On the cultivation of bacteria / by Edgar M. Crookshank.

With the Author's Compliments

Cultivation of Bacteria

by

E. M. Crookshank

5.

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CONTAINING ITS

TRANSACTIONS AND PROCEEDINGS

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INCLUDING

EMBRYOLOGY AND HISTOLOGY GENERALLY.

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Fig. 1. Cover-glass impression-preparation from a plate-cultivation. (fuchsine). Zeiss' AA. Oc. 2.

BACILLUS FIGURANS.

Fig. 2. The same preparation. Zeiss' 1/8. o.i. Oc. 4.

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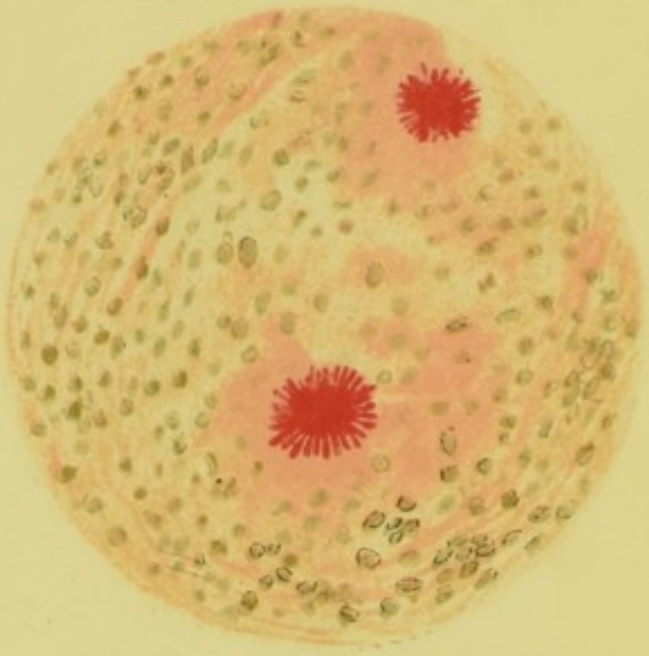
Fig. 1. From a section of a maxillary tumour in a cow.
Weigert's method. (Orseille and gentian-violet). Zeiss' 1/12. o. i. Oc. 4.



ACTINOMYCES.

Fig. 2. From a section of the lung of a cow.
Weigert's method. (Orseille and gentian-violet.) Zeiss' 1/12. o. i. Oc. 2.

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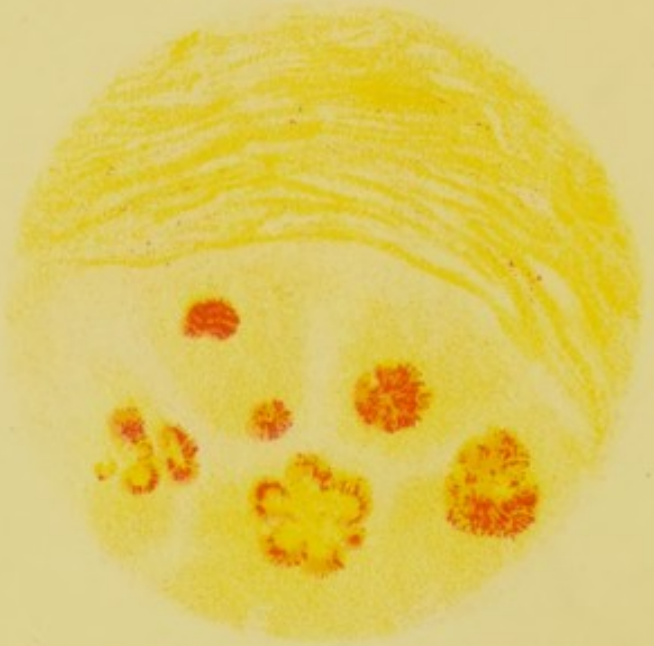

Fig. 1. From a section of a maxillary tumour in a cow. Plaut's method (Magenta and picric acid). Zeiss' AA. Oc. 4.

ACTINOMYCES.

Fig. 2. The same preparation. Zeiss' Ic. o.1. Oc 2.

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IV.—*On the Cultivation of Bacteria.*

By Edgar M. Crookshank, M.B. Lond., F.R.M.S.

(*Read 9th December*, 1885.)

Plates III.–V.

In the course of my remarks this evening upon the cultivation of bacteria, I shall touch upon several points which are well known to the Society. They will, however, lead me to bring forward many facts of extreme interest, and I trust of importance also, in that they disclose fresh fields for micro-biological research.

As is well known, there has been given during the last few years, more especially on the Continent, a very wide-spread stimulus to the study of bacteria. This is due in great measure to the encouraging results which have been obtained by employing the improved methods recently introduced for investigating micro-organisms.

The methods of cultivation on solid media have in many laboratories taken the place, almost entirely, of the old methods in which nutrient liquids were employed. I shall draw attention to some of the advantages offered by solid media, which may explain the reason for this change.

In the first place, the most essential thing in order to study the life-history of a particular micro-organism is to obtain and to maintain a "pure-cultivation." In the case of the pathogenic bacteria, this is emphasized by Koch as follows. Koch maintains that to prove satisfactorily that a particular micro-organism is the cause of a disease—

Firstly.—The micro-organism must be found in the blood, lymph, or diseased tissues, of man or animal suffering from, or dead of the disease.

Secondly.—The micro-organism must be isolated from the blood, lymph, or tissues, and cultivated in suitable media. These *pure cultivations* must be carried on through successive generations of the micro-organism.

Thirdly.—A *pure cultivation* thus obtained must, when introduced into the body of a healthy animal, produce the disease in question.

Lastly.—In the inoculated animal the same micro-organism must again be found.

Now, in the case of liquid nutrient media, it was no easy matter to obtain and maintain a pure cultivation.

If a drop of liquid containing several kinds of bacteria be introduced into a nutrient liquid, we have a mixed cultivation from

the very first: if then we require to isolate one species from the rest, the expenditure of much time is involved.

For example, to attain this object it was proposed, in the method of fractional dilution, to add sterilized nutrient fluid until there was an average of less than one germ to each drop of the fluid. If, then, fresh portions of sterilized nutrient fluid be inoculated with a single drop from the diluted mixture, some portions would in all probability receive no microbes, others would receive one or two, and others, again, one or more microbes of the same species. Then the growth of these microbes would give a pure cultivation of a particular species. It is obvious how complicated this process is, and how much the result would depend upon chance.

If, on the other hand, the mixture was left as a mixture, then the door was open to all sorts of conclusions. Some bacteria being unable to develope in the presence of others, or a change of temperature, or a change effected by the micro-organisms in the nourishing soil, allowing one form to predominate over another, the idea could arise that the various kinds of bacteria were but developmental forms of one and the same micro-organism. Further, very probably contamination of such cultivations led to the belief in the transformation of a harmless into a pathogenic bacterium.

In the case of solid cultivating media, on the other hand, the possible contamination of the nourishing ground by the gravitation of germs from the air is guarded against, not by elaborate apparatus or ingenious devices, but by the simple fact that test-tubes, flasks and other vessels can be inverted, and are inoculated from below.

The great secret of success in Koch's methods of cultivation consists in that we are able, from a mixture of micro-organisms, to isolate the individual species and establish a pure cultivation of each distinct form. By the same method, which is remarkable for its simplicity, if by any possibility contamination has occurred, we can separate the adventitious microbe and regain a pure cultivation.

This is accomplished in the following manner. A test-tube containing sterilized nutrient gelatin is warmed, and the liquefied jelly is then inoculated with a platinum needle from the mixture of bacteria, in such a way that the individual micro-organisms are distributed throughout the liquid medium. The liquid is then poured out upon a plate of glass, and allowed to solidify. The individual bacteria, instead of moving about freely as in a liquid medium, are fixed in one spot, where they develope individuals of their own species. In this way colonies are formed, each possessing its own characteristic biological and morphological appearances; if an adventitious germ fall upon the cultivation

during the few moments it is exposed to the air, it grows exactly upon the spot upon which it fell, and can be easily recognized as a stranger.

To maintain the colonies isolated from one another during their growth, and free from contamination, it is only necessary to thin out the micro-organisms sufficiently, and to limit the exposure of the plates to the air to as short a time as possible, both during their preparation and during their subsequent examination.

The result will depend upon the way in which the thinning or fractional cultivation has been carried out, and the colonies will be found to develope in the course of a day or two, the time varying with the rapidity of growth of the micro-organism and the temperature of the room.

If we have prepared three plates, we shall commonly find that the lower plate will contain a countless number of colonies which, if the micro-organism liquefies gelatin, speedily commingle and produce in a very short time a complete liquefaction of the whole of the nutrient medium. In the middle plate or "the first thinning," the colonies will also be very numerous; while in the uppermost plate, "the second thinning," the colonies are completely isolated from one another, with an appreciable surface of gelatin intervening. The microscopical appearances of the colonies can perhaps best be observed by placing the plate upon a slab of blackened plate glass, or upon a porcelain slab if the colonies are coloured.

The microscopical appearances are examined by placing a selected plate upon the stage of the Microscope, and it is better to have a larger stage than usual for this purpose. The smallest diaphragm is employed, and the appearances studied principally with a low power.

The morphological characteristics of the micro-organisms of which the colony is formed can then be examined in the following way. A platinum needle bent at the extremity into a miniature hook is held like a pen, and the hand steadied by resting the little finger on the stage of the Microscope. The extremity of the needle is steadily directed between the lens and the gelatin without touching the latter, until on looking through the Microscope it can be seen in the field above, or by the side of the colony under examination. The needle is then dipped into the colony, steadily raised, and withdrawn. Without removing the eye from the Microscope, this small operation may be seen to be successful, by the colony being disorganized or completely removed from the gelatin. It is, however, not easy to be successful at first, but with practice this can be accomplished with rapidity and precision. A cover-glass preparation is then made in the usual manner, by rubbing the extremity of the platinum needle in a droplet of

sterilized water, previously placed on the perfectly clean cover-glass. This, when dry, is passed three times through the flame of a Bunsen burner or a spirit-lamp, and stained with a drop of fuchsin or methyl-violet solution.

From the micro-organisms transferred to the cover-glass before it is dried and stained, from any remnants of the colony which was examined, and from other colonies bearing exactly similar appearances, inoculations should be made in test-tubes of nutrient gelatin and agar-agar. In this way pure cultivations are established, and the microscopical appearances of the growth in test-tubes can be studied.

The slower growth of the micro-organisms in solid media, and the greater facility afforded thereby for examining them at various intervals and stages of development, is an additional point in favour of these methods; and the characteristic microscopical appearances so frequently assumed are, more especially in the case of morphological resemblance or identity, of the greatest importance.

The colonies on plates of nutrient gelatin (examined with a low power) of *Bacillus anthracis*, or of *Proteus mirabilis*, the cultivations in test-tubes of nutrient gelatin of the bacillus of septicæmia in mice, and the brilliant and curious growth of *Micrococcus indicus* upon nutrient agar-agar may be quoted as examples in which the appearances in solid cultivations are absolutely pathognomonic.

As an example of the importance of these microscopical appearances in the case of morphological resemblance or identity, I need only refer to the comma-bacillus of Koch. This bacillus closely resembles in form the comma-bacillus of cholera nostras, and the comma-bacillus of the mouth, as well as a curved bacillus described as occurring in old cheese. From all these bacilli the bacillus of Koch is distinguishable by its mode of growth in nutrient gelatin when cultivated in test-tubes and on glass plates.

No one, so far as I am aware, has yet been able to demonstrate the existence of a curved bacillus, *which is exactly similar both morphologically and biologically* to the comma-bacillus of Koch. We owe, therefore, to the methods of cultivation on solid media that the presence of this bacillus serves as a reliable index to the existence of Asiatic cholera, although it may bear no causal relation whatever to the disease.

There are other facts brought to light by studying bacteria by the method of cultivation on the surface of nutrient gelatin. Not only do the colonies differ in size and colour, but sometimes the shapes assumed by the groups of bacilli are very characteristic. These appearances can be very readily demonstrated by making what is called in German a "Klatch-präparat"; by this method, we

can study the relative position of the individual micro-organisms one to another, and in some cases very beautiful preparations result. A perfectly clean cover-glass is carefully deposited on a plate, or potato-cultivation, and gently and evenly pressed down. One edge is then levered up with a needle, and the cover-glass lifted off by means of forceps. The preparation is then allowed to dry, passed three times through the flame, and stained as already described. In the case of plate-cultivations, especially where no liquefaction has taken place, the growth is bodily transferred to the cover-glass, and a vacant area mapped out on the jelly corresponding exactly with the form and size of the cover-glass which was employed.

In illustration of this method, I would call attention to a bacillus occasionally present in the air, of which I have been unable to find any written description, and for which I would suggest the name *Bacillus figurans.* (Plate III. figs. 1 and 2.)

In plate-cultivations this bacillus produces a cloudiness which gradually creeps over the surface of the gelatin. If a preparation is made in the manner I have just described, this growth is found to consist of rods which vary considerably in length. These rods lie parallel to one another, and form rows or chains which become twisted at intervals into the most curious convolutions, from which offshoots are continued in various directions. These long shoots or processes become in turn at intervals twisted into varying shapes and figures. If nutrient jelly in a test-tube be inoculated with a platinum needle charged with the bacilli, the growth appears in the form of windings on the free surface which are visible to the naked eye, from these fine filaments spread downwards into the substance of the jelly. Cultivated on a sloping surface of nutrient agar-agar the filaments spread transversely from the central streak, giving a feathery appearance.

Cheshire and Cheyne have described a peculiar mode of growth of the *Bacillus alvei* in plate-cultivations, and Hauser has photographed the peculiar grouping of certain bacteria connected with decomposition.

An interesting phenomenon which Hauser has also observed in connection with the last-mentioned bacteria, is the peculiar individual movement which they possess on solid media. This can be most conveniently studied by cultivating the bacilli in a glass capsule. The bacilli often move singly, or meet and progress in pairs, or form chain-like processions; possibly the movements are accounted for by the existence of a film of liquid as they are observed only on solid media containing less than ten per cent. of gelatin.

We may also apply the method of plate-cultivation to the examination of water, and to studying the bacteria which exist in

the soil or in food-substances, which can be sprinkled over the surface of the gelatin, and the colonies which develope studied as already described.

Lastly, if these biological appearances may be taken with other characteristics into consideration in the determination of species, we have a basis for a classification of bacteria into species, of which at present we stand in need.

These methods of artificial cultivation assist us also in determining the position in the scale of fungi of certain micro-organisms which is at present doubtful. In illustration of this, and in order to bring to your notice the specimens before you, I shall, in conclusion, say a few words with regard to the fungus *Actinomyces.*

Actinomycosis is a disease occurring not uncommonly in cattle, but very rarely in man. For the accounts of it, we are indebted chiefly to the writings of Bollinger, Israel, and Ponfick. The disease is caused by a parasite known as *Actinomyces*, or the "ray-fungus." The parasite appears in the form of a rosette, composed of club-shaped elements, and these rosettes are colourless or of a yellowish or yellowish-green tinge, and visible to the naked eye.

The fungus is believed to gain an entrance to the animal by the mouth, being taken in with the food, possibly through the medium of a wound of the gum, or a carious tooth. In whatever manner it has gained access to the living organism, it sets up inflamation, resulting in the formation of a new growth, composed chiefly of round cells, which resembles a tuberculous nodule. These nodules may break down and suppurate, or they may go on increasing in size; fibrous tissue developing between the nodules, large tumours eventually result, containing purulent cavities and excavations. In the slimy detritus, the little pale-yellow grains of fungus can be detected. In cattle, the lower jaw is usually affected, and then the upper jaw and neighbouring parts. The organism may also occur in nodular tumours of the pulmonary, subcutaneous, and intermuscular tissue; it is the cause of "wooden tongue," and has also been variously described, before its true nature was understood, as bone-canker, bone-tubercle, osteo-sarcoma.

In man the pulmonary formations tend to break down early, forming fistulæ and sinuses, with the clinical characters of empyema. In one case, there were symptoms of chronic bronchitis with fœtid expectoration. In other cases, the disease originating in the lung, spread to the prævertebral tissues. If the fungus attacks bones, it produces caries. This has been observed to occur in the bodies of the vertebræ. In another group of cases, the disease has been described as commencing in the intestinal canal. The parasite has also been detected in the crypts of the tonsils of healthy pigs, and a similar, if not identical, fungus in a diseased condition of the spermatic duct of the horse. The disease has been

transmitted from cattle to cattle by inoculation, and a rabbit infected by means of a piece of actinomycetic tumour from a human subject, introduced into the peritoneal cavity.

Until quite recently, *Actinomyces* has been classed as a hyphomycete, and the flask-shaped structures regarded as gonidia. From recent cultivation experiments, Bostrom regards the latter as a result of a degenerative stage in the life-history of the fungus. Inoculations of nutrient gelatin in the form of plate-cultivations and inoculations on blood serum and nutrient agar-agar, were made, it is claimed, with success. The cultures developed in five to six days, and best at a temperature of 33–37° C. Nutrient gelatin was not liquefied. The appearances of the cultivations were described as quite characteristic; a whitish granular appearance first occurs, followed after a few days by little yellowish-red spots which coalesce in the centre; in time the periphery also becomes dotted with little yellow-centred masses. The fungus thus cultivated has been described on examination as corresponding with the form found in man and animals, and further, at one stage to consist of thread-forms, short rods, and cocci. From these observations, Bostrom has come to the conclusion that *Actinomyces* should be classed with the bacteria, forming one of the *Cladothrix* group, and possibly closely allied to the *Streptothrix Försteri* of Cohn.

In conclusion, I would draw attention to the preparations of this fungus which are placed under the Microscopes on the table. These preparations have been stained by methods somewhat recently introduced. Very beautiful results can be obtained by either the methods of Weigert or Plaut. By the first-mentioned, sections are immersed in solution of orseille for one hour. They are then rinsed in alcohol, and placed in a solution of gentian-violet which is employed as a contrast stain. (Plate IV. figs. 1 and 2.)

In Plaut's method, the sections are placed in Gibbes' solution of magenta warmed to 45° C. They are then rinsed in water, and *after-stained* in concentrated solution of picric acid, for from five to ten minutes. After this they are immersed in water five minutes, laid in 50 per cent. alcohol fifteen minutes, passed through absolute alcohol and clove oil, and preserved in Canada balsam. (Plate V. figs. 1 and 2.)*

* Plates III.–V. have been taken from Dr. Crookshank's book on 'Practical Bacteriology' (see *infra*, Microscopy β.), the original stones having been kindly placed by him at our disposal for that purpose.—Ed. J.R.M.S.

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