# THE INFLUENCE OF THE CULTURE MEDIUM ON THE GERMINATION OF ANTHRAX SPORES,

## WITH SPECIAL REFERENCE TO DISINFECTION EXPERIMENTS.

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In the determination of the germicidal action of various disinfectants, it is customary, after treating emulsions of the organisms or of their spores, or silk threads which have been soaked in such emulsions, with the disinfectant agent for the required time, to test the vitality of the organisms or of the spores by inoculating traces of the treated emulsion or of the treated thread (with or without preliminary washing or other treatment) into broth culture media. If a growth ensues, presumably the organisms or the spores have not been killed; while if there be no growth, presumably the organisms or the spores have been killed.

One of us (R. T. H.), in testing the germicidal action of certain emulsified disinfectants of the higher coal-tar series, noticed that if similar sub-cultures of the treated material were made both in broth and on the surface of sloped agar, it was only with the *weaker* disinfectant solutions that a growth was obtained in broth; with stronger concentrations, no growth was obtained in broth sub-cultures, even after incubation for so long as nine days or more, while with the same material and concentrations growths were frequently obtained on surface agar. That is to say, while the broth cultures indicated that the anthrax spores had been killed by the particular solutions, the agar

cultures proved that this was not the case and that many of the spores still retained vitality. The broth used in the experiments was ordinary nutrient peptone broth[1] and was a perfectly satisfactory culture medium when inoculated directly with anthrax spores, always giving good growths in the controls, which were made for every experiment. That is to say, if broth is inoculated with a considerable number of untreated anthrax spores, growth ensues; when, however, the spores are either few in number, or are partially de-vitalised by a disinfectant, they do not as a rule develop in broth. These observations have induced us to make further experiments on this point.

## Part I.

First of all the effect of five well-known potent emulsified disinfectants was tested on a suspension of anthrax spores. A virulent anthrax bacillus was grown on the surface of agar for (1) 3½ days and (2) three weeks, the growths were emulsified in sterile water so as to form a distinctly opalescent suspension when viewed in an ordinary test-tube (15 mm. diameter). The emulsions were filtered through sterile filter paper to remove masses, and one drop of the emulsion was added to each cubic centimeter of the various disinfectant solutions, of which 5 c.c. were employed for each test. Spores of two different ages were used because it is well known that old spores are more resistant than young spores.

The five disinfectants employed all have a Ridead-Walker carbolic coefficient of from 12 to 20 for the typhoid bacillus. Each disinfectant was employed in three different strengths, namely 2 per cent., 5 per cent. and 10 per cent. The time of exposure of the anthrax spores to the disinfectant was in some cases one hour, in others three days, and the temperature was maintained at about 20° C. After the period of exposure, broth, and surface agar, tubes were inoculated [each tube with one standard (3 mm.) platinum loopful], and were then incubated at 37° C. up to 72 hours if necessary. The results obtained are given in the Tables (I, II, and III) of the experiments at the end of this section. It will be seen from these that with the young anthrax spores (3½ days) with an exposure of one hour to the action of the disinfectant solutions, the 2 per cent. solutions of all the disinfectants gave no growth in broth, but were all positive (*i.e.* gave a good growth) in the agar; with the 5 per cent. and 10 per cent.

[1] Whether made with "Lemco" or with meat seems to make no difference.

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solutions no growth was obtained in either the broth or agar sub-cultures. (See Table I.) From this experiment it is obvious that had the broth cultures been taken as the index of the destruction of the anthrax spores, the 2 per cent. solutions would have been regarded as sufficient, whereas the agar cultures demonstrated that this was not the case. Still more striking were the results obtained with the older spores (*i.e.* three weeks old). Table II shows the results with an exposure of one hour to the 2 per cent. disinfectant solutions. It will be seen that *not one* broth culture gave a growth, while *all* the agar cultures were positive. Table III shows the results obtained with the old spores (three weeks old) with an exposure of no less than three days

## TABLE I.

Disinfectant allowed to act for one hour.

Young anthrax culture used ($3\frac{1}{2}$ days old).

| Disinfectant | Strength | Broth | Agar | Strength | Broth | Agar | Strength | Broth | Agar |
|---|---|---|---|---|---|---|---|---|---|
| A | 2 % | – | + | 5 % | – | – | 10 % | – | – |
| B | 2 % | – | + | 5 % | – | – | 10 % | – | – |
| C | 2 % | – | + | 5 % | – | – | 10 % | – | – |
| D | 2 % | – | + | 5 % | – | – | 10 % | – | – |
| E | 2 % | – | + | 5 % | – | – | 10 % | – | – |

– = no growth; + = growth of anthrax.

## TABLE II.

Disinfectant allowed to act for one hour.

Old anthrax culture used (three weeks old).

| Disinfectant | Strength | Broth | Agar | Strength | Broth | Agar | Strength | Broth | Agar |
|---|---|---|---|---|---|---|---|---|---|
| A | 2 % | – | + | 5 % | – | + | 10 % | – | + |
| B | 2 % | – | + | 5 % | – | + | 10 % | – | + |
| C | 2 % | – | + | 5 % | – | + | 10 % | – | + |
| D | 2 % | – | + | 5 % | – | + | 10 % | – | + |
| E | 2 % | – | + | 5 % | – | + | 10 % | – | + |

## TABLE III.

Disinfectant allowed to act for three days.

Old anthrax culture used (three weeks old).

| Disinfectant | Strength | Broth | Agar | Strength | Broth | Agar | Strength | Broth | Agar |
|---|---|---|---|---|---|---|---|---|---|
| A | 2 % | – | + | 5 % | – | + | 10 % | – | – |
| B | 2 % | – | + | 5 % | – | + | 10 % | – | – |
| C | 2 % | – | + | 5 % | – | + | 10 % | – | – |
| D | 2 % | – | + | 5 % | – | – | 10 % | – | – |
| E | 2 % | – | + | 5 % | – | + | 10 % | – | + |

at room temperature (17° C. to 20° C.). Again, not a single broth culture gave a growth, whereas all the agar sub-cultures with the 2 per cent. solutions were positive; with the 5 per cent. solutions, only Disinfectant D was negative, but with the 10 per cent. solutions all were negative except Disinfectant E.

## Part II.

Under regulation of the British Home Office, horse hair from certain countries, *e.g.* China, Siberia, Russia, may only be manipulated after being submitted to some process of disinfection, either to steam disinfection, or to some disinfecting agent "under conditions of concentration and temperature of the disinfectant, and duration and manner of exposure of the material to it, and otherwise, as are certified to secure the destruction of anthrax spores in all parts of all horse hair subjected to the process" (Factory and Workshop Orders 1901. For the use of Horse hair, 1907, No. 984).

"Certified" in the above means certified by the head of a Bacteriological Laboratory, and the Home Office accepts no responsibility for such certification.

We have tested two of the processes certified as sufficient under the above regulations. The first one tested specifies immersion of the material in a solution consisting of one part of Disinfectant A in 100 parts of water at a minimum temperature of 15·5° C. and a minimum duration of exposure of one hour; large bundles of material to be opened out. The second one tested specifies immersion in a solution consisting of 0·5 per cent. of soft soap, 0·5 per cent. sodic carbonate and Disinfectant B, 1 in 120: minimum temperature 20·2° C., minimum duration of exposure one hour; large bundles of material to be opened out and soaked in water previous to treatment. We have submitted these processes to experimental investigation. Silk threads and horse hair, in the form of single hairs, and small pledgets of unwashed sheep's wool, after preliminary sterilisation by heat, were soaked in aqueous emulsions of anthrax spores of varying age, and were then dried in dishes at 37° C. The materials thus prepared were employed in the experiments detailed below.

*Process No.* 1.

Disinfectant A, 1 % *solution, acting for one hour.*

(*a*) HORSE HAIR. Soaked in a suspension of 12-days-old anthrax spores, dried for ten minutes, and placed in the disinfectant solution for one hour at 20° C. The hairs were then placed in broth and agar tubes and allowed to remain in the 37° C. incubator for 18 hours.

Result { Agar +
Broth –

Result after 72 hours' incubation same as above.

(*b*) WOOL. Same procedure as in (*a*) but wool not allowed to remain on agar, only passed over the surface. Wool allowed to remain in broth.

Result { Agar –
Broth –

(*c*) SILK. Soaked in a suspension of four-days-old anthrax spores. Procedure as in (*a*).

Result { Agar +
Broth –

(*d*) SILK. Soaked in a suspension of 12-days-old culture spores. Procedure as in (*a*).

Result { Agar +
Broth –

(*e*) HORSE HAIR. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (*a*) except allowed to dry for 36 hours at 37° C.

Result { Agar +
Broth –

(*f*) WOOL. Soaked in a suspension of 12-days-old anthrax spores, then dried. Procedure as in (*a*).

Result { Agar –
Broth –

(*g*) WOOL. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (*a*).

Result { Agar + (slight)
Broth –

(*h*) WOOL. Soaked in a suspension of three-weeks-old anthrax spores. Disinfectant was then allowed to act for one hour, after which the wool was thoroughly dried, and finally placed in agar and broth tubes.

Result { Agar +
Broth –

Disinfectant A, 1 % *solution, acting for two hours.*

(*i*) SILK. Soaked in a suspension of 12-days-old anthrax spores; disinfectant then allowed to act for two hours at 20° C.

Result { Agar +
Broth –

(*j*) HORSE HAIR. Soaked in a suspension of 12-days-old anthrax spores. Procedure as in (*i*).

Result { Agar +
Broth –

Disinfectant A, 2 % *solution, acting for one hour.*

(*k*) Horse Hair. Soaked in a suspension of three-weeks-old anthrax spores; disinfectant then allowed to act for one hour at 20° C. Treated hair then inoculated on to slope agar and allowed to incubate for 18 hours at 37° C.

Result. Agar +

(*l*) Silk. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (*k*).

Result. Agar +

Disinfectant A, 2 % *solution, acting for two hours.*

Silk. Soaked in a suspension of 12-days-old anthrax spores; disinfectant then allowed to act for two hours at 20° C. Treated silk finally inoculated on to slope agar tubes and allowed to incubate for 18 hours at 37° C.

Result. Agar +

*Process No.* 2.

Disinfectant B solution.

(*a*) Silk. Soaked in a suspension of 12-days-old anthrax spores; disinfectant then allowed to act for one hour at 20° C. Agar and broth tubes then inoculated and incubated at 37° C. for 18 hours.

Result { Agar +
Broth –

After 48 hours' incubation,

Result { Agar +
Broth +

(*b*) Wool. Soaked in a suspension of 12-days-old anthrax spores, then allowed to dry in 37° C. incubator overnight; disinfectant then allowed to act for one hour at 20° C., wool dried again (as before) and finally inoculated into broth and agar tubes.

Result { Agar +
Broth –

(*c*) Horse Hair. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (*a*).

Result { Agar +
Broth –

After 48 hours' incubation,

Result { Agar +
Broth +

(*d*) Silk. Soaked in a suspension of four-days-old anthrax spores. Procedure as in (*a*).

Result { Agar +
Broth +

(*e*) Wool. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (*a*).

Result { Agar +
Broth –

From the foregoing experiments it will be seen that neither 1 % solution of Disinfectant A nor the Disinfectant B solution is effective in destroying anthrax spores. The difference is also well brought out between agar and broth media as agents for testing the vitality of the spores, the agar cultures being almost always positive, while the broth cultures were generally (only three exceptions) negative. Even a 2 per cent. solution of Disinfectant A acting for two hours (double the concentration and time recommended) was inefficient to destroy the anthrax spores.

## PART III.

Recently a method has been devised by Mr Seymour-Jones for the disinfection of anthrax-infected skins and hides. It consists in soaking the skins for 24 hours in a solution containing 1 % formic acid (90 per cent. strength) to which is added mercuric chloride sufficient to make a 1–5000 solution. After this treatment the skins are transferred for an hour or so to a saturated solution of common salt in water. This process has been the subject of an exhaustive investigation by Constant Ponder[1], who reports favourably upon it. We have tested the process on artificially infected material, soaking the material after treatment with the disinfecting solution in ammonium sulphide so as to render inert any adherent mercuric chloride. The result has been that silk threads and horse hair give no growth on agar or in broth, but sheep's wool has invariably given growth on agar (but not in broth), showing that the anthrax spores had not been killed.

It is difficult to explain why the wool is not disinfected, while the silk threads and hair are disinfected, by this process. It may be that the disinfecting solution is unable to penetrate into the interior of the wool pledgets on account of adherent grease. We thought that entangled air might play a part, but the results were the same even if the vessels are partially exhausted of air, so that the entangled air-bubbles are removed.

## SUMMARY.

The foregoing experiments conclusively show that a broth medium is quite unsuitable as a test culture medium to determine the vitality of anthrax spores in disinfection experiments, whereas agar is a suitable

[1] A report to the Worshipful Company of Leather-sellers on the incidence of anthrax amongst those engaged in the hide, skins and leather industries, with an inquiry into certain measures aiming at its prevention. Published by the Worshipful Company of Leather-sellers, London, 1911.

and delicate medium for the purpose, even when considerable traces of the disinfectant are carried over with the inoculation.

The reason for this inefficiency of broth is not obvious. We thought that it might be due to the absence of bacillar forms in the sporing material, but the emulsion of spores heated to 80° C. for 15 minutes and then inoculated directly into broth gave good growths.

Absence of oxygen might be another factor, but the results were the same when splinters of sterilised wood infected with anthrax spores were treated. The wood floated on the surface of the broth and so was subjected to a free supply of oxygen, yet no growths were obtained in broth when the splinters were soaked in the disinfectants, while good growths were obtained on agar. The control splinters gave good growths in broth. It may be that the anthrax spores are partially de-vitalised by the action of the disinfectant and that in this condition broth is a comparatively unsuitable culture medium for them. Prolonging the time of incubation of the broth cultures up to 10 or 14 days makes no difference. If a culture in broth shows no growth in 48 hours, a growth hardly ever appears with more prolonged incubation. Nor is this superiority of agar over broth as a culture medium confined to the emulsified disinfectants employed in these experiments, for similar results have been obtained with phenol, and with formaldehyde, the latter both in the fluid (formalin), and in the gaseous, conditions.