liquefied. Those plates inoculated from subcultures from ammonia solutions developed very numerous colonies, which produced no liquefaction. These colonies corresponded to those that grew round the particles of magnesia. To find if these colonies had any relation to the organisms I was in search of, small pieces of gelatine on which colonies were observed, were cut out by means of a platinum needle and inoculated into test-tubes containing the ammonia solution. Out of 8 tubes so inoculated one showed signs of oxidation of the ammonia at the end of 3 months. From this oxidised ammonia solution plates were poured, and these exhibited the same species of organism as already noted.

To the naked eye the colonies presented at first a whitish and polished appearance. After keeping the plates some days these colonies became pale yellow, and this colour deepened later on. Under the A. A. Zeiss they were seen to be irregular in shape, with a brown centre and white margin.

Gelatines prepared from diverse soils gave the same result, namely, a pure culture of an oval organism morphologically similar to the nitroso-bacterium.

Agar Plates.

With this medium, as with gelatine, I commenced by using agar prepared with beef broth.

In continuing my researches, beef broth was replaced by sterile soil extracts, and finally agar was prepared with the solution of salts which I had always used as a cultivation medium, namely, a solution consisting of 1 % ammonium sulphate, 1 % potassium phosphate, and 1 % magnesium carbonate in distilled water.

Beef broth agar was inoculated with ammonia solutions that had oxidised directly from soil. These plates showed moulds, yeasts, and various bacilli, numerous oval forms being present with others.

A large number of agar plates were then poured from subcultures of the oxidised ammonia solutions. These plates showed in all cases a large predominance of an oval micro-organism, and in many instances a pure culture of this species. It corresponded to that already seen on the gelatine plates. After 6 days at room temperature the colonies appeared to the naked eye as white, iridescent growths varying in size. Some days later they became lemon coloured, and later yellow. Under the A. A. Zeiss the colonies were seen to have a brown centre, the colour fading at the margin.

Beef broth agar plates were also poured from the solution that had oxidised from the piece of gelatine plate containing colonies, already mentioned. These plates gave precisely the same result as above.

The further experiments made with agar were carried out in the hope of proving that this was either the nitroso-bacterium or a parallel organism which was morphologically similar but physiologically different. To this end beef broth agar plates which showed a pure culture of the oval organisms were taken, and pieces containing these colonies were inoculated into sterile ammonia solutions. 53 such tubes were inoculated and 20 showed oxidation of the ammonia, after two months as a rule.

Since these pieces of agar plates so inoculated might have produced oxidation from nitroso-bacteria which were present but not growing, control experiments were made by inoculating beef broth agar plates with solutions which would produce a pure culture of the colonies of the oval bacillus. These plates were then kept until the colonies had developed, and pieces of the agar removed where no colonies were seen after careful search with the microscope, and were inoculated into sterile ammonia tubes and kept for a period of from 10 weeks to 4 months. This experiment was made 19 times but on no occasion was any oxidation set up in the ammonia solutions inoculated. Hence we have:

Agar plate with colonies: Inoculated 53, Oxidised 20.

Agar plate without colonies: Inoculated 19, Oxidised 0.

As before mentioned, agars were also prepared from various soil extracts. Among these may be mentioned watery extracts from garden soil, watery extract from old manure heaps, watery extract from Thames mud; and these together with any other variety of soil obtainable were used to prepare agars, which I hoped and trusted would form a suitable means of growing the nitroso-bacterium.

These agars yielded precisely similar results to those already mentioned.

Being unsuccessful in obtaining oxidation of the ammonia from a single colony taken from bouillon agar I took a single colony from

a silica plate and grew it on sloping bouillon agar. When I had obtained good growth I subcultured it into an ammonia solution that was in an artificial filter containing sterile soil.

This filter consisted of two glass cylinders each about 1 inch in diameter, plugged at one end with wool and at the other end with perforated india-rubber corks. Through the perforation in these corks glass tubes were fitted and connected by means of a piece of india-rubber tubing about 8 inches in length. The cylinders were thus connected in such a way that not only could fluid pass from one cylinder to the other, but either cylinder could be raised or lowered at pleasure. Into one of these cylinders, sterile soil to the depth of $1\frac{1}{2}$ inches was placed, and on to this soil the ammonia solution was poured in sufficient quantity to saturate the soil and also allow of the presence of about 50 c.c. of the fluid, which would either remain above the soil, or on raising this cylinder would flow through the soil into the other, empty cylinder. The whole filter was then sterilised. The cylinder containing soil and ammonia solution was then inoculated with the agar culture before mentioned. Every day the cylinder which contained the soil was either raised or lowered. By raising this cylinder the fluid would flow from the soil into the empty cylinder, leaving the soil in a condition that would allow of its aeration. On lowering the cylinder the fluid would flow back into the soil again. This was done daily and the soil thus alternately aerated and moistened without being exposed to contamination from extraneous organisms.

The ammonia in solution in this filter was oxidised in 10 weeks, a control filter showing no change. No other experiments were made after this manner on this occasion.

The plates which I have been most successful with in growing the nitroso-bacterium were prepared from ammonia agar. The medium consisting of ammonium sulphate, 1 g.; potassium phosphate, 1 g.; distilled water, 1 litre.

The salts were dissolved and agar added to $1\frac{1}{2}\%$, the whole boiled up and prepared as ordinary agar. After sterilising the agar some sterile carbonate of magnesia was added, the amount being roughly 1 % by weight, the exact amount being of no consequence.

As will be seen, this agar corresponded in composition to the ammonia solution used for the ordinary cultures, save for the presence of the $1\frac{1}{2}\%$ agar. It has a slightly lower melting and coagulation point than bouillon agar.

Although this ammonia agar was the medium that I have been most successful with, yet it had certain disadvantages. The presence of the particles of carbonate of magnesia added to the difficulty in examining the plates, the colonies being closely associated with it and assuming a somewhat similar appearance under the A. A. Zeiss.

On this medium also the colonies of the nitroso-bacterium were very difficult to pick up; when a platinum needle was dipped into them they broke up, and neither specimen nor culture could be satisfactorily obtained. In order to be successful the colony had to be entirely removed by digging it out.

Having melted the agar and allowed it to cool to as low a temperature as was practicable, I inoculated 3 tubes in the usual way to obtain an original and two dilution plates. The culture that was inoculated was either a few drops of an oxidising ammonia solution or a piece of ammonia plate which contained colonies that had already undergone this process. On such plates the colonies developed in a few days at room temperature, and good oxidation of the ammonia could occur in as short a time as 3 weeks on the original plate. The dilution plates at times also showed nitrification a few weeks or months later, but in these plates the process was naturally much slower, when it did occur, on account of the fewer nitroso-bacteria present.

Inoculation of strongly Nitrifying Solutions.

This was done on 20 occasions. Of these plates so inoculated 16 original plates showed oxidation. The time varying from 3 weeks to 3 months.

The ammonia in the dilution plates oxidised less readily, and only 3 out of 40 poured showed this change.

Inoculation of small pieces of Ammonia Plates that showed oxidation.

This was repeated 35 times. Of the plates so inoculated 15 showed oxidation of the ammonia in from 1 to 2 months. From the oxidised ammonia agar plates pieces containing colonies were placed in ammonia solutions. After a lapse of time these solutions showed oxidation of the ammonia, and then from them ammonia agar plates were inoculated. This was done 6 times, dilution plates being also poured. All the original plates in these 6 experiments showed oxidation of the ammonia in from $1\frac{1}{4}$ to $4\frac{1}{4}$ months. None of the dilution plates showed any oxidation, although the same colonies could be observed on them.

Description of Colonies occurring on ammonia agar plates.

The plates were opened as a rule 1 month after inoculation, not sooner, to avoid the drying up of the medium.

In the plates that showed oxidation of the contained ammonia the colonies were numerous. They occurred in two forms, those which grew on the surface, and those in the depth. The colonies on the surface could not be seen by looking directly on to the plate, but if it were held almost on a level with the eyes they appeared as dull, colourless ground-glass-like growths 1 mm. or more in diameter. Under the A. A. Zeiss they could be seen by careful arrangement of light and focussing to be finely granular colonies with a dentate or almost moss-like margin. The colonies in the depth could only be seen as points to the naked eye. Under the A. A. Zeiss they exhibited the following characteristics, the centre had a flaky or lumpy appearance, and was of a pale reddish-brown colour; from this centre the colony

spread indefinitely and almost invisibly, its colour being lost, only a faint brown shade and a granular appearance was seen; this spreading margin extended in some instances to a neighbouring colony. At times the granular margin assumed a filamentous character, giving the colony a spider-like appearance. The very tiny colonies had the same flaky or lumpy appearance, but one could not see any spreading margin; their outline appeared to be more definite and regular.

Specimens made from all the above forms of colonies showed micro-organisms morphologically similar to the nitroso-bacterium.

All the ammonia agar plates that showed oxidation contained great numbers of them. The plates which showed no oxidation contained few or none, or else (as was seen on one or two occasions) they were very poorly developed all over the plate.

I also endeavoured to inoculate single colonies on sloping ammonia agar in tubes, but of several tubes so inoculated one only showed good oxidation of the ammonia; this occurring in 9 months. From the centre of this tube, which showed oxidation of the ammonia through its whole bulk, a tiny piece was taken and inoculated into beef broth agar, and plates poured. In 3 days at room temperature enormous numbers of colonies, some 3,000,000 or more, were seen under the A. A. Zeiss. They were sherry coloured, and had a sharp outline but no definite shape. The dilution plates showed greyish-white, polished, semi-translucent colonies which after some days developed to 1 mm. or more in diameter. After 1 month they became yellow in colour, as already noted in former experiments with beef broth agar. These colonies were made up of oval bacilli morphologically similar to the nitroso-bacterium.

Potato used as Culture medium.

I have inoculated potatoes from ammonia solutions showing oxidation, on several occasions. Profuse growth always occurred, the colour being usually yellow. On 10 occasions subcultures were made in ammonia solutions; oxidation occurred twice.

Cultivations of the Nitroso-bacterium at 37° C.

I have made a few experiments with cultures containing nitroso-bacteria at this temperature. Ammonia solutions were inoculated with earth; these showed oxidation in about a month. Ammonia solutions were then inoculated with strongly nitrifying solutions and incubated, but these failed to show any evidence of oxidation. It would seem therefore that 37° C. is not well suited to these bacteria.

Anaerobic Cultures.

Tubes subjected to this form of culture failed to give any evidence of oxidation of the ammonia contained in them.

Finally I would mention that the length of time during which I have been engaged in this work was due in the first place to the fact that the functional power of the nitroso-bacterium is only displayed when the micro-organism is in considerable quantity, and that it is easily lost. It has frequently occurred that cultures known to contain this species failed to show any oxidation of the ammonia in which they were inoculated. This happened in about 30 % of the solutions, and at least 100 plates. Secondly, the time required by the bacterium to oxidise ammonia was lengthy; two months was the average time, and there were several instances in which 4, 5, 6, 9, and even 12 months were required.

Summary of Results.

In the first place cultures of the nitroso-bacterium were developed in inorganic solutions. These carried to a 5th dilution exhibited practically a pure culture of this species.

Secondly, cultures of the nitroso-bacterium were inoculated into solutions containing small quantities of organic matter. In these they were able to oxidise the ammonia present. It was found that a culture developing in the presence of small quantities of organic matter was better able to oxidise the ammonia in higher percentages of organic matter than a culture taken directly from an inorganic solution.

In growing this species on plates silica jelly has the disadvantage of being difficult to prepare. It was 18 months before I obtained a satisfactory plate. This jelly grows the nitroso-bacterium well, but other species can also develop colonies on it. Ammonia agar also grows the species, as shown by the oxidation of the ammonia, and the colonies assume a characteristic form.

As to beef broth agar and gelatine, these media grew colonies of a micro-organism similar to the nitroso-bacterium from oxidised ammonia cultures. Pieces of these plates containing such colonies in pure culture frequently oxidised ammonia in solutions. On the other hand pieces of such plates showing no colonies never produced any oxidations of the ammonia. Furthermore the single colonies from silica and ammonia agar plates, which oxidised the ammonia in the media

into which they were sub-cultured, grew well on beef broth agar and gelatine.

From the above results I have come to the following conclusions:

(1) That the nitroso-bacterium grows well on any ordinary medium.

(2) That the supposed parallel organism is no other than the nitroso-bacterium itself.

(3) That in the presence of large percentages of organic matter the nitroso-bacterium, although growing very profusely, loses for a time the power of converting ammonia into a nitrite.

In conclusion I beg to offer my best thanks to Dr Blaxall, Bacteriologist to the Government Lymph Laboratories and Westminster Hospital Medical School. On his advice I undertook this work, and through his kindly help I was enabled to carry out the little that I have done in contributing to the study of the micro-organisms associated with the nitrifying process, whose life history is as yet but imperfectly known and whose functions are of such far-reaching importance.