FURTHER NOTES ON THE CULTURE OF THE NITROSO-BACTERIUM.

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This research was begun in 1895, and since then, as opportunity offered, investigations have been carried out on the nitroso-bacterium. My first paper[1] gave particulars of the cultivation of this micro-organism. Following, in the first instance, the methods adopted by Winogradsky[2], of growing it on inorganic media, a solution was used containing ammonium sulphate 1 per 1000, potassium phosphate 1 per 1000 and magnesium carbonate 1 per 100, and for plate culture silica jelly was prepared to which nutrient salts were added. The results of these experiments, using organic media as controls, showed clearly that the nitroso-bacterium would grow on ordinary media prepared with bouillon. Following this up in my second paper[3] I used agar as a jelly instead of silica, and found that the presence of some broth or urine added to a water agar increased the activity of this micro-organism. I observed also that it was especially active when developed in calcium sulphate blocks used in Petri dishes.

In this paper I shall give more details of the life history of the nitroso-bacterium and its power of nitrification. As I have already in my earlier papers examined its action in many sorts of organic media, I shall confine myself to those easily prepared and at the same time suitable for its development.

The cultures of nitroso-bacteria used in my earlier experiments were of English origin and obtained from rich garden soils, humus, sand, etc., which nitrified satisfactorily in a 1 per 1000 ammonia solution and at times a little stronger as 1 in 500, or very occasionally 1 in 200.

For further work I wished if possible to obtain a stronger culture, and through the kindness of Colonel Prain, Director of Kew Gardens, I obtained samples of soils from some highly cultivated areas in the tropics. These I received in 1911, and after 2 years' work I obtained cultures that would nitrify 1 in 50 ammonium sulphate either in solution or in plates with agar.

The following media were used:

A solution 1 in 50 ammonium sulphate and 1 in 50 potassium phosphate in tap water, to which was added at the time of inoculation chalk to 1 in 50 or more; this medium will be called Am. K.

Urine both undiluted and diluted with tap water.

Urine allowed to become ammoniacal by keeping for about a fortnight at 22° C., both undiluted and diluted with tap water.

Peat extract, prepared from peat collected in woods by boiling for some hours in water

[1] Fremlin, H. S. (1903), *J. of Hygiene*, **3**, 364.

[2] Winogradsky, M. S. (1890–92), *Ann. Inst. Pasteur*, **4** and **5**; *Arch. Sci. Biol. de St Pétersb.* **1**.

[3] Fremlin, H. S. (1914), *J. of Hygiene*, **14**, 149.

https://doi.org/10.1017/S0022172400009967 Published online by Cambridge University Press

sufficient to cover it, and afterwards straining through a cloth; the peat residue left on strainer being used as a base in which to cultivate a large amount of nitroso-bacterium culture.

Watery extract of decomposed rabbit, prepared by opening up a freshly killed rabbit, leaving for a fortnight in sufficient water to cover it, at room temperature, and straining off the liquid.

1 per cent. mannite in 1 in 1000 Am. K.

A jelly composed of 4 per cent. potato starch to which 1 in 50 Am. K. solution was added.

Bouillon agar.

The object of using each medium was twofold, (1) to develop a good quantity of very active culture, (2) to develop nitrification on dilution plates and so isolate an active nitroso-bacterium in pure culture. In order to develop a quantity of liquid culture Woodhead flasks were used; these flasks possess the advantage of having a large area available for culture purposes and a small opening for inoculation so that inoculations can be made readily without fear of contamination. They were partly filled with peat residue and powdered chalk and then sterilised, and sterile culture medium was added at the time of inoculation. When nitrification was complete, that is when no ammonia reaction to Nessler could be obtained, the excess fluid was poured off and more culture medium, usually 40 c.c., added. Such a culture lasted from 2 to 4 years when working satisfactorily. For the further development of nitroso-bacterium in quantity in agar, a 1 in 50 Am. K. agar was used, and when nitrification was complete more of the medium was poured on. The ability of this micro-organism to grow through agar I described in my second paper.

1 *in* 50 *ammonium sulphate and* 1 *in* 50 *potassium phosphate solution in tap water.*

This was used as a culture medium both in test-tubes and Woodhead flasks. In test-tubes holding 10 c.c. of medium, complete nitrification usually occurred in $2\frac{1}{2}$ months. About 70 per cent. nitrified completely, 20 per cent. developed some nitrification, 10 per cent. failed. I have used Woodhead flasks with this medium for many years for developing a good quantity of culture. Complete nitrification occurred in 78 per cent., and 22 per cent. failed; they required on an average $2\frac{2}{3}$ months to nitrify the contents, and the life of a culture was about 4 years as a rule.

1 *in* 50 *ammonium sulphate and* 1 *in* 50 *potassium phosphate agar.*

These plates I have used for 14 years to maintain the activity of the nitroso-bacterium. They are poured before inoculation and allowed to set, then 3 or 4 grm. of an active culture is inoculated into the centre of the plate. I have made many such plates, of these 46 per cent. showed complete nitrification, 48 per cent. did not completely nitrify, and 6 per cent. failed to establish any nitrification. The average time for complete nitrification was 4 months.

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Urine.

Sterile undiluted urine was used in test-tubes alone, and in contact with peat and chalk in Woodhead flasks. The test-tubes contained 10 c.c. of urine and the Woodhead flasks a few c.c. more urine than the amount required to saturate the peat residue. Both test-tubes and flasks were inoculated with a good amount of active culture. The urine soon became ammoniacal but even after a year no nitrification occurred.

Urine with water agar in various percentages.
10 per cent. urine in water agar.
Plates inoculated in centre.

54 per cent. nitrified completely, 10 per cent. partially, 36 per cent. failed, average time for complete nitrification 2⅔ months.

50 per cent. urine in water agar.

30 per cent. nitrified completely, 40 per cent. partially, 30 per cent. failed, average time for complete nitrification 4 months.

Although undiluted urine as used above was not suitable for nitrification when a culture was added to it, yet if to a well-developed and active culture in a Woodhead flask 25 to 50 c.c. of fresh undiluted urine were added, complete nitrification would take place, and by removal of the excess fluid and addition of more urine further nitrification could be obtained. Complete nitrification occurred on an average in 3⅔ months, and the culture would remain active for about 2½ years. As mentioned in my last paper, a calcium sulphate block in a Petri dish in which nitroso-bacteria are active contained sufficient culture to nitrify rapidly a small quantity of urine. Also a filter bed of peat and chalk in which these micro-organisms have developed and shown active nitrification can be used as a urinal, and all urine flowing through it is so changed that no further decomposition is observable in it, the filtrate remains odourless and contains a large amount of nitrites and nitrates.

Mr R. E. Garrod, M.A., F.C.S., who kindly analysed the urine filtrates, was of opinion that the change was due to the conversion of the ammonia into ammonium nitrite and nitrate. During the War I fitted up many such filters as urinals, utilising cresol drums, horse-troughs, dust-bins, etc., for this purpose. Each was fitted with a perforated false bottom to allow of free aeration. The space below being tapped and the "filtered" urine drawn off. These utensils were used by forty men for a year or so and worked quite well. From time to time some of the nitrified peat was removed and was replaced by fresh peat so that I obtained a bulk of nitrified peat for further use. When the men left I had probably 5 cwt. of this material. Sawdust can be used instead of peat but it is not so reliable, probably on account of the many sorts of trees from which it may come, also when saturated with water 1 lb. of the sawdust that I used only took up 2 lb. 9½ oz. of fluid, whereas 1 lb. of the peat took up 3 lb. 14 oz. The power of the nitroso-bacterium to hold up ammonia was observed when such a filter was washed to remove the nitrates and nitrites;

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after these had been removed a large amount of ammonia not yet nitrified remained, and several further washings failed to remove it, whereas with a control filter to which a large amount of ammonium solution was added the ammonia was readily removed in a few washings.

Ammoniacal urine.

Although the nitroso-bacterium did not develop freely in fresh urine in tubes or flasks, yet if one kept urine for a fortnight or so, at 22° C., until it became strongly ammoniacal (roughly 0·6 grm. to 100) it proved an excellent medium for cultivating this micro-organism and could be used both undiluted and diluted as a liquid and agarised.

As a liquid.

40 c.c. in matured cultures in Woodhead flasks completely nitrified in from a week to a fortnight; it was then poured off and a further 40 c.c. added.

Plates.

20 *per cent. ammoniacal urine in* 80 *per cent. water agar.*

Inoculated with a loop of culture for dilution purposes.

Original plates.

Of these 62 per cent. nitrified completely, 38 per cent. nitrified partially. Those that nitrified completely required about 2 months.

First dilution plates.

66 per cent. nitrified well in 3 months.

25 *per cent. ammoniacal urine in* 75 *per cent. water agar.*

These were also inoculated with a loop of culture for dilution purposes.

Original plates.

75 per cent. nitrified completely, 25 per cent. only nitrified partially.

First dilution plates.

33 per cent. nitrified in 3 months.

50 *per cent. ammoniacal urine in* 50 *per cent. water agar.*

These were used for central inoculation only; 70 per cent. completely nitrified, 15 per cent. partially, 15 per cent. failed.

Ammoniated broth agar.

This was prepared by exposing broth at 22° C. for a fortnight when it became strongly ammoniacal, the fluid was then sterilised, and 1 per cent. agar added. Plates were poured and inoculated in the centre with active culture.

Plates.

50 per cent. nitrified completely, 25 per cent. partially, 25 per cent. failed.

1 *per cent. mannite in* 1–1000 *Am. K. solution.*

This was tried both in solution and as plates. The addition of mannite was found useful by Winogradsky[1]. In my experiments 19 months were

[1] Winogradsky, M. S. (1925), *Ann. Inst. Pasteur.*

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required to effect complete nitrification in the solution, and in plates nitrification was not complete. Calvert finds that 0·1 per cent. sucrose can be purified to meet standard sewage effluent[1].

4 per cent. potato starch with 1 *in* 50 *Am. K. solution.*

In the few experiments made, nitrification occurred in 80 per cent., the average time being $6\frac{1}{2}$ weeks. Some of the plates developed numerous colonies of a mould ? *Monilia.*

Bouillon agar.

This medium was used both as a culture medium for the development of nitrification, and as a means of isolating individual species occurring in the nitrifying cultures.

As a culture medium.

The plates were centrally inoculated with active nitrifying culture. Of 58 plates so inoculated, 55 per cent. nitrified completely, 4 per cent. partially and 41 per cent. failed to show any nitrification after being kept for periods of from $6\frac{1}{2}$ months to 1 year.

The attempt to cultivate the nitroso-bacterium is beset with delays from the first; a good nitrifying soil inoculated in an Am. K. solution only produces a certain percentage of active nitrifying cultures. Dilutions of these again are only in part successful. Further, each culture takes 2 months or so to nitrify the ammonia completely, so that it is only after a year or more that one has satisfactory nitrification of the fourth or fifth sub-culture, by which time one hopes to have excluded the majority of the other species present in the soil. The isolation of the nitroso-bacterium itself is much more difficult to obtain.

First of all it is necessary to obtain a plate dilution that shows good nitrification; and as the nitroso-bacterium develops better and more rapidly from a massive inoculation, a loopful of culture carried to dilution plates frequently fails; when successful it usually takes three months and often much longer. Such plates have to be carefully sealed to keep them from drying up. Melted paraffin wax run round with a brush answers this purpose, but renders plates difficult to open. For those only requiring a month or two, vaseline answers fairly well, and the plate can then be easily opened. Having a dilution plate, giving good nitrification, with scattered colonies that show the characteristic tiny oval forms, sub-cultures were carried on by cutting out separate colonies. The percentage of success, however, was small and fairly dilute media were necessary. 1 in 1000 Am. K. solution and agar and 1 in 4 ammoniacal urine agar were those used. Some colonies were inoculated on to $\frac{1}{2}$-inch square pieces of the agar, making little plates, and kept in a Petri dish. A colony setting up nitrification was transferred either to culture medium on a plate or to a liquid medium. If a plate of one colony was nitrifying poorly, it could sometimes be stimulated into greater activity by dropping $\frac{1}{2}$ c.c. or so of a semi-

[1] *Report of Water Pollution Research Board,* 1927–8.

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liquid mixture of 2 per cent. Am. K. solution and chalk on the plate here and there. The culture nitrifying from a single colony was then tested for its purity on bouillon agar plates, and then one found not the nitroso-bacterium but what appeared to be an almost pure culture of some other species! What had happened? Was the nitroso-bacterium present as a polymorphic species or had it been overwhelmed and destroyed by the more rapidly growing species? After much plate investigation impression preparations taken from dilution plates cleared the matter up, showing that the nitroso-bacterium almost always grows in association with some other species! In all the cultures of a single colony in which nitrification occurred I have never found the nitroso-bacterium alone. The micro-organisms accompanying it are usually bacilli but not of one species, one commonly occurring developed large, grey, moist, semi-translucent colonies consisting of Gram negative bacilli 2–4 μ in length, not growing at 37° C., and liquefying gelatine, not growing on potato. Another particularly annoying associate was a small spindle-shaped bacillus, which developed colonies not at first distinguishable from those of the nitroso-bacterium, and appearing as tiny droplets. Besides these, *B. mesentericus*, a sarcina, a coccus, and moulds as *Aspergillus*, *Penicillium* and a ? *Monilia* appear at times; any one of which might be the commonest species on a plate poured to observe species. On the hyphae of the ? *Monilia*, colonies of the nitroso-bacterium were to be found.

Another peculiarity of the nitroso-bacterium should be mentioned. In the ordinary method of plating, by inoculation of the agar in a test-tube before plating, a large number of the colonies grow below the surface of the medium. In order to obtain surface colonies a modification of the technique of plating was adopted; agar plates were made and allowed to set before inoculation. They were then inoculated with suspensions of the organism in water spread over the plate. When finally isolated this bacterium occurs as a micro-organism measuring 0·5 μ by 0·6 μ; it stains readily with the usual stains, such as carbol fuchsine in $\frac{1}{2}$ a minute and methylene blue in 2 minutes, and is Gram positive. Motility is only very slight in the many examinations that I have made. On gelatine slope cultures the lemon-yellow growth formed streaks as the gelatine liquefied slowly. Potato shows good polished lemon-yellow growth; grows well on sloping bouillon agar as a transparent greyish growth, becoming citron-yellow in a week to ten days. On agar plates the colonies appear as colourless and translucent growths about 1 mm. across on the fourth day and look like small drops of water; they become lemon-yellow a few days later and increase somewhat in size. Under slight magnification they are seen to be irregular in outline, translucent, with a raised centre, citron-yellow in colour and a broad colourless ground-glass-like margin, reminding one of a poached egg. Under a Zeiss AA. lens the colonies are coarsely granular and show a citron-yellow centre and colourless margin. These colonies are further characterised by a tenacious nature that renders it difficult to pick up the material on a platinum needle. On obtaining some of this material from the

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colony it is not easy to emulsify it on a slide. This is overcome by slight warming when the colony melts as it were and a large amount of jelly-like material spreads readily over the surface. On examining a specimen one finds the nitroso-bacteria spread out separately; this separation between the individuals is found in all the specimens made, in contact preparation or otherwise, and renders it easy to find this species when it occurs in a mixed colony. Colonies develop equally well on urine agar and Am. K. agar but are colourless and translucent.

The detection of the nitroso-bacterium rests on the following points:

It produces nitrites in ammoniacal solutions. Isolation is carried out by the removal of single colonies from plates showing good nitrification. As the nitroso-bacterium is usually associated with another species, further plate sub-cultures from these single colonies are necessary, and it is advisable to obtain these by diluting with water and spreading the dilutions on set agar plates.

Summary.

The nitroso-bacterium inoculated into sterile urine does not produce nitrites. If, however, urine be added to an active culture of this micro-organism nitrites are rapidly developed.

A nitroso-bacterium culture developed in bulk in peat and chalk can be used as a urinal. After passing through this urinal all ammonia in urine appears to have been converted into ammonium nitrite and nitrate. No odour is noticeable when left to evaporate at either 37° C. or at room temperature.

Urine rendered ammoniacal before use and then sterilised is also a good medium for the growth of the nitroso-bacterium.

The nitroso-bacterium is difficult to isolate because (1) when colonies are developed on a dilution plate, sufficiently spaced to allow of certainty in sub-culture, nitrification only rarely takes place in the sub-culture, and when it does take place some months are required for the production of a measurable amount of nitrite, and (2) the nitroso-bacterium usually grows in association with some other more rapidly growing species. The colonies of this other micro-organism often appear to be pure, but when growth has proceeded for some days individual colonies of the nitroso-bacterium may appear in a certain percentage of the plates.

(*MS. received for publication* 26. IV. 1929.—Ed.)

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