The etiology of tuberculosis

1882 • Robert Koch

Koch, Robert. 1882. Die Ätiologie der Tuberkulose. *Berliner Klinischen Wochenschrift,* No. 15, April 10, 1882, pages 221–230. First presented at a meeting of the Physiological Society of Berlin, March 24, 1882.

THE DISCOVERY OF VILLEMIN THAT tuberculosis can be transmitted to animals has been confirmed a number of times, but has also been opposed on seemingly good grounds, so that up until recently it has not been possible to state for certain whether tuberculosis is an infectious disease or not. Since then, Cohnheim and Salomonsen, and later Baumgarten, have achieved success by inoculation in the anterior chamber of the eye, and Tappeiner has been successful with inhalation. These studies have shown without a doubt that tuberculosis must be counted amongst the infectious diseases of mankind.

If the importance of a disease for mankind is measured from the number of fatalities which are due to it, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera, and the like. Statistics have shown that 1/7 of all humans die of tuberculosis. . . .

The nature of tuberculosis has been studied by many, but has led to no successful results. The staining methods which have been so useful in the demonstration of pathogenic microorganisms have been unsuccessful here. In addition, the experiments which have been devised for the isolation and culture of the tubercle virus * have also failed, so that Cohnheim has had to state in the newest edition of his lectures on general pathology, that "the direct demonstration of the tubercle virus is still an unsolved problem."

In my own studies on tuberculosis I began by using the known methods, without success. But several casual observations have induced me to forego these methods and to strike out in a new direction, which has finally led me to positive results.

The goal of the study must first be the demonstration of a foreign parasitic structure in the body which can possibly be indicted as the causal agent. This proof was possible through a certain staining procedure which has allowed the discovery of characteristic, although previously undescribed bacteria, in organs which have been altered by tuberculosis. . . .

The material for study was prepared in the usual manner for the study of pathogenic bacteria. It was either spread out on cover slips, dried, and heated, or cut into pieces after dehydration with alcohol. The cover

* [The word "virus" as used here means "infective agent."]

slips or pieces were placed in a dye solution which contained 200 cc. distilled water with 1 cc. of a concentrated alcoholic solution of methylene blue. They were shaken and then 0.2 cc. of 10% potassium hydroxide added. This mixture should not give a precipitate after standing for days. The material to be stained should remain in this solution for 20–24 hours. By heating this solution at 40°C. in a water bath, this time can be shortened to ½ to 1 hour. The cover slips are then immersed in a freshly filtered aqueous solution of vesuvin for 1–2 minutes, and then rinsed in distilled water. When the cover slips are removed from the methylene blue, the adhering film is dark-blue and strongly overstained, but the treatment with vesuvin removes the blue color and the films seem light brown in color. Under the microscope the structures of the animal tissues, such as the nucleus and its breakdown products, are brown, while the tubercle bacteria are a beautiful blue. Indeed, all other types of bacteria except the bacterium of leprosy assume a brown color. The color contrast between the brown colored tissues and the blue tubercle bacteria is so striking, that the latter, although often present in very small numbers, are quite easy to find and to recognize.

The tissue slices are handled differently. They are removed from the methylene blue solution and placed in the filtered vesuvin solution for 15–20 minutes and then rinsed in distilled water until the blue color has disappeared and a more or less strong brown tint remains. After this, they can be dehydrated with alcohol, cleared in clove oil and can be immediately examined under the microscope in this fluid or first placed in Canada balsam. In these preparations the tissue components are brown, and the tubercle bacteria are a most distinct brown.

Further, the bacteria are not stained exclusively with methylene blue, but can take up other aniline dyes with the exception of brown dyes, when they are treated at the same time with alkali. However, the staining is not so clear as with methylene blue. Further, it can be shown that the potassium hydroxide solution can be replaced with sodium or ammonium hydroxide, which shows that it is not the potassium which is especially important, but the strongly alkaline properties of the solution which are necessary. . . .

The bacteria visualized by this technique show many distinct characteristics. They are rod-shaped and belong therefore to the group of Bacilli. They are very thin and are only one-fourth to one-half as long as the diameter of a red blood cell, but can occasionally reach a length as long as the diameter of a red cell. They possess a form and size which is surprisingly like that of the leprosy bacillus. . . . In all locations where the tuberculosis process has recently developed and is progressing most rapidly, these bacilli can be found in large numbers. They ordinarily form small groups of cells which are pressed together and arranged in bundles, and frequently are lying within tissue cells. They present in places a picture similar to that in tissue which contains leprosy bacilli. Many times the bacteria occur in large numbers outside of cells as well. Especially at the edges of large, cheesy masses, the bacilli occur almost exclusively in large numbers free of the tissue cells.

As soon as the peak of the tubercle eruption has passed, the bacilli become rarer, but occur still in small groups or singly at the edge of the tubercle mass, with many lightly stained and almost invisible bacilli, which are probably in the process of

dying or are already dead. Finally they can disappear completely, but this complete disappearance occurs only rarely, and then only in such sites where the tuberculosis process has stopped completely. . . .

Because of the quite regular occurrence of the tubercle bacilli, it must seem surprising that they have never been seen before. This can be explained, however, by the fact that the bacilli are extremely small structures, and are generally in such small numbers, that they would elude the most attentive observer without the use of a special staining reaction. Even when they are present in large numbers, they are generally mixed with finely granular detritus in such a way that they are completely hidden, so that even here their discovery would be extremely difficult. . . .

On the basis of my extensive observations, I consider it as proven that in all tuberculous conditions of man and animals there exists a characteristic bacterium which I have designated as the tubercle bacillus, which has specific properties which allow it to be distinguished from all other microorganisms. From this correlation between the presence of tuberculous conditions and bacilli, it does not necessarily follow that these phenomena are causally related. However, a high degree of probability for this causal relationship might be inferred from the observation that the bacilli are generally most frequent when the tuberculous process is developing or progressing, and that they disappear when the disease becomes quiescent.

In order to prove that tuberculosis is brought about through the penetration of the bacilli, and is a definite parasitic disease brought about by the growth and reproduction of these same bacilli, the bacilli must be isolated from the body, and cultured so long in pure culture, that they are freed from any diseased production of the animal organism which may still be adhering to the bacilli. After this, the isolated bacilli must bring about the transfer of the disease to other animals, and cause the same disease picture which can be brought about through the inoculation of healthy animals with naturally developing tubercle materials.

The many preliminary experiments which helped to solve this problem will be passed over, and only the final method will be described. The principle of this method is based on the use of a solid, transparent medium, which can remain solid even at incubator temperature. The advantage of a solid medium for bacteriological research in the production of pure cultures has been discussed by me in an earlier paper.* This same procedure has led to the solution of the difficult problem of the pure culture of the tubercle bacillus and is further proof of the value of this method.

Serum from cow or sheep blood, which is obtained as pure as possible, is placed in cotton-plugged test tubes and heated every day for six days, one hour per day at 58°C. Through this procedure it has been possible in most cases to completely sterilize the serum. This serum is then heated for a number of hours at 65°C., until it has solidified completely. The serum appears after this treatment as an amber-yellow, perfectly transparent or lightly opalescent, solid gelatinous mass. When this is placed for a number of days in the incubator, no bacterial colonies develop. . . . In order to obtain a large surface for the culture, the serum is allowed to harden while the test tubes are in a slanted position. . . .

* [See page 101.]

On this solidified blood serum, the tuberculous materials are placed in the following manner.

The simplest way, and one which is almost always successful, is by the use of an animal which has just died of tuberculosis, or by the use of an animal suffering from tuberculosis which is killed for this purpose. First the skin of the breast and abdomen is laid to the side with a flamed instrument. Then the ribs are cut in the middle with a flamed scissors and forceps, and a portion of the ribs are removed without at the same time opening the abdominal cavity. The lungs are then to a great extent uncovered. The instruments used here are now discarded and freshly sterilized ones taken up. Single tubercles or particles about the size of a millet seed are quickly cut out of the lung tissue and immediately carried over to the surface of solidified serum in a test tube, with the use of a flamed platinum wire. Naturally the cotton plug should only be exposed to the air for the shortest possible time. In this way, a number of test tubes, perhaps 5–10, are inoculated with tuberculous material. Such a large number are prepared because even with the most careful manipulations, not all test tubes can remain free of accidental contamination. . . .

These test tubes are now placed in an incubator and are kept there for a long time at 37–38°C. In the first week, no noticeable changes take place. Indeed, if bacteria develop in the first days, either around the inoculum or away from it, these usually white, gray, or yellowish droplets, which often bring about the liquefaction of the serum, are due to contamination, and the experiment is a failure.

The growth of the tubercle bacilli can first be seen by the naked eye in the second week after seeding, ordinarily after the 10th day. They appear as very small dots, dry and scale-like. This growth arises from the material inoculated, and if the tubercle has been spread around extensively on the surface, then a large amount of growth ensues, while if the tubercles have remained in small patches, then the bacterial growth is less extensive. If there are only very few bacilli in the inoculum, then it is hardly possible to free the bacilli from the tissue and have them growing directly on the nutrient medium. . . . With the help of low magnification, 30–40 power, the colonies of the bacilli can already be seen at the end of the first week. . . .

The growth of the culture ceases after several weeks, and a further increase probably does not occur because the bacilli have lost their own power of movement,* and only spread because of the slow reproduction of the bacilli, being pushed forward on the surface, and because of the slow growth of the bacilli, this spread can only occur to a small extent. In order to keep such a culture going, it must be brought onto a new medium 10–14 days after the first inoculation. This is done by removing several of the small scales with a flamed platinum wire, and transferring them to a fresh, sterilized serum slant, where the scales are broken up and spread out as much as possible. Further scaly, dry masses then develop which coalesce and cover more or less of the surface of the serum, depending upon the extent of the seeding. In this way the culture can be continued.

The tubercle bacilli can also be cultured on other nutrient substrates, if the latter possess similar properties to the solidified serum. They are able to grow on a solidified gel which remains solid at incubator temperature,

* [The tubercle bacillus is not motile.]

prepared by adding agar-agar * to a meat infusion or peptone medium. However, on this medium the bacilli form only irregular small crumbs, which are not nearly so characteristic as the growths on blood serum.

Originally I cultivated the tubercle bacilli only from lung tubercles of guinea pigs which had been infected with tubercular material. Therefore the cultures from various sources had first to pass through the intervening stage of the guinea pig before they were obtained in pure cultures. In this way there was a possibility for error, in the same way as in the transfer of a culture from one test tube to another. This might occur through the accidental inoculation of other bacteria into the animal, or through the appearance in the guinea pig of spontaneous tuberculosis. In order to avoid such errors, special precautions are necessary, which can be deduced from observations on the behavior of this spontaneous tuberculosis.

From hundreds of guinea pigs that have been purchased and have occasionally been dissected and examined, I have never found a single case of tuberculosis. Spontaneous tuberculosis develops only occasionally and never before a time of three or four months after the other animals in the room have been infected with tuberculosis. In animals which have become sick from sponstaneous tuberculosis, the bronchial glands become quite swollen and full of pus, and in most cases the lungs show a large, cheesy mass with extensive decomposition in the center, so that it occasionally resembles the similar processes in the human lung. . . . Animals that have been inoculated with tuberculosis show a completely different picture. The place of inoculation of the animals is in the abdomen. close to the inguinal gland. This first becomes swollen and gives an early and unmistakable indication that the inoculation has been a success. Since a larger amount of infectious material is present at the beginning, the infection progresses much faster than the spontaneous infection, and in tissue sections of these animals, the spleen and liver show more extensive changes from the tuberculosis than the lungs. Therefore it is not at all difficult to differentiate the artificially induced tuberculosis from the spontaneous tuberculosis in experimental animals. From a consideration of these facts, it can be concluded that the development of tuberculosis in an experimental animal is due to the action of inoculated material, when a number of guinea pigs are purchased and inoculated at the same time in the same way with the same material, and kept separated from other animals in their own cage, and when they show the development of the characteristic tuberculosis symptoms of inoculated animals in a short period of time.

In this way, a substance can be tested for its virulence by inoculating four to six guinea pigs with it, after making use of all precautions, such as previously disinfecting the site of inoculation, using sterile instruments, etc. The results are uniformly the same. In all animals which are inoculated with fresh masses containing tubercle bacilli, the small inoculation site has almost always coalesced on the next day, then remains unaltered for about eight days, then forms a little nodule which may enlarge without breaking open, although it most often changes into a flat, dry abscess. After about two weeks, the inguinal glands and axillary glands on the side where the inoculation has occurred enlarge

* [Koch did not at this time seem to be aware of the superiority of agar as a solidifying agent.]

until they are the size of peas. From then on the animals become progressively weaker and die after four to six weeks, or are killed in order to exclude the later development of spontaneous tuberculosis. In the organs of all of these animals, and most especially in the spleen and liver, the recognizable changes due to tuberculosis occur. That these changes in the guinea pigs are only due to the inoculation of material containing the tubercle bacilli, can be see from experiments in which inoculation was performed with scrofulous glands or fungus masses from joints, in which no tubercle bacilli could be found. In these cases, not a single animal became sick, while in the animals inoculated with bacilli-containing material, the inoculated animals always showed an extensive infection with tuberculosis after four weeks.

Cultures of tubercle bacilli were prepared from guinea pigs which had been inoculated with tubercles from the lungs of apes, with material from the brain and lungs of humans that had died of miliary tuberculosis,* with cheesy masses from phthisistic lungs, and with nodules from lungs and from the peritoneum of cows affected with bovine tuberculosis. In all these cases, the disease processes occurred in exactly the same way, and the cultures of bacilli obtained from these could not be differentiated in the slightest way. In all, 15 pure cultures were made of tubercle bacilli, four from guinea pigs infected with ape tuberculosis, four with bovine tuberculosis, and seven with human tuberculosis.

In order to answer the objection that the nature of the bacilli was changed through the preliminary inoculation into guinea pigs, so that they became more similar, experiments were set up to cultivate tubercle bacilli directly from spontaneous cases in man and animals.

This was successful a number of times, and pure cultures have been obtained from the lungs of two people with miliary tuberculosis, as well as one with cheesy pneumonia, twice from the contents of small cavities in phthisistic lungs, once from cheese-like mesenteric glands, twice from freshly removed scrofulous glands, twice from lungs of cows with bovine tuberculosis, and three times from the lungs of guinea pigs that had suffered spontaneous tuberculosis. All of these cultures were quite similar and also resembled those that had been isolated through the preliminary guinea pig inoculation, so that the identity of the bacilli occurring in the various tuberculous processes cannot be doubted. . . .

Up until now my studies have shown that a characteristic bacillus is always associated with tuberculosis, and that these bacilli can be obtained from tuberculous organs and isolated in pure culture. It now remained to prove the most important question, namely, that the isolated bacilli were able to bring about the typical tuberculosis disease process when inoculated again into animals. . . .

The results of a number of inoculation experiments with bacillus cultures inoculated into a large number of animals, and inoculated in different ways, all have led to the same results. Simple injections subcutaneously, or into the peritoneal cavity, or into the anterior chamber of the eye, or directly into the blood stream, have all produced tuberculosis with only one exception. Further, the infection was not limited to only isolated nodules,

* [An acute, systemic form of the disease.]

but depending upon the size of the inoculum, large numbers of tubercles were produced. . . .

A confusion with spontaneous tuberculosis, or an accidental infection with tubercle virus in the experimental animals, is excluded for the following reasons: (1) Spontaneous tuberculosis or accidental infection cannot develop in so short a time into the extensive eruption of tubercles experienced here. (2) The control animals, which were handled in exactly the same way as the inoculated animals, remained healthy. (3) The typical picture of miliary tuberculosis does not occur when guinea pigs or rabbits are injected with other substances. . . .

All of these facts taken together lead to the conclusion that the bacilli which are present in the tuberculous substances not only accompany the tuberculosis process, but are the cause of it. In the bacillus we have, therefore, the actual tubercle virus.

Comment

The scientific world quickly recognized the importance of this work of Koch, and it was widely acclaimed. We must consider it his masterpiece and the culmination of all the work he had done before. We can see the evolution of his work clearly through the last four papers. This evolution is all the more remarkable when we remember that in 1876, only six years previously, Koch published his first work on anthrax. In those six years he developed a series of new techniques, and it was these techniques which enabled him to discover the tubercle bacillus.

Several properties of the tubercle bacillus make it an organism that is extremely difficult to work with, and it is remarkable that Koch achieved such quick success in his experiments. The organism is extremely tiny, being at any rate a tenth the size of the anthrax bacillus. It is very difficult to stain successfully, due to a waxy layer on its cell surface. Further, it is a very slow-growing organism and requires several weeks for good growth on solid media. Thus Koch had to be extremely persistent in his work. If he had thrown out his cultures after one week, he would have been unsuccessful. It was necessary to have patience and a faith that tuberculosis was an infectious disease.

Koch was also fortunate that the strain of tubercle bacillus that is pathogenic for humans can be transferred so readily to guinea pigs. Without an experimental animal which showed characteristic symptoms upon inoculation with tuberculous material, his work would have been much harder. He might have cultured the organism successfully, but the actual proof that this organism was the causal agent for tuberculosis would have been much more difficult. It should be noted that in this paper he does not have a final proof that the organism he has isolated in pure culture is really the cause of human tuberculosis. This could only be done by making inoculations in humans. Since this cannot be done, we can only infer that the isolated organism causes the human disease. Such a dilemma is always with the investigator of human diseases. He must learn to live with it

The differential staining of Schizomycetes in tissue sections and in dried preparations

1884 • Christian Gram

Gram, C. 1884. Ueber die isolirte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. *Fortschritte der Medicin*, Vol. 2, pages 185–189.

(I WISH TO THANK HERR DR. RIESS, Director of the General Hospital of the city of Berlin for the facilities and equipment to perform the following studies.)

The differential staining method of

Koch and Ehrlich for tubercle bacilli gives very excellent results either with or without counter-staining, since the bacilli stand out very clearly due to the contrast effect.

It would be very desirable if a similar method for the differential staining of other Schizomycetes were available which could be used routinely by the microscopist.

My studies—as associate of Herr Dr. Friedländer in the morgue of the city hospital in Berlin—have attempted to demonstrate cocci in tissue sections of lungs of those who have died of pneumonia as well as in experimental animals. As Friedländer has already mentioned briefly in his paper on the micrococci of pneumonia, I have discovered by experimentation a procedure for the differential staining of pneumococci. In my procedure the nucleus and other tissue elements remain unstained, while the cocci are strongly stained. This makes them much easier to locate than previously, since in ordinary preparations from pneumonia patients, where such a large amount of exudate occurs, they are impossible to see.

Further studies on the usefulness of this method for other Schizomycetes has gradually shown that this method is an almost general method for all tissue sections and dried preparations. . . .

For staining the ordinary aniline-gentian violet solution of Ehrlich is used. The appropriate sections must be carried up to absolute alcohol and taken from this directly into the dye solution. They should remain in the dye for 1–3 minutes (except tubercle bacilli, which should remain as usual 12–24 hours). Then they are placed in a solution of iodine-potassium iodide in water (iodine 1.0 part, potassium iodide 2.0 parts, water 300.0 parts) with or without a light rinse with alcohol and allowed to remain there for 1–3 minutes. During this time, a precipitate forms, and the previously dark blue-violet sections now become dark purple-red. (Footnote: This purple-red color is not soluble in water but dissolves very easily in alcohol. The chemical studies will be continued at a later time.) They are then placed in absolute alcohol until they are completely decolorized. It is well to change the alcohol once or twice during this step. Then the sections are cleared as usual in clove oil, in which the rest of the dye is dissolved. The nucleus and fundamental tissue is stained only a light yellow (from the iodine) while the Schizomycetes, if any are present in the preparation, are an intense blue (often almost black). The intensity of the staining has not been equaled by any of the current staining methods. This presents another great advantage of our method. It is possible after the decolorization in alcohol to place the sections for a moment in a weak solution of bismarck brown or vesuvine, and then dehydrate again with alcohol, in order to achieve a counterstain. The nucleus will appear brown, while the Schizomycetes will remain blue.

In this way it is possible to prepare doubly-stained preparations that are just as excellent as those of the tubercle bacillus made after the method of Koch and Ehrlich. Permanent preparations in Canada balsam-xylene or gelatine-glycerol remain unchanged after 4 months.

This method is very quick and easy. The whole procedure takes only a quarter-hour, and the preparations can remain many days in clove oil without the Schizomycete cells becoming decolorized.

It is also useful for dried preparations. It is performed exactly as for

tissue sections, except that one treats the cover slip just like a section.

I have tried many times different aniline dyes (rubine-aniline, fuchsin-aniline and simple gentian violet solution), but without success. In addition, tincture of iodine or potassium iodide solution, as opposed to iodine-potassium iodide solution, are also ineffective, since the Schizomycetes then are also decolorized. When the sections are treated with water or dilute alcohol, the results are variable. . . .

I. After iodine treatment, the following forms of Schizomycetes are not decolorized by alcohol.

(a) The coccus of bronchial pneumonia (19 cases). . . .

(b) Pyogenic Schizomycetes (9 cases). . . .

(c) Cocci of a liver abscess . . . (1 case). . . .

(d) Cocci and small bacilli in circumscriptive infiltration of the lungs . . . (1 case). . . .

(e) Cocci of osteomyelitis (2 cases). . . .

(f) Cocci of suppurative arthritis following scarlet fever (1 case). . . .

(g) Cocci of suppurative nephritis following cystitis (3 cases). . . .

(h) Cocci of multiple brain abscesses following empyema (2 cases). . . .

(i) Cocci of erysipelas (1 case). . . .

(k) Tubercle bacilli (5 cases). . . .

(l) Anthrax bacilli (3 cases) (in mice). . . .

(m) Putrefactive Schizomycetes (bacilli and cocci). . . .

II. The following forms of Schizomycetes are decolorized in alcohol after iodine treatment.

(a) 1 case of bronchial pneumonia with cocci that formed capsules. . . .

(b) 1 case of bronchial pneumonia with cocci that did not form capsules. . . .

(c) Typhoid bacilli (5 cases) are decolorized either with or without iodine treatment very easily by alcohol. I have attempted to leave the sections in the dye for as long as 24 hours but without any better results.

I would like to make one closing remark. The behavior of the cell nucleus and the Schizomycetes to aniline dyes in other methods are almost identical, whereas with the present staining method a very distinct difference is visible.

Studies on Schizomycetes have been significantly improved by the use of this method. It is because of this that I publish my results, although I am well aware that they are brief and with many gaps. It is to be hoped that this method will also be useful in the hands of other workers.

Editor's note. I would like to testify that I have found the Gram method to be one of the best and for many cases the best method which I have ever used for staining Schizomycetes.

Comment

Presented here is the first report of the bacteriological staining method most widely used today. As first devised by Gram, the method was useful in staining bacteria in tissue sections. In his time this was an important discovery, because studies of the pathogenesis of different species of bacteria was just in its infancy. The first of Koch's postulates (see page 116) was that the suspected causal organism should always be found in association with the disease. However, this

presupposed a method for staining the minute bacteria in lesions so that they could be adequately visualized. Because of the fact that many bacteria exhibit the peculiar staining reaction which Gram describes here, it was possible to detect them much easier with his method.

For many years the main use of the Gram stain has been to differentiate species of bacteria. In the present paper, Gram describes several organisms that were not stained by his technique. We would call these Gram-negative, and the number of Gram-negative bacteria is probably larger than the number of Gram-positive bacteria. The Gram stain is one of the first procedures learned by beginning bacteriology students and is one of the first procedures carried out in any laboratory where bacteria are being identified. Its importance to bacterial taxonomy is therefore obvious.

The mechanism of the Gram stain is still a partial mystery. As Gram himself noted, the iodine-potassium iodide solution is essential in the reaction. We know that this solution must follow, and not precede, the gentian violet. We know that the iodine and the gentian violet form a complex inside the cell (Gram also noted this complex formation) which is insoluble in water but is soluble in alcohol. Apparently Gram-positive bacteria are those which are able in some way to keep the alcohol from reaching this insoluble complex. We know that the Gram stain is not an all-or-nothing phenomenon, but that quantitative variations in Gram-positivity exist between different species, and within the same species during different parts of the growth cycle or under different environmental conditions. We know that only intact cells are Gram-positive, so that cells which are even gently broken become Gram-negative. We know that bacterial protoplasts, devoid of cell wall, are still Gram-positive, indicating that it is probably the semipermeable membrane which is somehow involved in the reaction. Finally, we know that Gram-positivity is restricted almost exclusively to the bacteria, with only a few other groups, such as the yeasts, exhibiting this reaction. We can truly say that the implications of Gram's discovery have been widespread.

The root-nodule bacteria

1888 • Martinus W. Beijerinck

Beijerinck, M. W. 1888. Die Bacterien der Papilionaceenknöllchen. *Botanische Zeitung*, Vol. 46, pages 725–804.

SINCE ROOT NODULES HAVE BEEN discussed widely in recent years, I assume that their structure and morphological properties are quite well known. However, since I am going to present a new viewpoint on these bodies, a short discussion of their relationships would seem useful.

Following Brunchorst, I shall call the characteristic bodies within the root nodules "bacteroids." However, to avoid any ambiguity, let me say that I consider these bacteroids to develop from a species of bacteria which enters the roots from the outside, and not an autonomous formation of the protoplasm of the plant, as Brunchorst believed. I shall call this bacterial species *Bacillus radicicola.* The bacteroids are derived from bacteria by a metamorphic process, have lost their ability to reproduce, and function in the plant as proteinaceous bodies. They are derived from normal *Bacillus radicicola* by a stepwise loss in their power of reproduction. . . .

Bacteria that are still capable of growth on gelatin plates can be isolated in large numbers from the very young root nodules, as well as from the actively growing regions of older root nodules. The bacteria in the nodules eventually lose their ability to multiply on gelatin plates.

At the end of the period of growth, the root nodules can behave in one of two ways. They can lose completely their protein content through dissolution, in which case the whole nodule including the bacteroids that have been formed from the bacteria becomes emptied, and the protein material is used by the plant for growth. Alternatively, the nodules can be weakened through extensive growth of bacteria, in which case large numbers of bacteria remain alive within the cells and make use of the nodule as a habitat for growth and nutrition. As soon as the dissolution begins, it is ordinarily impossible to culture bacteria from these nodules, . . . while in the second case it is always very easy to culture bacteria. The two processes are not always sharply differentiated. . . .

In sterilized soil, the nodules do not develop. For this experiment, I used a closed sheet-iron container, in which the soil could be heated extensively in a double-boiler on a stove. Small leguminous plants, such as *Vicia hirsuta* and *Lathyrus aphaca*, could germinate and form roots quite easily in this container, and when the atmosphere was kept moist with boiled canal water, these plants could mature and form flowers and seeds without the slightest trace of nodules. This result has been previously reported

also by Frank in 1879. . . . Even a short heating is sufficient to prevent nodule formation, showing that spore-forming bacteria in the soil are not responsible for the process.

From these results one can conclude that the nodules develop because of an infection by an organism that is killed by boiling water temperature. *Bacillus radicicola*, which is always present in soil, and which I have always been able to culture from nodules, does not form spores and is killed by 100°C., and is therefore the infective agent. . . .

Before discussing the bacteriological properties of the nodules in detail, I would like to describe briefly the culture conditions which have been used in this study.

Since *Bacillus radicicola* is an aerobe, I could use the plate technique of Koch in these studies. For my culture plate, I used the bottom of a glass dish which had a ground glass lid.* The gelatin was poured directly in the container. The gelatin was either inoculated before pouring by mixing into the gelatin pulverized nodules that had been carefully surface-sterilized before grinding, or the gelatin was inoculated after solidifying by spreading on the surface water that contained the test material, or finally, it was inoculated by streaking test material on the surface of the gelatin. Since *Bacillus radicicola* grows very poorly when imbedded in the gelatin, culture on the surface is preferable. . . . If one is not acquainted with *Bacillus radicicola*, it is necessary to begin a culture by washing a nodule in alcohol, burning off the alcohol, disrupting the nodule in water, and pouring the aqueous suspension on plates. Amongst the large number of *Bacillus radicicola* colonies, the occasional contaminants are readily identified and can be avoided when a pure culture is sought.

Meat extract-peptone-gelatin is too rich a medium for the initial culturing of nodule bacteria, since if growth occurs at all it is too slow. . . . More rapid growth of active forms takes place only on a poorer nutrient medium, such as an extract of the leaves of leguminous plants containing 7% gelatin. To this extract should be added 0.25% asparagine and 0.5% sucrose. . . .

The nutrient medium should not be too acid, since even 2 or 3 ml. of 1*N* acid per 100 ml.† prevents growth completely. Even Cohn's nutrient solution is too acid for *B. radicicola*. Alkaline and neutral reactions are also harmful, and I have found that for *Bac. rad.* from *Trifolium repens*, that the optimum growth was obtained when 0.6 ml. of 1*N* malic acid per 100 ml. was used. Growth is best at room temperature, while no growth occurs above 47°C., but growth is still fairly good between 0° and 10°C.

To prevent moisture from accumulating on the surface of the glass lid and making observations difficult, I have found it useful to incubate the dishes upside down. In this way, cultures can be incubated for weeks with no accumulation of water droplets and no drying out. . . .

From culturing nodule bacteria from a wide range of different leguminous plants, I have convinced myself that only one species of bacteria is found, which I have characterized by the name *Bacillus radicicola*. . . .

However, I must state that the isolates from different species of plants are always quite similar, although not necessarily always identical. This is

* [From Beijerinck's illustration, his culture dish seems to be quite similar to a Petri dish.]

† [The concept of *p*H to express hydrogen ion concentration was not devised until many years later.]

especially noticeable in the primary culture on gelatin, deriving directly from the nodule. It is probable that this variability is due to the influence of the nodule material present, which has exerted an influence on the bacteria. However, the inheritability of these observed differences makes it necessary to indicate these different cultures by different variety names. . . .

It is hardly necessary for me to add that the surface of the nodules harbors large numbers of soil bacteria of numerous types, which will appear in the cultures unless the nodules are carefully surface-sterilized. If the nodules are cleaned first with water, then with alcohol, and the alcohol quickly burned off in a flame, one almost always obtains pure cultures of nodule bacteria when this material is streaked onto plates. . . .

On a nutrient medium made from stems of *Vicia faba, Bacillus radicicola* forms colonies that are not spreading, white, hyaline, or turbid, hemispherical, and variable in size. Isolated colonies are usually very small, about 0.25 mm. in diameter but often smaller, and first can be seen only with a magnifying glass. The largest colonies are watery and faintly turbid, while the smaller ones are more firm and opalescent, while the smallest are usually spheres, which can be lifted in one unit from the gelatin. . . .

The large watery colonies consist in all cases of a mixture of rods and swarmers. In such colonies almost all the cells are motile, and the very smallest rods and double rods seek oxygen at the edge of the preparation. Except for occasional very long forms, the thick rods usually measure 4 microns long by 1 micron thick. . . .

The swarmers which occur in all preparations are exceedingly small, belonging to the smallest living beings which have been described. Exact measurements for these swarmers . . . indicate dimensions of 0.9 microns long and 0.18 microns thick. . . .

Because of the size of these swarmers and the known dimensions of the holes in the pericambial cells of the plant root, it seems possible that these bacteria could pass through the pericambial cells into the root without first inducing or requiring a lesion for their entry. . . .

The rapid movement of these forms is dependent on oxygen. In an Engelmann gas chamber with carbon dioxide or hydrogen, movement ceases, and begins again when oxygen is readmitted. Movement is not prevented in distilled water.

Although these swarmers are very small, they seem to move in the same way as rods, by the use of flagella. Although I could not see these flagella directly, I saw often the type of movement well known to microscopists, which consists of a backing up or rotating on invisible threads, and I also saw the characteristic snake-like movement of inert particles, as they were moved back and forth by the flagella of the swarmers. . . .

I conclude these general observations on *Bacillus radicicola* with the observation that this organism does not have any fermentative powers, and no special oxidizing or reducing ability, either of potassium nitrate, indigo blue, or induline. However, it attacks hydrogen peroxide rapidly with the liberation of oxygen. Spore formation has never been observed, and freezing and drying are not lethal. In liquid nutrient media, the cultures always die between 60 and 70°C., and often much lower.

It seems to me premature to consider the question of whether the bacteria have any influence on the protoplasm of the plant, and whether the formation of nodules is due to the

excretion of some special product by the bacteria, or is due only to the nutritional conditions of the bacteria. . . .

Although the nodules cannot be considered as plant organs in the usual sense, such as roots, stems and leaves, these nodules seem to show such an extensive analogy to organs that the existence of some sort of physiological function in the plant seems quite likely. This possibility seems further likely from the observation that the protein content of the bacteroids of these nodules is apparently utilized by the plant under normal conditions. . . .

The accumulation of protein in the nodules and its later utilization seems to vary amongst the various species of legumes. It seems to be the most important in the annual herbaceous legumes, where the development of the nodules and the later utilization of their protein seems to be a regular event. . . . However in the woody forms the nodules usually appear late and at irregular times, and often are missing completely. . . .

Although it may seem improbable a priori that an organism with such weakly developed chemical processes as *Bacillus radicicola* would be able to oxidize ammonium salts to nitrate, or convert free atmospheric nitrogen into protoplasmic protein, it seems to me that such a conclusion should be verified by experimentation. . . . However, the results up until now have been completely negative.

The formation of nitrate was determined in nitrate-free liquid medium, as well as in agar medium, in which ammonium sulfate or asparagine were present as nitrogen sources. *Bacillus radicicola* . . . develops quite luxuriously in these media at 25°C., but at the end of the incubation period neither nitric acid nor nitrates could be detected. . . . I must conclude therefore that this species is different from the nitrifying bacteria of other authors. Further, neither nitrous oxide nor free nitrogen gas were formed in significant quantities by action on ammonium salts or asparagine, since no gas bubbles developed within nutrient gelatin.

Further, it was not possible to demonstrate the fixation of free nitrogen gas. When the nitrogen content was determined by Kjeldahl's method on an agar medium that contained no added nitrogen compounds except for the nitrogen of the agar itself, after 14 days no detectable increase in nitrogen could be observed. The growth of the organism in this agar culture was fairly extensive, but eventually stopped, apparently when the nitrogen compounds of the agar were used up. When a nutrient medium was used containing some asparagine, in which very good growth occurred, there was no indication of any increase in bound nitrogen. . . .

The possibility remains that there is only a very slow fixation of nitrogen, which could be detected only after several months, . . . or else it may be, as Frank has postulated, that the fixation of nitrogen is connected in some way with growth of the bacteria in the green plant. . . .

Because of the simple nutritional requirements of the organism, the symbiotic relationship with the leguminous plants seems all the more surprising, since except for sugar, its nutrient requirements are just as simple as those of the plant itself, and we know of many nonsymbiotic organisms, such as *Bacillus* [*Pseudomonas*] *fluorescens*, which have just as simple requirements. . . .

If we examine all of the data, mostly negative, which is available to us regarding the chemical requirements of these bacteria, this seems to indicate

well the extremely complicated situation we have in the symbiotic relationship which exists in the nodule. When the living plant cell must live with another organism which is actually a part of its protoplasm, it is then necessary that a subtle balance must exist between the growth of the plant and the growth of the bacterium. It seems to me hardly likely that a bacterium which has strong chemical abilities, such as the ability to fix nitrogen, or to convert ammonium salts into nitrate, would be suitable in the development of such a delicate equilibrium. Therefore only an organism like *Bacillus radicicola*, which is similar in its chemical properties to that of the protoplasm of the plant cell, would be suitable for such a symbiotic relationship. . . .

Comment

Beijerinck became interested in root nodules of plants through his earlier work on leaf galls. These leaf galls are caused by larvae of certain insects which have hatched on the leaf, and apparently secret substances which stimulate the growth of the leaf and make tumor-like galls in which the larvae live. Because of his knowledge of the cause of these galls, it was probably easy for Beijerinck to postulate a similar cause for the development of root nodules in leguminous plants. He then proceeded through careful bacteriological studies to isolate the bacterium in these nodules. This group of bacteria is classified today in the genus *Rhizobium*.

Beijerinck found it difficult to determine the function of these organisms in the root nodules. The fixation of free nitrogen seemed an obvious possibility, and he made a number of atttempts to demonstrate that his pure cultures would carry out this process. However, the root-nodule bacteria are not able to fix nitrogen when they are free-living, but only when in symbiotic relationship with the plant. The reasons for this are still not understood today and would make a challenging research area. The free-living nitrogen fixing bacteria, such as *Azotobacter* sp., were later discovered by Beijerinck and Winogradsky (see page 237), and their ability to fix nitrogen is much easier to demonstrate, since the process can be carried out in pure culture.

Beijerinck's work on the root-nodule bacteria was a pioneer effort in a difficult field of endeavour, and his work opened the way for later studies that showed clearly that nitrogen fixation does take place when the bacteria are living symbiotically within the plant. The importance of this process in agriculture is very great, and most of the fixation of nitrogen that occurs under natural conditions is carried out by the symbiotic organisms, rather than by the free-living bacteria.

On the nitrifying organisms

1890 • S. Winogradsky

Winogradsky, S. 1890. Sur les organismes de la nitrification. *Comptes rendus de l'Académie des Sciences*, Vol. 110, pages 1013–1016.

BEFORE SUMMARIZING THE WORK ON nitrification which has occupied me for the past year, I would like to recall several of my previous works which were the point of departure for the present report.

Besides the organisms which are the subject of the present note, two groups of organisms have been studied which have the ability to oxidize inorganic substances. I have designated them by the names sulfur bacteria and iron bacteria.

The first group live in natural waters which contain hydrogen sulfide and do not grow in media lacking this substance. This gas is absorbed extensively and oxidized by their cells and is converted into sulfur granules. These latter are in turn degraded and sulfuric acid is excreted. The second group are able to oxidize iron salts, and their life is also closely connected with the presence of these compounds in their nutrient medium.

My efforts to elucidate the physiological significance of these phenomena have led me to the concept that these inorganic compounds are the fermentable materials (in the largest sense of the word) in the life of these beings, instead of the organic materials which are the fermentable substances for the large majority of microbes. This concept leads to the logical conclusion, confirmed by experience, that these beings comprise a group with certain physiological properties which can be summarized as follows. All of the energy necessary for their vital activity would be furnished by the oxidation of mineral substances, and their dependence on organic compounds for growth would be quite slight. In addition, inorganic compounds of carbon which are not utilizable by other organisms that lack chlorophyll would be used by them as a source of carbon.

The remarkable work of MM. Schloesing and Müntz has thrown light on the role of lower organisms in the process of nitrification. However, although their work makes it highly likely that a special agent exists for nitrification, they have not succeeded in demonstrating the process away from the soil, which is a natural medium with a wide variety of microorganisms. The principal requirement

for all microbiological experiments today is the isolation in pure culture of the agent responsible for the process. Because of the difficulties involved, a number of workers have failed to isolate the nitrification ferment, so that the conclusion of MM. Schloesing and Müntz concerning the existence of this ferment has not been confirmed by bacteriologists and botanists.

This question must be clarified first. I have found that the failures of my predecessors are due to the fact that they used media which had been solidified with gelatin, such as are used so often today for the isolation and culture of microbes. The nitrifying organisms will not grow on such media, so that if a mixture of microbes taken from a soil that is in the process of nitrifying are placed in such a medium, all of the organisms that are active die, and one only isolates those which are ineffective. It is possible, with some difficulty, to eliminate one by one all of the foreign species and to obtain pure and in large numbers the nitrifying species, by using a medium that is favorable to it but unfavorable to the other organisms. These cultures are able under the usual microbiological experimental conditions to carry out the nitrification process just as intensely as M. Schloesing has recently shown it to occur in the soil.

This organism has been more difficult to experiment with than any of the other very delicate organisms which I had previously worked with. However, its physiological properties not only confirm my conclusions, but have revealed a new fact which I would like to report to the Academy.

I applied to this study the ideas which I had already acquired concerning the nutrition of organisms which oxidize mineral substances. I cultivated the nitrifying microbe from the beginning in a liquid which did not contain organic matter, but only a natural water that was very pure. Since the addition of organic compounds did not seem to promote its growth, I have used for its culture a mineral solution that is completely devoid of organic carbon. Although this medium does not have any other carbon compounds in it but carbonic acid and carbonates, the action of the nitrifying organism has not diminished in its intensity over several months.

We must conclude that this organism is able to assimilate carbon from carbonic acid, and this conclusion is confirmed by the amounts of organic carbon in the cultures. This demonstrates that there has been an accumulation of organic carbon by the action of this organism.

The nitrifying organism, which is colorless, is able to synthesize completely its cell substance from carbonic acid and ammonia. It carries out these syntheses independently of the light, and without other sources of energy than the oxidation of ammonia. This new fact is contradictory to that fundamental doctrine of physiology which states that a complete synthesis of organic matter cannot take place in nature except through chlorophyll-containing plants by the action of light.

It is hardly likely that the nitrifying organism exhibits a chlorophyllous action, since a release of oxygen has never been observed. Another hypothesis, that it is an amide, perhaps urea, that is the first stage in the synthesis occurring in this organism, seems to me to be the only plausible one.

Further studies on the physiology and morphology of the nitrifying organism are in progress.

Comment

Winogradsky was able to show in a clear way for the nitrifying organisms that they obtained their energy from the oxidation of ammonia and use this energy for the assimilation of carbon dioxide. His earlier studies on the sulfur and iron bacteria had pointed this way, but these organisms had proven harder to work with. This discovery is really one of the most important in physiology, since it shows, as Winogradsky realized, that carbon dioxide is convertible into organic carbon without the intervention of light energy through chlorophyll. With the addition here of a third group of bacteria that could obtain energy from the oxidation of inorganic compounds, the chemosynthetic bacteria appeared to be fairly common.

The process of nitrification turned out to be more complicated than it appears here, and Winogradsky was instrumental in clarifying this picture. He described two genera of bacteria, one which oxidized ammonia to nitrite, and the other which oxidized nitrite to nitrate. This process is important agriculturally, since ammonia is easily lost from the soil, while nitrate is more stable and serves as a good nitrogen source for plants. As he mentioned, the isolation of these organisms in pure culture was quite difficult, mainly because the soil is so rich in bacteria that other forms, which grow much faster than the nitrifying bacteria, will take over on agar plates containing organic media. Further, the nitrifying bacteria seem to be inhibited by organic matter, so that it is necessary to find a substitute for agar or gelatin. Winogradsky later did this, using silica gel, and succeeded in this way in isolating pure cultures of each of the nitrifying bacteria. He was then able to demonstrate this process in pure culture and show that a different organism was responsible for each stage.

The biochemical aspects of chemosynthetic organisms are just beginning to be worked out. We know that the process of carbon dioxide fixation in the sulfur bacteria is quite similar to the process in green plants, using the same enzyme systems. The difference is in the source of energy. The sulfur and nitrifying bacteria derive their energy from the oxidation of these inorganic compounds, and these oxidations are coupled to phosphorylation, giving ATP. The energy from ATP is used in the process of carbon dioxide fixation. Only a small amount of modern work has been done on these interesting organisms, and many new things remain to be discovered.