A Method for Isolating and Cultivating the *Mycobacterium enteritidis chronicae pseudotuberculosae bovis*, Johne, and Some Experiments on the Preparation Of a Diagnostic Vaccine for Pseudo-tuberculous Enteritis Of Bovines[1, 2]

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The disease of cattle described under the name of pseudo-tuberculous enteritis, or Jöhne's disease, is a serious affection which causes considerable losses to farmers and stockowners throughout the British Isles and Europe. Clinically, it is characterized by a slow progressive emaciation and chronic diarrhea, which causes the milk-yield in cows to fall off, and often ends in death. B. Bang states that some cases show no diarrhea, though the affected animals die; he also states that the annual losses from this disease on some of the large farms in the Islands of Denmark may amount to 5 to 8 percent of the total head of cattle. The disease may affect bovines of all ages (usually from 3 to 6 years, Meissner ([33])), but as the period of incubation has been shown to be very long, it is not usually recognized in animals which are less than a year old. It often appears in pregnant cows, and becomes aggravated after calving.

On post-mortem examination the lesions are found to be confined to the bowels and the surrounding lymphatic glands. The mucous membrane of the small intestine is seen to be very much thickened—in some cases it may be three or four times the normal. It is thrown into characteristic folds or corrugations, and may show areas of congestion. The large intestine and cæcum often present the same lesion. The affected glands are enlarged and œdematous, but show no caseation.

Scrapings taken from the thickened mucous membrane, and stained by Ziehl-Neelsen's method, as a rule show enormous numbers of acid-fast bacilli, though in some cases they are less numerous, and in others may be very difficult to discover.

In sections of an affected intestine the bacilli are found to be most numerous near the surface, i.e., towards the lumen of the bowel, but they are also found in the villi and in the deeper layers. The increase in thickness is seen to be due to the formation of new connective tissue. "The tissue is filled with large epithelioid cells, surrounded by small round lymphatic cells, and in some cases with giant cells" (B. Bang).

The presence of an acid-fast bacillus, not

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[3] From the Laboratories of the Brown Institution, University of London.

to be distingushed microscopically from the tubercle bacillus, in the thickened mucosa of the bowels of cattle suffering from chronic diarrhea, was first shown in 1895 by Jöhne and Frothingham (1), who considered the condition to be a form of tuberculosis, and with them Koch was in agreement.

In 1881 J. Hansen and P. H. Neelsen, of Holland, pointed out the thickening and corrugation of the mucous membranes of the intestines of certain cattle dying from chronic diarrhea, while Hurtrel d'Arboval, in 1826 (Dict. de Med. et de Chirurg. Vét.), under the head of chronic enteritis in cattle, described conditions which might well have been due to the micro-organism now known as Jöhne's bacillus. Bouley and Reynal do not seem to have recognized it as a special form of enteritis (Dict. de Chirurg. Méd., et d'Hygien Vét., 1860).

The disease is prevalent in many countries. Van der Sluys (3) and Markus (5) have described its occurrence in Holland, Liénaux and van den Eeckhout (7) in Belgium, B. Bang (14) in Denmark, and Borgeaud (8) in Lausanne.

Jöhne described the first case in Dresden, and Bongert (12), Meissner (33), and others have reported many further cases in Germany. Fréger (13), Matthis (15), and Lechlainche (18) in France, and Horne (24) in Norway, have also recognized and conducted experimental work on this disease.

In North America, the first case was described by Pearson in Pennsylvania, in 1908, and since then it has been recognized by Beebe (25), of Minnesota, and others.

In 1906 B. Bang (37), of Copenhagen, demonstrated the disease, and showed microscopic preparations of the diseased gut and glands before the National Veterinary Association at Liverpool; he suggested that many cases of chronic diarrhea ascribed to various intestinal strongyles were really due to Jöhne's bacillus, and predicted that the disease would be found in Great Britain, as he had in his own experience found it in tubercle-free cattle imported from Jersey.

In 1907 McFadyean (19) described cases in this country occurring among Shorthorn, Sussex, and Jersey cattle, and, later, observed one case in a deer. In 1909 Stockman, whilst investigating a disease of sheep in Scotland, known locally as "Scrapy" or "Scrapie," found acid-fast bacilli in lesions corresponding to those of Jöhne's disease. Previous to this, in 1907, Liénaux attempted to inoculate the disease in sheep, but was apparently unsuccessful.

In 1909, in the Report of the United States Bureau of Animal Industry, mention is made by Dr. J. Mohler of an attempt to cultivate the bacillus of Jöhne's disease from specimens received from California, but it is clear from the details given that the specific bacillus was not grown.

Many attempts have been made to produce this disease experimentally in cattle and in the usual laboratory animals. As far as can be ascertained, in no animal, except cattle, has inoculation proved successful. Meissner and Trapp (34) state that they have only been able to produce the disease in calves. This they have done, as also has B. Bang, by intravenous and intraperitoneal inoculation of large quantities of infected material, and also by feeding calves with considerable quantities (1 to 3 lbs.) of the mucous membranes of the intestines of cattle dying from this disease. Experiments of a similar nature on mice, guinea-pigs, rabbits, sheep, and goats have all proved negative. In many instances the results have caused confusion, since the material used has been taken from animals also suffering from tuberculosis, and this strengthened the theory that the condition was a form of tuberculosis affecting the bowels and surrounding lymphatic glands, but showing no tendency to caseation. B. Bang, by feeding a calf with 300 gm., of affected material, found that the animal showed signs of diarrhea in eight months, and this evidence of the long period of incubation has been supported by other observers.

All writers on this disease state that the causative micro-organism cannot be cultivated outside the animal body. Meissner (33), however, obtained on a decoction of grass (*Phleum pratense*) and glycerine agar a pure culture of an acid-fast bacillus which Koch and Rabinowitsch (17) declared to

be identical with the bacillus of avian tubercle. Mettam also, in a private communication, states that he has sub-cultured for 12 generations an acid-fast micro-organism obtained from a cow suffering from Jöhne's disease, and that it agrees in every respect with the avian tubercle bacillus. These must be regarded as cases of accidental contamination with the avian tubercle bacillus.

Most authors agree that animals suffering from this disease give no reaction with ordinary diagnostic tuberculin, but O. Bang ([38]) has obtained in some cases a more or less decided reaction with avian tuberculin. In this country Male ([39]), of Reading, has since used avian tuberculin prepared by Stockman. He tested 19 cattle, giving 5 c.c. doses of tuberculin, and in four cases obtained a reaction of 3.6°, 3°, 4°, and 2.8° F. respectively. He does not state, however, whether the pre-inoculation temperature given is that of the morning or evening, and it must be remembered that both Meissner and Mettam have obtained avian tubercle bacilli from the intestine of cows infected with Jöhne's bacillus.

In June, 1910, we started some experiments with the object of cultivating Jöhne's bacillus and of preparing a diagnostic vaccine from the culture obtained. A preliminary note on the results of this work was included in a paper by one of us ([41]) on the cultivation of the lepra bacillus of man, published in 1910.

We have to thank Mr. Brennan de Vine, of Birmingham, and Mr. D. Hamilton, of Hamilton, for the pathological specimens used in these experiments.

Portions of infected intestine and glands were obtained in as fresh a condition as possible, but owing to the time taken in transit, some of the specimens were found to be contaminated in the deeper tissues, and it was found necessary to kill off these contaminations with a solution of ericolin—a method which was originally devised for isolating tubercle bacilli ([42]). The gut and glands were thoroughly washed in water, and the surface of an infected area seared with a hot spatula; microscopic films were made from the tissues beneath to prove the presence of the specific bacillus. Small pieces of tissue were then removed with sterile scissors and rubbed over the culture medium to be tested, either directly, or indirectly after being incubated in a 1 per cent watery solution of ericolin for one to two hours at 37° C.

The first case received from Mr. de Vine, on June 15, 1910, showed the typical lesions of pseudo-tuberculous enteritis, and contained a large number of acid-fast bacilli. The specimen was fresh, and cultures were made directly on to all the ordinary laboratory media. Fresh extracts of various glands and organs (including the intestine) of normal bovines were also prepared, and sterilized by passing through a Doulton white filter. The extracts thus sterilized were placed in sterile tubes plugged with cotton wool, and inoculated with the diseased material. Small sterile portions of bovine organs were also obtained, placed in sterile tubes, and inoculated. The above media were tested in various combinations, both with and without glycerin, cholesterin, various sugars, fresh blood, and other substances. The cultures were made aërobically and anaërobically at 39° to 40° C. On none of these media were we able to obtain any definite growth of the specific bacillus.

Some experiments were also conducted to test the possibility of an ultramicroscopic virus working in symbiosis with Jöhne's bacillus. Extracts of bovine intestine infected with the disease were prepared and passed through a Doulton white filter. The sterile filtrate so obtained was added to the various media, and the whole inoculated with portions of intestine containing living Jöhne's bacilli. These experiments all gave negative results.

From the experiments conducted on this case we came to the conclusion arrived at by most other workers, namely, that the specific bacillus would not grow on any artificial medium known to bacteriologists, and that if successful cultivation were to be achieved some new medium would have to be prepared. We considered also that the failure of growth of the specific bacillus must be due, either to some substance in the medium acting as a poison, or to the absence of some material or foodstuff nec-

essary for its vitality and growth.

On considering the question further, we were struck by the apparent close relationship existing between this micro-organism and the tubercle bacillus; and since the bacillus of pseudo-tuberculous enteritis and the tubercle bacillus both grow in the same species of animal, we considered it highly improbable that there could be any substance in the ordinary laboratory media which would act as a poison to the one bacillus and not to the other. This possibility was accordingly excluded, and we were forced to conclude that the failure to grow the bacillus must be due to the absence of some necessary foodstuffs.

Considering again the apparent close relationship between the tubercle bacillus and the bacillus of pseudo-tuberculous enteritis, and the fact that both these bacilli live in the bodies of bovines, we judged it probable that they would require the same chemical substances for building up their protoplasm, certain of which substances could be elaborated from artificial media by the tubercle, but not by the bacillus of pseudo-tuberculous enteritis—in other words, that the latter bacillus has lived a pathogenic existence from such remote ages, that it has lost the original power of its wild ancestor—whatever bacillus that may have been—and can no longer build up all its necessary foodstuffs outside the animal body.

It was thought probable that if these substances could be obtained ready formed and added to some good artificial medium (Dorset's egg medium), the bacillus would grow, and further, that these substances might be elaborated by allied micro-organisms, such as the tubercle bacillus, and even stored up as reserves in their envelopes. On this reasoning, which led to the successful cultivation of the lepra bacillus of man ([41]), we decided to prepare media containing these allied bacilli which had been killed by heat.

We had at the time in our possession about three hundred strains of tubercle bacilli, mostly isolated from human tuberculous material on Dorset's egg medium. A number of these cultures were taken, and after the necessary sub-cultures had been made, they were killed by steam. The growth was then scraped off, taking care to avoid any admixture of the medium which might contain the waste products of the bacillary growth and be toxic to the bacillus of pseudo-tuberculous enteritis. More recently we have found this precaution to be unnecessary. The growth of tubercle bacilli thus obtained was ground in a mortar with glycerine and saline, steamed for half an hour, and added to the yolk and white of new laid eggs in the following proportions: Egg 75 parts, 0.8 sodium chloride in re-distilled water, 25 parts; these were thoroughly mixed, and to the mixture were added tubercle bacilli 1 percent and glycerine 5 percent. This medium was placed in sterile test-tubes, these were plugged with cotton wool and heated in a hot water bath at 60° C. for one hour on three successive days, the tubes being incubated at 37° C. for 6 to 12 hours in the intervals between steaming. Finally the tubes were inspissated in slopes at 85° to 90° C.

A second case of pseudo-tuberculous enteritis was now obtained from Mr. de Vine. Specimens of intestine and glands were received on July 28, 1910. Both the intestine and glands showed the typical characters of the condition, and a large number of Jöhne's bacilli were present in various parts of the tissues. Unfortunately, owing to the hot weather prevailing at the time, the specimens on delivery had commenced to decompose, but, in spite of this, we prepared some cultures in the manner previously described, both directly, and indirectly after treating with ericolin solution. The cultures were made on several of the media tested with the first case, as well as on a number of tubes of the special tubercle bacillus medium. The tubes were capped with gutta-percha tissue and incubated at 39° to 40° C. After two days' incubation all the direct cultures were badly contaminated, yet those inoculated with ericolinized material showed only a few contaminating colonies. Sub-cultures were made from uncontaminated areas of most of the latter tubes on to fresh tubes of the same medium, but, owing to the small amount of the tubercle bacillus medium

then prepared, only one of these tubes was sub-cultured—this was one made from a gland. Films from these sub-cultures were prepared at intervals of about four or five days, and examined microscopically. After 19 days the sub-culture on the special medium showed quite definite evidence of multiplication, the bacilli had grown larger and thicker, they were well stained, and were present in large close masses. Sub-cultures were made from this tube on fresh tubes of various media, including one tube of the special tubercle bacillus medium. These were examined at intervals as before, and the sub-culture on the special medium showed microscopic evidence of growth in 10 days. Both the first and second sub-cultures showed growth visible to the naked eye after four weeks, which gradually increased, and reached a maximum in about eight weeks.

These tubes were easily sub-cultured on to fresh tubes of the same medium, but on none of the ordinary laboratory media were we able to get any evidence of growth.

The third case of pseudo-tuberculous enteritis was obtained from Mr. Hamilton. Specimens of intestine, but no glands, were received on September 23, 1910. They showed the typical lesions of the disease, and a very large number of Jöhne's bacilli were present in the tissues. When delivered, the specimens had already commenced to decompose, but from them cultures were made as previously described, both directly, and indirectly after treatment with ericolin solution, on various media, including some tubes of the tubercle bacillus medium. The tubes were capped with gutta-percha tissue and placed in an incubator at 39° to 40° C. The results were the same as in Case No. 2; all the direct cultures were badly contaminated, and those tubes which had been inoculated with material previously treated with ericolin solution grew only a few contaminating colonies. Of the latter, the cultures on the tubes of special medium were sub-cultured from uncontaminated areas on to a number of fresh tubes of various media, including the special medium. The sub-cultures on the ordinary media remained sterile, but those on the tubercle bacillus medium grew Jöhne's bacillus in pure growth, and were, without difficulty, repeatedly sub-cultured on to fresh tubes of the special medium. Naked eye evidence of growth was present in the first sub-cultures after about six weeks.

The fourth case was obtained from Mr. de Vine, a specimen of intestine being received at the Institution on January 26, 1911. It showed the typical lesions of pseudo-tuberculous enteritis, and a large number of the specific bacilli were present in the lesions. Since the specimen was quite fresh, cultures were made as previously described from the ileum, cæcum, and ileo-cæcal valve directly on to nine tubes of the special tubercle bacillus medium, these were capped and placed at 39° to 40°C. After three weeks' incubation two tubes were found to be contaminated, whilst the remainder were covered with extremely minute colonies of Jöhne's bacillus without any contaminations; the cultures grew well, and were sub-cultured without any difficulty on to the special medium. Sub-cultures taken on to Dorset's egg medium, glycerine agar, and various other media, gave no growth.

Case 5 was obtained from Mr. Hamilton, and was received at the Institution on February 8, 1911. The specimen, consisting of ileum and ileo-cæcal valve, showed the typical lesions of pseudo-tuberculous enteritis, and a considerable number of acid-fast bacilli were present in the lesions. Cultures were made from several parts of the specimen directly on to 12 tubes of Dorset's egg medium. They were taken in the manner already described, but as the specimen was fresh on arrival, previous treatment with ericolin solution was unnecessary. The tubes were capped with gutta-percha tissue, and placed in the incubator at 39° to 40°C. On the following day they were examined and found to be free from contaminating colonies, so the tiny pieces of tissue were removed from three of the tubes and placed on to three tubes of the special tubercle bacillus medium. These were capped and placed with the other tubes in the incubator at 39° to 40° C. Six weeks later the three tubes of special medium showed a few tiny colonies of

Jöhne's bacillus. Compared with the previous cases the rapidity of growth was very slow and was slight in amount, due, as was proved later, to the unsuitability of the particular strain of tubercle bacillus incorporated in the medium. Sub-cultures from these tubes on to tubes of a fresh batch of tubercle bacillus medium grew well. All the original cultures on Dorset's egg medium remained sterile, as also did sub-cultures from the special medium on to Dorset's egg medium.

Four strains of Jöhne's bacillus having been isolated on media containing dead tubercle bacilli, we next proceeded to test them on slightly modified media. We found that growth was not nearly so good in the absence of glycerine, but the exact percentage most suitable for the growth of Jöhne's bacillus has not yet been determined, although we have reason to believe that about 4 percent by volume gives the best results. A higher percent sodium chloride solution can be used in preparing the medium, without any detrimental effect.

We found also that it was better to dry the growth of the tubercle bacillus after killing it and before making it up into medium, a fact which may be due to the formation of cracks or breaks in the continuity of the covering, enabling the essential substance to diffuse more easily into the medium. Experiments showed that ½ to 1 percent of the dried tubercle bacillus was the most suitable quantity to add. To obtain the best results, the dried bacilli should be ground up with the glycerine which has been mixed with an equal quantity of 0.8 percent saline, and the remainder of the saline added later. The emulsion so obtained should then be steamed for 15 minutes, and, when cool, added to the egg. The probable explanation for this is that the glycerine acts as a solvent for the essential substance, and some experiments to be described later tend to confirm this suggestion. We also tried some media similar to the above, in which the normal alkalinity of the egg was wholly or partially neutralized by hydrochloric acid; these were found to be unsuitable. This proves the necessity of maintaining a distinctly alkaline reaction.

In another series of experiments the egg was replaced by various other substances, such as broth or agar. These, as a rule, did not give such good results, although ordinary glycerine peptone bouillon, made distinctly alkaline, and containing ½ to 1 percent of dried tubercle bacilli, gave a fairly satisfactory growth. This, with other experiments to be described later, proved that Jöhne's bacillus can grow quite well in the absence of albumen.

We next proceeded to test our strains of Jöhne's bacilli on media in which the dead tubercle bacillus was replaced by various other micro-organisms. We soon found that some strains of human tubercle bacilli were more suitable than others; and, further, that if the human tubercle bacillus was replaced by the bovine type, no growth of Jöhne's bacillus took place, and that this was so even when sub-cultured from strains which had been growing outside the animal body for a year. Several strains of tubercle bacilli isolated from cats were also tested, but gave negative results.

The question then arose as to whether these results were due to the absence of some substance in the bovine tubercle bacillus, or to the presence of some toxic substance not found in the human type. This was tested by preparing four batches of medium, one containing ½ percent of dried human tubercle bacilli, another ½ percent of dried bovine bacilli, a third ½ percent of both the human and bovine types, and a fourth ¼ percent of both types. Several tubes of each were inoculated with pure cultures of Jöhne's bacillus and incubated at 39° to 40° C., with the following results: Growth took place on the medium containing the human type and on the two containing both types, but no growth took place on that containing only the bovine type. This experiment proves fairly conclusively that the unsuitability of the bovine type of bacillus is not due to the presence of any toxic body in its substance; otherwise no growth would have taken place in the media containing the mixture of the two bacilli. We may note, however, that we have not tested many strains of the bovine bacillus, and it is possible that Jöhne's bacillus will grow on some bovine strains, or on those strains which have been

described as occupying an intermediate position between the typical human and typical bovine bacilli; but we have no evidence that this is so.

Whatever this difference between the two types of bacilli may be due to, it does not in our opinion necessarily represent an important biological difference; it is probably physiological in nature, and may be due to the presence or absence of some reserve food material existing or otherwise outside the strictly vital portion of the bacillus, or it may be due to some fat, wax, or other covering material preventing this substance from being utilized by Jöhne's bacillus. In the light of some recent experiments the latter possibility seems improbable, as we have been unable to extract any substance suitable for the growth of Jöhne's bacillus. These experiments are being continued.

While in this paper we cannot enter into the controversy concerning the relationship between the human and bovine types of tubercle bacilli, yet, incidentally, we venture to remark, that in spite of all that has been written in this country, we are not yet convinced that the human and bovine types are only slightly different varieties of one and the same micro-organism. In this connection the difference between the two bacilli described above may be worthy of note and further investigation.

The failure to obtain any growth of Jöhne's bacillus on media containing tubercle bacilli isolated from bovines and cats led us to seek for other acid-fast bacilli which might act as substitutes for the tubercle bacillus of man, and two bacilli at once suggested themselves. As we have already remarked, O. Bang has shown that avian tuberculin may cause some reaction with pseudo-tuberculous enteritis of bovines, and the possibility of the avian tubercle bacillus and Jöhne's bacillus being closely allied is at once obvious.

Accordingly we prepared several batches of medium containing the avian tubercle bacillus in place of the human type. On this medium our strains of Jöhne's bacillus usually grew, but only slightly, and the medium proved to be quite unsuitable for practical purposes.

The other micro-organism which suggested itself was the timothy-grass bacillus. From very remote times this bacillus must have been repeatedly ingested by bovines in their food, and it seems quite possible that it may be the wild ancestor and originator of Jöhne's bacillus which now infests the intestine, causing pseudo-tuberculous enteritis. Batches of medium containing ½ percent of this bacillus in place of the human tubercle bacillus were prepared, placed into tubes, and sterilized in the manner previously described. A number of these tubes were inoculated with pure cultures of Jöhne's bacillus and incubated at 39° to 40°C. as before. These cultures grew quickly and well, the growth being better than on any of the media containing the human tubercle bacillus. A slight growth was visible along the needle track after incubation for one week, and after six weeks the growth closely resembled that of a bovine tubercle bacillus recently isolated from the animal body. A full description of the cultural characters of the bacillus will be given later. The smegma bacillus of Moeller, the nasenschleim bacillus of Karlinski, and the fish tubercle bacillus of Dubard were then tested in place of the human tubercle bacillus; each type was added to the medium in quantities of ½ to 1 percent of the dried powdered growth. The first two media gave satisfactory results, but were not quite so good as media containing the timothy-grass bacillus. The fish tubercle bacillus medium gave negative results, but so far only one batch of this has been tested.

The butter bacillus of Rabinowitsch was also found to be unsuitable. Certain blastomyces and non-acid-fast bacilli which have been recently investigated also gave negative results.[4]

In the above experiments it must be noted that we were testing the various media with vigorous growing strains of

[4] Subsequent experiments have shown that the following acid-fast bacilli can also be used in the medium, and give good results:—*B. Pseudoperlsucht*, Moeller; *B. aus Harn*, Marpmann; *B. aus Butter*, Grassberger. No positive results have yet been obtained with the Tobler group of acid-fast bacilli (January 29, 1912).

Jöhne's bacillus, some strains of which had been growing outside the animal body for nearly a year. The question now arose as to whether we should have obtained the same good results with such micro-organisms as the timothy-grass bacillus, had we started by inoculating the media directly with bovine tissue infected with Jöhne's bacillus, instead of with cultures which had been growing outside the animal body for a considerable period.

To test this point Mr. de Vine kindly sent us a further specimen of diseased gut, and this, our sixth case, was received at the Institution on July 28, 1911. The specimen was delivered in a fresh condition, and showed the appearance of pseudo-tuberculous enteritis, most marked near the ileo-cæcal valve; the disease was in an early stage, and the thickening of the intestine was quite moderate. Films were made from the ileum and the ileo-cæcal valve, but only a very few Jöhne's bacilli could be found, even after searching for some considerable time. Small pieces of tissue were removed aseptically in the manner previously described and inoculated on to six tubes of Dorset's egg medium, and on to two tubes of special medium containing ½ percent of dried timothy-grass bacillus. All were capped with gutta-percha tissue and inoculated at 39° to 40° C. as with the previous cases.

After 48 hours one of the tubes of special medium was found to be contaminated and was discarded. After five weeks' incubation films were made from the tubes and examined microscopically. Those taken from the cultures on Dorset's egg medium showed no acid-fast bacilli, but that taken from the remaining tube of special medium, made with dead timothy-grass bacilli, showed some small clumps of acid-fast-fast bacilli presenting the characters of Jöhne's bacillus. Accordingly sub-cultures were made from this tube on to fresh tubes of the same medium and on to fresh tubes of Dorset's egg medium. All the tubes were capped and placed in the incubator at 39° to 40° C. Films were now made at intervals of about a week from the various tubes, and without describing all in detail it will be sufficient to note that the bacillus found on the original tube of special medium continued to grow on this and on all the sub-cultures made on to the timothy-grass bacillus medium, and on media containing the human tubercle bacillus, but that the sub-cultures on Dorset's egg medium remained sterile. The bacillus isolated resembled in every way the bacilli isolated from the four previous cases, and the cultural characters were also the same.

From the above experiments it is clear that Jöhne's bacillus will grow on media containing the dead timothy-grass bacillus, not only after it has been cultivated in the laboratory for a considerable period, but also when taken direct from the diseased gut of cattle.

Having determined the various acid-fast bacilli most suitable for the growth of Jöhne's bacillus, an attempt was made to extract the essential substance from certain of these bacilli. The timothy-grass bacillus was chosen, chiefly because it gave the best results in the above experiments, also because it is harmless to man and grows quickly on simple media, thus enabling a large quantity of growth to be obtained in a short time.

Dr. W. Bulloch kindly gave us a quantity of this bacillus, besides various dead and dried tubercle bacilli, which latter had been given to him by Prof. Bang about eight years previously. Many of these had already been extracted by Bulloch and MacLeod ([40]) when investigating the acid-fast properties of the tubercle bacillus. The different bacillary powders were made up into media, the tubercle bacillus of our original medium being replaced by one or another in quantities of ½ percent. Tubes of each were inoculated with a fresh culture of Jöhne's bacillus, and the results may be summarized as follows:

Dried timothy-grass bacilli gave very good results.

Dried human tubercle bacilli gave good results, but inferior to the timothy grass bacillus.

Dried bovine tubercle bacilli gave negative results.

Dried swine tubercle bacilli gave negative results.

Dried tubercle of uncertain source, freed from wax and fat, gave negative results.

Dried tubercle of uncertain source, freed from wax, fat, and proteid, gave negative results.

The dried timothy-grass bacillus and the dried human bacillus were found to be equally good when previously autoclaved in normal saline for 30 minutes at 120° C. The above results prove conclusively that the essential substance contained in these bacilli is comparatively stable, remaining undiminished in timothy grass and human tubercle bacilli which had been dried and killed eight years previously, and also after they had been autoclaved.

Some further experiments were now made: I gm. of dried timothy-grass bacilli was taken and extracted with 20 c.c. of 0.8 percent sodium chloride and 4 c.c. of glycerine. The mixture was autoclaved for half an hour at 120° C. and passed through filter paper. The filtrate was then added to the white and yolk of hens' eggs in the proportion of one part of filtrate to three parts of egg. Another batch of medium was prepared by taking the residue of the timothy-grass bacillus, washing it repeatedly with normal saline, filtering it and drying the residue. This residue was made up into medium, the tubercle bacillus of the original tubercle egg medium being replaced by ½ percent of the residue of the timothy-grass bacillus. Further batches of medium were prepared by extracting the dried timothy-grass bacillus with distilled water, the necessary quantities of sodium chloride and glycerine being added after extraction and filtration. The residue was treated as before.

We found that Jöhne's bacillus grew on the medium containing the glycerine saline extract, and on that containing the residue. It also grew on the residue after extraction with distilled water, but it failed to grow on the medium containing the distilled water extract. From these results we judge that the essential substance is only very slightly, if at all, extracted by distilled water, but that it is soluble in a glycerine saline solution, although from the above it is clear that some of the essential substance remained in the residue.

A further series of experiments was made, using ethyl alcohol as our solvent. Two grammes of dry timothy-grass bacillus were powdered, placed in a Soxhlet apparatus with 100 c.c. of absolute alcohol, and extracted for three hours. The residue was dried in an incubator, and the alcohol evaporated to dryness, leaving a dark yellowish sticky mass. The extract and residue were then weighed separately, and it was found that the original weight of the bacilli was reduced from 2 gm. to about 1.25 gm., the difference being represented by the extract. Media were prepared with the extract and residue thus obtained, the tubercle bacillus of our original medium being replaced by 1 percent of the extract or residue. Other batches of these media were thus prepared, some of which contained only ¼ or ½ percent of the extract or residue. Tubes from each batch were inoculated from young growths of Jöhne's bacillus, and incubated at 39° to 40° C.

Good growth was obtained on all the media containing the extracts, but, as a rule, there was none on the residues.

These experiments prove that the substance in the timothy-grass bacillus essential for the growth of Jöhne's bacillus is extracted by hot ethyl alcohol. As is well known, if this hot alcoholic extract is allowed to cool, a white flocculent precipitate forms, and can be removed by filtration. The clear colored filtrate, when evaporated to dryness, leaves a thick oily residue which becomes firmer on cooling. Part of this residue is soluble in hot and cold chloroform, leaving an insoluble liquid portion which floats on the surface of the chloroform, but is soluble in water.

Media prepared with any one of these different parts of the alcoholic extract give positive results with Jöhne's bacillus, the best being that which is insoluble in chloroform.

So far these extracts have not been purified, and it is possible that the essential substance contained in each portion is identical.

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CONCLUSIONS

From the experiments detailed in this paper it is possible to deduce certain conclusions, the most important of which are the following:

1. The acid-fast bacillus present in cases of pseudo-tuberculous enteritis of bovines, and known as Jöhne's bacillus, fails to grow outside the animal body on any of the artificial media at present used by bacteriologists.

2. The bacillus shows no definite growth on fresh bovine tissue or fresh extracts of bovine tissue removed aseptically and placed into sterile tubes.

3. There is no evidence that Jöhne's bacillus grows in symbiosis with an ultramicroscopic virus.

4. The specific bacillus will grow on media containing the dried and powdered growth of certain acid-fast bacilli which have been previously killed, and this is so even when the dead bacilli have been kept for a period of eight years, and subjected to a temperature of 115° C. in the autoclave for 1 hour.

5. The most suitable bacillus to incorporate in the medium is the timothy-grass bacillus, and to a somewhat less degree the smegma bacillus of Moeller and the nasenschleim bacillus of Karlinski. The human type of tubercle bacillus is also good, but on media containing the avian type Jöhne's bacillus grows very slightly, if at all. With the few bovine strains tested in media we were unable to get any definite evidence of growth with Jöhne's bacillus. Tubercle bacilli isolated from cats also gave negative results.

6. The essential substance or substances necessary for the growth of Jöhne's bacillus can be extracted from the various acid-fast bacilli which give positive results by means of hot ethyl alcohol.

7. We have isolated Jöhne's bacillus from five consecutive cases of pseudo-tuberculous enteritis, and have proved the morphological and biological characters of the bacilli isolated to be identical in every respect.

8. The bacilli isolated produce no lesions in mice, rats, guinea-pigs, rabbits, pigeons, or hens, if given by the mouth or inoculated into the peritoneal cavity or into a vein or subcutaneously.

9. The specific bacillus, when inoculated intravenously or given by the mouth to bovines, reproduces pseudo-tuberculous enteritis in the animal, and this cannot be distinguished from the original disease either clinically during life or post mortem. Further, the bacillus can be recovered from the lesions in the intestine of the inoculated animal, and shows characters in every way identical with the bacilli isolated from the original cases.

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