FURTHER OBSERVATIONS ON NITROSO-BACTERIA.

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In a former paper[1] I discussed the methods that I employed to cultivate the nitroso-bacterium. To this end both fluid and solid media were used.

I obtained my cultures in the first instance by inoculating small particles of garden soil into a solution advised by Winogradsky[2] consisting of 1 per 1000 ammonium sulphate, 1 per 1000 potassium phosphate, and 1 per 100 magnesium carbonate. The nitroso-bacteria developed in 70 % of the tubes and required as a rule two months to nitrify. After carrying on a strongly nitrifying culture through six successive ammonia solutions the culture appeared to be almost pure, from microscopical examination.

Continuing to use ammonia solutions I tried the effect of the presence of small quantities of organic matter in them[3]. The organic matter tried was introduced as either beef broth, urea, or peptone, commencing in quantities so small as 1 per 11,000. I used stronger solutions as nitrification developed, and in the case of beef broth was able to obtain nitrification when as much as 10 per 100 was present in an ammonia solution. Neither peptone nor urea was so favourable, although nitrification occurred in presence of small quantities.

I also employed solid media. Those used were silica jelly, as advised by Winogradsky, agar, and gelatine. Cultures used for inoculation were obtained from the strongly nitrifying ammonia solutions above

[1] Fremlin. *Journal of Hygiene*, III. 364–379, 1903.

[2] Winogradsky. *Ann. de l'Inst. Past.* IV. 1890, and V. 1891; also *Arch. des Sci. Biol. de St Pétersb.* I. 1892.

[3] A. Stutzer. *Centralblt. f. Bakt.* Abt. 2, Bd. VII. 1901.

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mentioned. Silica jelly with nutrient salts was a good medium, as also was ammonia agar (made from 1 per 1000 ammonium sulphate, 1 per 1000 potassium phosphate, and 1 per 100 magnesium carbonate and $1\frac{1}{2}$ % agar). I was able to obtain individual colonies both from silica and ammonia agar plates; these colonies inoculated into ammonia solutions in three tubes set up nitrification. When nitrification occurred in this solution I subcultured into bouillon agar plates and found that large numbers of colonies developed. Both bouillon agar and gelatine were also used as media. Cultures of the nitroso-bacteria from nitrified inorganic media were inoculated into these and plates poured; these plates developed a pure culture of a bacillus similar to the nitroso-bacterium. Pieces of the plates were inoculated into ammonia solutions. This was done 53 times, and of these inoculated tubes, 20 or 37 % showed nitrification. Again 19 pieces were taken from the bouillon agar plates where no colonies were observed and inoculated into ammonia solutions; in none of these did nitrification occur.

From all these results I came to the conclusion that the nitroso-bacterium can grow on ordinary media.

Since the publication of my former paper further evidence of nitroso-bacteria developing in organic matter has not been wanting. The following are results obtained by some of the workers on this subject.

(1) Coleman[1] came to the conclusion that peptone and urea in 0·5 % solution were inimical to nitrification.

(2) Niklewski[2] found that nitrite bacteria cannot develop in even dilute urine but are found and can develop well in manure. He says further that the nitrite bacterium is the same as that isolated from soil by Winogradsky and Omeliansky; but that these observers failed to grow it in organic matter, either from inoculating the organism in too small quantities, or from the unsuitability of their cultures.

(3) Stevens and Withers[3], in a large series of experiments, found that organic matter in large amount in soils is not inimical to nitrifying bacteria.

(4) Temple[4] obtained the most active nitrification in soils by adding cultures of nitrifying bacteria grown in manure.

(5) Fischer[5] used blood mixed with soil to assist nitrification.

[1] Coleman. *Centralblt. f. Bakt.* Abt. 2, Bd. xx. 484.
[2] Niklewski. *Ibid.* Abt. 2, Bd. xxvi. 388.
[3] Stevens and Withers. *Ibid.* Abt. 2, Bd. xxvii. 169.
[4] Temple. *Ibid.* Abt. 2, Bd. xxxiv. 204.
[5] Fischer. *Landw. Jahrbuch.* Bd. xli. 1911.

Reagents used for testing.

The tests for ammonia, nitrites and nitrates were made by a drop of fluid or a small piece of culture to one drop of the reagent on a white glazed plaque.

For ammonia—Nessler's test.

For nitrites—(1) Potassium iodide and starch solution with acetic acid; (2) sulphanilic acid and naphthylamine; (3) diphenylamine in sulphuric acid.

For nitrates—Diphenylamine in sulphuric acid.

For nitrates in presence of nitrites—Boil in saturated solution of ammonia chloride, then test with diphenylamine.

The diphenylamine solution is convenient for both nitrates and nitrites; when the solution contains nitrates without nitrites the blue colour does not develop instantaneously as it does with nitrites.

The Brucine test in my hands was not satisfactory. I obtained an equal reaction with either nitrites or nitrates.

In continuing my observations I have used cultures obtained from garden soil collected in Kent in most instances. In those cases in which they were cultivated from sewage mention is made of the fact. A culture is developed in a solution of ammonium sulphate, potassium phosphate and magnesium, or calcium carbonate in tap water. The ammonium sulphate and potassium phosphate may be in a strength of 1 per 1000 or as high as 1 in 200. I do not weigh the carbonate but certainly use quite 1 in 100 and probably much more. For some years I used light carbonate of magnesia as an alkaline carbonate, but finding that calcium carbonate (chalk) was equally good and more readily obtainable, I have used it instead in most cases for the past two or three years.

In these experiments the cultures were developed in a laboratory that has a mean annual temperature of 17° C., varying from 19·6° in the summer to 14·1° in the winter. An incubator was not used as I required a good deal of space for the cultures.

In this paper I will try to show that the nitroso-bacterium, cultivated under certain conditions, can nitrify organic matter, including some ordinary laboratory media, and also urine. I will first describe its cultivation on ammonia agar, then the results obtained by adding organic matter to this medium. Following this I will give results obtained in agar plates containing a small amount of organic matter but no ammonia, and then in bouillon agar plates. Experiments will

then be recorded with calcium sulphate blocks used as a means of obtaining a large bulk of culture, and the action of such cultures on laboratory media and urine.

Cultivation on ammonia agar.

About 20 c.c. of ammonia agar, poured and allowed to set in a deep beaker about 5 cm. in diameter, were inoculated from a culture of nitroso-bacteria in ammonia solution that I had had for about a year in a filter of sand through which ammonia solution passed.

Nitrification occurred after some weeks. On to this nitrifying culture a layer of ammonia agar was poured to a depth of about $\frac{3}{4}$ inch; this layer also nitrified, and on examination it was found that the nitroso-bacterium was present in the culture medium. As oxidation proceeded layers of ammonia agar were added until the beaker was filled; the nitroso-bacterium was found in all the layers. The culture thus filling the beaker was used from time to time to inoculate other media and was refilled as necessary with fresh medium. Since the nitroso-bacterium appeared to grow through the medium I was led to try the same method with cultures in plates; here again I found the nitroso-bacterium in the upper layers. Control experiments with *P. aureus* showed me that a few bacteria are washed up when fresh medium is poured on so that this might account for the nitroso-bacterium in upper layers of jelly. In order to avoid any washing up of bacteria I poured thick layers of ammonia agar in sterile Petri dishes, and when they had set dropped them carefully on to cultures in plates. I found then that the nitroso-bacterium grew upwards through the added agar and permeated the whole medium in the plate. Following on from this knowledge, pieces of culture were placed in the centre of ammonia agar plates, and it was found that the nitroso-bacterium grew from this centre and spread about into all parts of the plate. It was necessary only to take a piece from the margin of a plate so inoculated, after nitrification was well established, and to place it in the centre of a fresh plate to obtain a subculture. In connection with this observation it is to be noted that as soon as nitrite reaction began it diffused rapidly through the medium.

The question then arose whether the presence of nitrite was essential to the satisfactory development of the nitroso-bacterium. To settle this point a strongly nitrifying ammonia agar culture was dialysed until all the nitrite had disappeared. Pieces were then placed in the centre of six ammonia agar plates to inoculate them. In 17 days very good

nitrification had developed in four of the six plates inoculated. From this it would appear that nitrite is not essential to the growth of this species.

Ammonia agar with added organic matter used as a medium.

Cultures of the nitroso-bacterium from inorganic solutions were inoculated into ammonia agar containing differing amounts of organic matter in order to observe if any difference occurred either with regard to length of time required to develop nitrous formation or in the amount of nitrite formed. The organic matter added was that contained in bouillon gelatine or bouillon agar; 5, 10, and 20 % of one or other of the above jellies were used in the experiments.

Ammonia agar with 5 % *bouillon gelatine.*

This proved to be an excellent medium. Ammonia agar cultures of the nitroso-bacterium were either inoculated into the liquid medium as ordinarily obtains in pouring plates or else a plate was poured, allowed to set, and a culture in ammonia agar placed in the centre. No difference was observed in the length of time required for nitrification, two, three, or four weeks being the most common, the difference between the cultures was that the one containing organic matter in the medium showed very much more marked nitrification than the cultures in ammonia agar without this addition and was indeed more marked than I had previously obtained in any medium.

Ammonia agar with 10 % *bouillon gelatine.*

This also was an excellent medium, good nitrous formation developed. The plates were inoculated in the centre. Nitrification occurred in three weeks.

Ammonia agar with 10 % *bouillon agar.*

Here again a very good medium was found for the growth of the nitroso-bacterium, and in those plates inoculated in the centre the growth could be seen as a thin layer spreading outward until in some parts of the plate it would reach the margin like *B. mesentericus*, but unlike this species the growth was observed to be in the depth of the plate rather than on the surface, and this was so although the plates were inoculated on the surface!

Ammonia agar with 20 % *bouillon agar.*

This medium was inoculated from the 10 % bouillon agar cultures; good nitrification developed in most of these plates. More time was required before nitrification developed, but it was very pronounced after it had started. At least six weeks were required before it was well established and the development was not so certain as in the plates containing the lower percentages.

Ammonia agar with 30 % *bouillon agar.*

Twelve plates inoculated from 20 % bouillon agar cultures. After a year one gave very good nitrite reaction.

Potassium phosphate agar with 5 % *bouillon gelatine as a medium.*

Since the ammonia agar combined with a small amount of organic matter was found to be a very satisfactory medium for obtaining very marked nitrification, I thought that probably the ammonia was so well oxidised because the nitroso-bacteria had some organic matter to live on. If this was the case the nitroso-bacteria did not necessarily want ammonia as a source of food supply.

I had made many attempts to obtain an enzyme from these bacteria but had not satisfied myself up to that time; now it occurred to me that a medium containing phosphate of potash and bouillon gelatine in agar would grow the nitroso-bacterium, but as no ammonia would be supplied, I could perhaps obtain an enzyme, extract it from the plate, and then test its action on ammonia in solution.

With this object 12 plates were poured of a medium containing:

1 % potassium phosphate,
1·5 % agar,
5 % bouillon gelatine.

When they were set I inoculated them in the centre with strong nitrifying ammonia agar plate cultures. The plates were paraffined up and left by accident for 9½ months. On opening, all save three had dried up. As a matter of routine I tested for ammonia and nitrite; to my amazement, although no ammonia was found, nitrification in all was intense!

Here was evidence that the organic matter was used and directly nitrified.

I now used this as a medium and numerous plates were poured. Nitrification occurred in nearly 50 %, sometimes it was intense, at others only poor. Several plates were inoculated with a soil, 88 % of these nitrified, but subcultures from these carried on to plates of the same medium did not nitrify, evidently not proving a suitable medium for continued activity.

Ordinary bouillon agar used as a medium.

The results obtained with plates containing bouillon gelatine caused me again to attempt the cultivation of the nitrifying bacteria on ordinary bouillon agar made from beef broth, 1 % peptone, 0·5 % sodium chloride, and 1½ % agar. I was the more inclined to this since I had found that inoculation in the centre of a plate with a good quantity of culture had proved eminently successful.

In these experiments about 20 c.c. of ordinary bouillon agar were melted and thoroughly mixed in a Petri dish with an alkaline carbonate; the jelly was then left to set. When stiff, it was ready for the inoculation. Inoculations were always made in the centre of the plate, 2 or 3 c.c. of culture being incorporated with the agar jelly by means of a sterile spatula. The plates were then left for 24 hours and afterwards carefully paraffined to check evaporation of moisture. I find it always necessary to paraffin up plate cultures as these are frequently kept for six months or even a year before they are finally discarded, since the nitrifying bacteria are often very slow to commence operations. I poured altogether 47 of these plates.

The cultures used were very carefully selected and consisted, first, of those that had already oxidised ammonia in urine and other organic media; secondly, one that had been cultivated in dilute and undiluted urine for four years; thirdly, those that had been cultivated in ammonia solution for varying times, no organic matter having been added.

The following gives the number in each group, with results:

29	5	16
were from cultures in various organic media.	of cultures in urines.	from cultures in ammonia solution, one for 4½ years, one for 3½ years, seven for 2 years, seven for 6 months.
Of these, 6 showed good nitrification.	Of these, 2 showed good nitrification.	Of above, 2 showed nitrification, viz. those of 4½ and 3½ years' culture.

In these 10 plates nitrification was set up in the first instance in seven weeks, but it was seven months before it developed in the ten.

The first change observed in the plates was the formation of free ammonia. This commenced within 48 hours, and a large quantity was soon formed. The nine plates inoculated with young cultures showed not only formation of ammonia but developed also a very disagreeable odour like that of a very foul cesspool; this was absent from the other plates, which only had an ammoniacal odour lasting for a time.

Calcium sulphate blocks used for cultivation.

A method of culture found very satisfactory was carried out by the use of calcium sulphate in blocks. These were prepared by moulding this material into blocks of a size to lie in a Petri dish and allow of half an inch or so of space round them. This space was filled by the culture medium into which inoculations for subculture were made. After the block was introduced the plate was sterilised, and sterile culture medium could then be poured on. A $3\frac{1}{2}$ inch Petri dish containing a block will hold about 15 c.c. of fluid. I inoculated cultures of nitroso-bacteria into these plates, and at first two months were required for complete nitrification. When this occurred the fluid was poured off and fresh added. Less time was taken after each change of the fluid until only a week was finally required. The object of these blocks was to have a substance which the nitroso-bacteria could permeate so that one could have a large amount of active culture for testing fluids which could be readily poured off and changed.

These cultures were most useful for observing the action of the nitroso-bacterium on organic media, their success being probably due to the large amount of culture as compared with the amount of medium tested, and also to the efficient aeration of such shallow dishes.

The disadvantages were (1) that the fluid medium was liable to overflow by capillary attraction round the edge, and (2) that all examinations of the culture exposed it to contamination.

These calcium sulphate blocks were used for testing the action of nitroso-bacteria on urine, milk, peptone water, peptone beef broth, and blood serum.

Urine as a medium.

In the first instance undiluted urine with an alkaline carbonate was poured into the plates containing active cultures of nitroso-bacteria in calcium sulphate blocks. This set up marked bacterial action, nitrification ceased, gas having a disagreeable odour was produced,

and a very large amount of ammonia was formed. This fluid was poured off and water added. After a few days if no nitrification had occurred and much ammonia was still present the fluid was again poured off and more water added. Nitrification commenced later on and was soon well established.

Equal quantities of urine and water were also tried, but here again much ammonia was formed and as a rule the fluid had to be poured off and water added before nitrification developed. In some cases nitrification had been checked from six weeks to four months.

Twenty of the nitroso-bacterium cultures in calcium sulphate blocks were used for these urine tests: of these, 13 cultures had been obtained direct from sewage and the other seven were either from potassium phosphate agar and gelatine plate cultures, or from strongly nitrifying solutions from soil in ammonia solution.

The urine used has been sterile, fresh unsterilised, and also very old, stale, and disagreeable non-sterile fluid. The stale was nitrified rather more rapidly than the fresh, but after two or three lots had been used in succession on the same block nitrification was not so good. A culture rendered less active in this way could be put right by pouring on a 0·5 % ammonia solution.

It was found that urine diluted with four or six parts of water was very suitable, no odour or gas formation followed. The first quantity poured on took about three weeks to nitrify at 18° C., but after that four days to a week was long enough.

When a culture was working well the ammonia increased markedly for two days, on the third day it had diminished, and by the fourth it was gone and nitrification was very good. The decanted fluid gave no reaction to Nessler in the cold, but on distilling free ammonia was found, and on adding alkaline permanganate to the remaining fluid a good quantity of albuminoid ammonia was obtained!

Experiments with undiluted urine.

Although I had found that water was necessary with calcium sulphate blocks, on speaking to Mr Colin Frye, he considered that water should not be necessary if sufficient oxygen were present. Following that advice I took cultures that were already active and had nitrified urine; these I arranged in three groups. The first group consisted of three cultures made up of chalk and calcium sulphate; these I heaped up into dome-shaped masses and made a depression in the centre of the

top of the dome. They stood in deep trays, the culture fluid being poured into the depression in sufficient quantity to soak the culture masses and overflow into the dish. These raised cultures were to allow of penetration of air. The urine was poured from one to the other of these almost daily to assist further in aeration.

The second group consisted of ten active nitrifying cultures in Woodhead flasks, each containing 50 c.c. of urine, and these were aerated by pouring urine from one to the other as the first. The third group had six active cultures in jars with loose-fitting lids, which were treated in the same way, the urine being changed from one to the other until nitrification was complete. To control these 19 I had 22 cultures in Woodhead flasks, to each of these 50 c.c. of urine were introduced and this just covered the culture; all of these were left undisturbed. After about two months nitrification had developed in all the 41 cultures, occurring not only in those especially aerated but also in their controls!

The first change was the formation of ammonia, this developed in large amount in the first few days and gradually lessened as nitrification developed. No difference in time was noted between the aerated and non-aerated cultures, but the heaped up masses cannot be fairly estimated in comparison with the others as evaporation caused necessity for adding more urine from time to time, and to prevent too great concentration water was also added; however, nitrification developed in about the same time.

Effect of ordinary laboratory media on cultures of the nitroso-bacterium in calcium sulphate blocks that had been used for nitrifying sewage and urine.

Milk.

Milk diluted with four, eight, or 16 parts of water or 1 in 200 ammonia solution was used as a culture medium for five cultures of nitroso-bacteria in calcium sulphate plates.

In the first experiments water was used with the milk, but ammonia did not develop and nitrification was checked.

The water not proving satisfactory, the ammonia solution was tried, so that ammonia would be present in good quantity.

Milk diluted with three parts ammonia solution and some magnesium carbonate was tried on two cultures: nitrification in one case was checked, the reaction being poor for five days, the ammonia was also used up and a thick pellicle covered the fluid; on addition of more

ammonia solution however good nitrification occurred in another 48 hours.

In the second culture the nitrite reaction was lost for a week and a thick pellicle covered the fluid; all ammonia also disappeared. This fluid was poured off and fresh ammonia solution used. Nitrite again developed and was very good by the 13th day.

Milk diluted with seven parts ammonia solution was used on three nitroso-bacteria cultures. In these also nitrification was suspended, but again returned and was very good in from one to three weeks; it was necessary to add ammonia solution to these cultures to re-develop the nitrifying action, but no milk was added again until nitrification was well established. The further addition of milk medium to these plates inhibited nitrification to a greater extent than was the case in the first instance. A culture giving a good nitrite reaction in the presence of milk in seven days required 15 on again adding the solution containing milk.

Milk diluted with 15 parts of ammonia solution did not appear to check nitrification when added for the first time, but, as before observed, the addition of a second amount to such a culture caused some days delay in the nitrifying process.

Peptone water.

This was the ordinary 1 % solution as prepared for bacteriological work. It was diluted with equal parts of 1 in 200 ammonia solution.

This medium was used for six cultures. In all of these nitrification was good on the second day and all ammonia was used up by the 5th day: this is about the time required to use up the ammonia in an inorganic solution. The further addition of the peptone water medium on pouring off the first was followed by almost complete cessation of nitrification in four of the six plates, with formation of bubbles in five of them, and a disagreeable odour was also noted in three.

In from nine to 18 days however nitrification was re-established in all, and in only one instance was it necessary to pour off the peptone medium and add ammonia solution to obtain this result.

Peptone beef broth.

This was tried on five nitroso-bacterium cultures in calcium sulphate.

A 10 % solution in water was tried twice but nitrification was not good.

A medium was then used consisting of one part beef broth and one part 1 in 200 ammonia solution with some magnesium carbonate. This was poured on five cultures. Nitrification was checked for four or five days, and bubbles were frequently noted; in one instance a faint disagreeable odour was noticed, however by the eighth day nitrification was very good. One nitroso-bacterium culture was subjected to the above beef broth medium for two months and during that time the fluid was nitrified, poured off, and a fresh amount added six times. At the end of this time the culture continued to be exceedingly good, in fact a 50 % beef broth in ammonia solution was well suited to the cultures if used now and then.

Blood serum.

This, diluted, was used on nine cultures of nitroso-bacteria in calcium sulphate blocks. The dilution was usually one part of serum to seven. Four of the cultures were treated with serum diluted with water, the other five had serum with 1 in 200 ammonia solution poured on them. Those treated with serum and water lost the nitrification, the fluid becoming foul and numerous bubbles forming; the addition of ammonia solution re-established nitrification in from nine days to four weeks, and the nitrites were then formed in good amount.

The five plates on which the serum diluted with ammonia were poured varied: in three, very good nitrification occurred on the fifth day, in the other two nitrification ceased for a few days but was very good again after 10 and 19 days respectively.

Calcium sulphate blocks.

Cultures in Anaerobic conditions.

Eleven calcium sulphate blocks containing cultures of nitroso-bacteria were placed in these conditions by enclosing the Petri dishes with their contained culture blocks in jars used for anaerobic culture methods.

The oxygen was extracted by means of pyrogallic acid in caustic potash solution.

For the experiment six of the cultures were surrounded with 10 c.c. of a 0·5 % solution of ammonium sulphate and potassium phosphate to which some magnesium carbonate was added. The other five cultures were surrounded with 10 c.c. of urine diluted with water to 1 in 4 to which magnesium carbonate was also added. These cultures were controlled by aerobic cultures with corresponding media.

RESULTS.

Cultures with ammonium sulphate.

No nitrites found after 14 days. Large quantity of ammonia present, but some ammonia taken up in first week.

The control cultures had formed nitrites and completely oxidised the ammonia some days before the anaerobic cultures were finally tested.

One part urine and three parts water.

No nitrites formed.

In first two or three days a good quantity of free ammonia formed, and this was present until the end of the experiment, which lasted for some days after the controls had completely oxidised the ammonia.

All above plates, on being removed from anaerobic jars and placed in a cupboard exposed to ordinary atmospheric conditions, rapidly oxidised the ammonia in their surrounding solutions.

These results confirm those made with the cultures in test tubes, the results of which were given in my last paper.

SUMMARY.

Interesting results were obtained with cultures in ammonia agar, from them it is evident that the nitroso-bacterium grows readily through this medium whether inoculated on the centre of a plate or when covered with the agar jelly itself. Further, the presence of 5 to 10 % of beef broth in the ammonia agar allows of better development.

Potassium phosphate agar containing bouillon gelatine but no free ammonia, on being inoculated with cultures of nitroso-bacteria, also developed nitrification; the organic matter was broken down, ammonia formed, and nitrites built up.

Nitrification in ordinary bouillon agar plates shows that organic matter does not necessarily form a barrier to the process; and further, that two of the cultures had only been fed with inorganic salts for three and four years respectively prior to the inoculation into the plates, is good evidence that gradual additions of organic matter are not absolutely necessary.

Calcium sulphate blocks allowed me to work with a larger amount of very active culture: from these it was found that urine was a very good culture medium indeed. Also that peptone water diluted with

water, or peptone beef broth, milk, or blood serum diluted with ammonium sulphate could be used as culture media; sometimes these checked nitrification for a time but it afterwards became well established.

The experiments with undiluted urine were wholly successful and stand as a further proof of nitrification in presence of organic matter.

From these results it is clear that the nitroso-bacterium is a very powerful nitrite-forming micro-organism, that it is not readily destroyed, and that a certain amount of organic matter is essential to its greatest development, but for this a large amount of culture must be used.

In conclusion I have to offer my deepest thanks to Sir William Power for the great help that he has given me and for the kindness that he has always shown; also to others who have most kindly advised me my grateful thanks are due.