

ATTEMPTED CULTIVATION OF *MYCOBACTERIUM* *LEPRAE*¹

BY BERNICE E. EDDY, PH. D.

Recently of the National Leprosarium, Carville, Louisiana

Attempts to cultivate *Mycobacterium leprae* have been made by many workers over a period of more than fifty years. Some of them have succeeded in cultivating acid-fast organisms, others have obtained nonacid-fast forms, and with still others the results have been negative. There seems at present to be no agreement as to whether the microorganisms that have been isolated are *M. leprae*, or whether that organism has ever been cultivated at all. For a historical review of the subject the reader is referred to the comprehensive summary of McKinley (12). The purpose of the present study was to repeat some of the work reported by others, and if possible to find some new medium upon which this organism would grow.

LEPROUS MATERIALS USED FOR CULTIVATION

The materials used in this work consisted of skin nodules from twenty cases of leprosy; spleen, liver or testicles from nine cases; sputum from six cases with laryngeal involvement; ascitic fluid from one case; and blood from fifty-six cases. Whenever possible cases were chosen that showed no clinical symptoms of tuberculosis. When nodular material was used, care was taken to select lesions which were not broken down or were not hard and fibrous.

The skin over a nodule was cleansed with iodine and alcohol, and a small cut was made through the surface of the skin and the cut surface scraped to obtain serum and cells, or the surface of the nodule was seared and the mass removed by an electric cautery. When the nodule was removed, it was seared on the under side and a cut made and scraped; the tissue was ground in a mortar and diluted with ordinary salt solution (0.85 percent sodium chloride), or it was cut with scissors into small pieces about 4 mm. in diameter. The spleen, liver, and testicular material was removed at necropsy, seared on the surface, and then treated in the same way as the excised nodules. Strict asepsis was maintained throughout.

Material was obtained from pustules by opening them with a scalpel and taking up the pus into a capillary pipette or on a cotton swab. Sputum was collected in paper containers before a meal and after the patient had rinsed out his mouth with water. The single specimen of ascitic fluid was removed aseptically and centrifuged, the sediment being used for inoculation. Blood was collected in 5 cc. amounts from

¹Published with the permission of the Surgeon General, U. S. Public Health Service.

an arm vein. Some specimens were mixed with 1 cc. of 1 percent sodium citrate, centrifuged at high speed, and the sediment was used. Other specimens, not citrated, were centrifuged at low speed and the layer of white cells was used.

Treatment of materials used.—Whenever possible the tissues (nodules, spleen, liver, testicle) and purulent material used were not subjected to treatment of any kind for the purpose of destroying commensal or contaminating bacteria. When these materials were so treated, the method of choice was 3 percent sodium hydroxide at 37° C. for 20 minutes, neutralizing with 6 percent sulphuric acid or 10 percent citric acid.

Other methods tried were: (a) 6 percent sulphuric acid at 37° C. for 20 minutes, neutralized with 1.3 percent sodium bicarbonate solution containing 3 percent of glycerol; (b) 2 percent acetic acid at 37° C. for 20 minutes, neutralized as above; (c) 6 percent acetic acid used in the same manner; (d) 0.1 percent aqueous gentian violet in the icebox over night, centrifuged and washed with salt solution; (e) 1 percent methylene blue employed similarly; (f) 0.1 percent ammonium hydroxide, also employed similarly; and (g) heat, at 60° C., for 20 minutes.

The sputa were treated with 3 percent sodium hydroxide at 37° C. for 20 minutes and neutralized with 6 percent sulphuric acid. Of the 56 samples of blood that were used, 23 were treated with 2 percent acetic acid at 37° C. for 20 minutes, centrifuged and washed with saline.

The treatment of tissues with acids was unsatisfactory because many of the bacilli planted on media after such treatment disappeared. Gentian violet and methylene blue, as used, were ineffective in destroying other microorganisms. Ammonium hydroxide or heating did not affect the morphology of the organism, but no advantage over the sodium hydroxide method was observed.

Microorganisms in the materials used.—The inocula prepared from skin nodules, spleen, liver, testicle, pus and sputum contained, as a rule, large numbers of *M. leprae*. A few separate mycobacteria and a few globi were seen in a stained preparation from the centrifuged ascitic fluid.

Of the 23 samples of blood which were concentrated, 2 showed mycobacteria in direct smears of the sediment. Of the 32 untreated samples of blood, 4 showed mycobacteria in smears from the white-cell layer. However, it is probable that organisms were present in more samples than those in which they were found. The numbers of organisms in the smears were small except in one sample, and in two instances some were found later from cultures (one inoculated with untreated white cells and one with treated concentrate) although none had been found in direct smears of the inoculum.

Smears from treated concentrates of blood from 105 cases of leprosy were examined and 14 (13 percent) showed mycobacteria. Smears of the white-cell layers of untreated blood were positive in 4 out of 34 cases (12 percent). With regard to the one specimen in which many organisms were found, another specimen taken a few days later showed only a few coccoid acid-fast and acid-fast debris; similar forms were also found a few weeks later on the surface of the culture media which had been inoculated with the first specimen. A few months later a third sample was examined, but no mycobacteria were seen in direct smears or obtained from cultures.

CULTURE MEDIA USED

Altogether, a very large number of culture media were used in this work. They are listed as briefly as possible.

1. Glycerol meat infusion broth.
2. Glycerol meat infusion agar.
3. Wherry's medium (17, 18).
4. Herrold's egg medium (5) containing 1 per cent oleic acid.
5. Nutrient ascitic agar containing bromocresol purple and 1 percent of one of: d-glucose, d-fructose, d-mannose, d-galactose, l-xylose, l-arabinose, rhamnose, maltose, trehalose, saccharose, lactose, aesculin, dextrin, salicin, melezitose, melibiose, raffinose, adonitol, duleitol, l-inositol, d-mannitol or d-sorbitol. (McCoy (11) reported that the growth of some strains of acid-fast bacilli from leprosy was more luxuriant when glucose was added to the medium.)
6. Petroff's medium with 1 percent of one of the carbohydrates, glucosides, or alcohols used in No. 5.
7. Trypsinized egg albumen medium, used by Duval (2).
8. Duval's medium T20 (2). Ringer's solution, 5 cc., containing 1.5 percent agar and 0.2 cc. of glycerol to which was added 2.5 cc. of a sterile filtered mixture containing equal parts of .01 percent cystine, .05 percent tyrosine, 2 percent leucine, 2 percent tryptophane and 5 percent dextrose in distilled water. On the surface of the slanted medium a few drops of trypsin were placed to digest the tissue in the inoculum. (It was reported after this medium was used (13) that cystine on standing in solution is converted to cysteine).
9. Duval's medium T21. This differed from No. 8 in that 0.5 cc. of sterile banana extract was substituted for the 0.5 cc. of 5 percent dextrose in the added mixture. The extract was prepared by soaking six minced, medium-sized bananas in 1,600 cc. of Ringer's solution in the ice-box over night, centrifuging, and filtering the supernatant liquid through a Seitz filter.
10. Duval's medium T22. Like Nos. 8 and 9 but containing no dextrose or banana extract.
11. Herrold's egg medium with 1 percent of a sterile filtered mixture of equal parts of saturated aqueous solutions of cystine, tyrosine, leucine and hydrolyzed casein containing tryptophane.
12. Long's synthetic medium (10) mixed with an equal part of trypsinized egg albumen.

13. Potato wedges moistened with 1 cc. of the solution of amino acids used in No. 11.
14. Francis' cystine agar ⁽⁸⁾ modified by substituting ascitic fluid for serum and adding 3 percent glycerol.
15. Francis' cystine agar differing from No. 14 in that 2 cc. of sterile filtered orange juice was added to 6 cc. of the melted medium at 45°C.
16. Francis' cystine agar without ascitic fluid.
17. Nutrient broth with 10 percent trypsinized egg albumen.
18. Glycerol nutrient broth containing 10 percent of the amino acid solution used in No. 11.
19. Glycerol nutrient broth with amino acid solution, put up in tubes in which 1 gm. of cotton had been placed on the bottom.
20. Tryptophane broth made from hydrolized casein ⁽³⁾ containing 10 percent of the amino-acid solution and 10 percent trypsinized egg albumen.
21. Dextrose tryptophane broth.
22. Minced chick embryo suspended in Tyrode's solution under aseptic conditions. (Used by McKinley and Verder.)
23. Minced chick embryo suspended aseptically in a liquid containing 1 percent dextrose and 0.85 percent sodium chloride.
24. Same as No. 23 except that the suspension was sterilized in the autoclave.
25. Petroff's medium modified by using eggs containing embryos instead of fresh eggs. The embryos were macerated and added to the mixture.
26. Oyster broth prepared by soaking 1 pint of minced oysters in 500 cc. of distilled water over night, heating in a boiling water bath for 20 minutes, filtering and adding 0.1 percent maltose, 1 percent oleic acid, 0.1 percent calcium phosphate, and 1 percent potato starch. Autoclaved after adjusting reaction.
27. Oyster agar, medium No. 26 with 1 percent agar.
28. Oyster agar with a few drops of sterile trypsin added to the slanted medium.
29. Oyster agar, same as No. 27 with 1 percent dextrose.
30. Oyster egg medium made by adding 200 cc. of autoclaved oyster broth (similar to No. 26 but containing 1 percent dextrose and 4 percent glycerol instead of 1 percent oleic acid) to six eggs opened aseptically, tubing and sterilizing by inspissating for 1 hour on three successive days, on the first day at 85°C. and on the two following days at 75°C.
31. Wherry's medium with a living 24-hour growth of *Escheridia coli* on the surface.
32. Wherry's medium with *E. coli* as above, killed by heating the tube in a slanting position at 80° C. for one hour.
33. Herrold's egg medium with living *E. coli*.
34. Herrold's egg medium with *E. coli* killed as in No. 32.
35. Herrold's egg medium with living *E. typhosa*.
36. Herrold's egg medium with killed *E. typhosa*.
37. Milk and egg medium with living *E. typhosa*. Prepared by sterilizing 150 cc. of whole milk containing 0.5 percent dextrose and 3 percent glycerol and adding aseptically the yolks of five eggs; sterilization as with medium No. 30.
38. Fish agar prepared by allowing 2.5 pounds of minced red snapper in 1,000 cc. of distilled water to decompose for several days, heating in boiling water for 20 minutes, filtering, adding 3 percent glycerol, 1 percent dextrose, 0.1 percent maltose and 1.5 percent agar, and autoclaving after adjusting the reaction.

39. Noguchi's medium (6) for spirochetes, in which Seitz-filtered ascitic fluid from a leprous patient was substituted for sheep, horse, or rabbit serum, both with and without the addition of sterile rabbit kidney.
40. Noguchi's medium as above except that the ascitic fluid had been heated in a water bath at 60° C. for several hours on each of three successive days.
41. Glycerol meat infusion agar with ascitic fluid.
42. Filtered ascitic fluid without additive.
43. Corper's potato medium (8) with 2 or 3 cc. of ascitic fluid.
44. Petroff's medium to each tube of which 4 or 5 drops of ascitic fluid was added to the water of syneresis.
45. Long's serum agar prepared by adding 6 cc. of serum from a leprous patient to 6 cc. of the melted agar at 45° C.
46. Glycerol urine meat infusion agar prepared by adding 2 cc. of filtered urine from a leprous patient to 6 cc. of melted glycerol meat infusion agar at 45° C.
47. Herrold's egg medium.
48. Petroff's medium with and without crystal violet.
49. Loewenstein's medium (9) with and without Congo red.
50. Petraganis' medium (16) with one-half the usual amount of malachite green.
51. Milk and egg medium, prepared like No. 37 but without *E. typhosa*.
52. Liver hormone egg medium prepared by soaking 1 pound of minced calf's liver in 1,000 cc. of water over night in the ice-box, heating in boiling water until the color changed to brown, adjusting the reaction and heating in a sterilizer at 100° C. for 1½ hours, loosening the clot and heating for another 1½ hours. The liquid was decanted, centrifuged, and 0.5 percent peptone, 0.5 percent sodium chloride, 6 percent glycerol, and 1 percent dextrose were added. To six eggs opened aseptically was added 200 cc. of the liquid; sterilization as in No. 30.
53. Casein egg medium. Glacial acetic acid was added to 2 quarts of whole milk until coagulation took place, and after filtering the casein residue was washed with water. Ten grams of the dried casein were added to 150 cc. of meat infusion agar, the reaction adjusted, the preparation autoclaved, cooled to 45° C., and the yolk of one egg added aseptically.
54. Potato egg medium. To six eggs opened aseptically were added 20 cc. of a solution of 1 percent dextrose, 0.1 maltose and 1 percent potato starch. Sterilization as in No. 30.
55. Corper's potato medium.
56. Corper's tissue-substrate microculture method for growing *M. tuberculosis* (1), using egg yolk or rabbit's blood.
57. Long's synthetic liquid medium.
58. Long's synthetic medium with 1 percent dextrose.
59. Long's synthetic agar.
60. Sohngen's medium as used by Frey and Hagan (3).
61. Dextrose meat infusion broth.
62. Rabbit's blood meat infusion agar.
63. Long's synthetic agar containing 1 percent dehydrated hog intestine according to Kendell (7). Approximately 400 gm. of minced hog intestine was treated with 1,600 cc. of 95 percent alcohol at 37° C. for two days, and extracted with 1,600 cc. 95 percent alcohol twice and 1,600 cc. benzine once. The residue was dried, ground to a powder, and used in preparing the medium, which was autoclaved.

64. Petroff's medium with 2 or 3 drops of 0.2 percent aqueous potassium iodide added to the water of syneresis.
65. Glycerol meat infusion agar containing 1 percent mucilage.
66. Gorodkow's medium (4) for cultivating yeasts.
67. Brain medium made by soaking 250 cc. of minced calf brain in 500 cc. of distilled water in the ice-box over night, heating in boiling water for 20 minutes, adding 3 percent glycerol, 1 percent dextrose and 0.5 percent sodium chloride, and autoclaving after adjusting the reaction.
68. Loeffler's blood serum medium.

The reaction of most of the media used was adjusted to approximately pH 7.6, though there were exceptions. Gorodkow's medium was approximately pH 3.8, some of the tryptophane broth pH 8.2, and the potato medium pH 5.0. The Francis cystine agar was adjusted to approximately pH 7.6 before the filtered orange juice was added (medium No. 15), so the final value was probably lower.

TREATMENT OF CULTURES

Inoculation of cultures.—Several different kinds of media were inoculated with each sample of material, and from two to as many as forty tubes of each kind of medium were inoculated; the usual number was six. All of the sputa were inoculated onto Petroff's medium as well as others. The blood specimens were inoculated onto Petroff's, Loewenstein's, or potato egg medium.

In the fear that some of the mycobacteria in the inoculum might be dead, some cultures were seeded with many of them. On the other hand, lest there might be present some toxic product of the organisms which might hinder their growth, other cultures were seeded with few of them in order to reduce the amount of any such hypothetical substance.

Evaluation of numbers of organisms.—In order to be able to compare the different cultures as regards the number of organisms present, the method of recording the numbers found in routine smears used at the National Leprosarium was employed in the present work. Smears were made of as uniform thickness as possible, stained with gently steaming carbol-fuchsin for 5 minutes or at room temperature for one hour, decolorized with 2 percent hydrochloric alcohol (instead of 3 percent as is used for *M. tuberculosis*), and counterstained with methylene blue. The number of mycobacteria present was recorded by letters as below. The morphological appearances of the individual organisms (i.e., multigranular, bipolar, monopolar and coccoid) were recorded by certain symbols.

Single acid-fast rods: A. One to five in a smear. B. More than five in a smear but less than one in a field. C. One to five in a field (average). D. From more

than five in a field to numbers difficult to count. E. Too many to count in the average field. F. Extreme numbers in every field.

Small globi: G. One to five in a smear. H. More than five in a smear but less than one in a field. I. One to five in a field. J. More than five in a field to numbers difficult to count.

Medium-sized globi: K. One to five in a smear. L. More than five in a smear but less than one in a field. M. More than one in a field.

Large globi: N. One to five in a smear. O. More than five in a field but less than one in a smear. P. More than one in a field.

Conditions of cultivation.—Cultures were maintained (a) under aerobic conditions, (b) under partial tension conditions as used by Wherry, (c) with increased carbon dioxide and oxygen as used by Soule and McKinley (14, 15), and (d) under anaerobic conditions. When cultures were put under modified atmospheric conditions, aerobic cultures were carried in parallel.

Wherry's partial tension method consists in attaching agar slants inoculated with *E. coli* to the culture tubes inoculated with *M. leprae*, the attachment being made with a short piece of rubber tubing in such a way as to permit free atmospheric exchange between the two tubes. In some cultures the original slant of *E. coli* was undisturbed during the entire period of incubation, while to others freshly inoculated colon bacillus cultures were attached at intervals of two or three days.

In the method of Soule and McKinley the culture is placed in a Novy jar, a vacuum is produced, and a gaseous mixture containing 10 percent carbon dioxide and 40 percent of oxygen is admitted.

Cultures were made anaerobic by Rockwell's method (pyrogallie acid and sodium bicarbonate) or by placing a layer of liquid petrolatum over liquid medium. Some cultures were also made in which cotton was placed in the bottom of a tube containing liquid medium, a method used for cultivating anaerobes.

The majority of aerobic cultures were closed with cotton plugs soaked in a mixture of nine parts of paraffin and one part of vaseline, with a hypodermic needle inserted in the plug to admit air. Other methods were also used. Some cultures were closed by flamed cotton plugs with lightly paraffined corks above them. Others were plugged with cotton and placed in a sealed museum jar containing a dilute solution of mercuric chloride, the liquid to supply moisture and the mercuric chloride to prevent the growth of molds.

The temperature of incubation was 35° to 37° C. in the majority of instances. Series of cultures were also incubated at room temperature, and a few cultures on Sohngen's medium were kept at 42° C.

Cultures were examined macroscopically and microscopically over a period of time from a few days to (two cases) over two years. Transplants were made at various intervals (usually of three to six weeks, but sometimes after a few days or after a few months), from cultures in which *M. leprae* could be found in smears. The trans-

plants were made on the same kinds of media as those used for the primary cultures, and on different ones.

RESULTS OBTAINED

In discussing the results obtained, a detailed analysis of the cultural findings in each case will not be made, but rather a review of the whole with reference to certain media and methods.

Macroscopically, on several of the solid slanted media there could be seen dewdrop-like elevations resembling colonies on the surface above the water of syneresis, but very similar elevations could be seen on some of the same media which had not been inoculated.

Microscopically, after a few days time the mycobacteria seemed to be more numerous and the globi larger than in the original inoculum. While it is possible that some growth-promoting substance was present in the tissue of the inoculum, it is not clear that the apparent increase noted could be interpreted as growth. Further increase was not observed. Some tissue cells or body fluids were always placed on the medium, regardless of the method used in preparing the material. In aerobic cultures at least, the tissue underwent autolysis, so that a loopful of autolysed material may have represented a concentration of the mycobacteria. Globi are easily broken up, and it was possible that in the autolysed material larger globi were seen because there was less chance for them to be broken up. The largest globus observed from a culture was 158 microns in diameter.

With regard to morphology of the single organisms, in the material used for inoculation they were usually multigranular or contained monopolar or bipolar granules. In some cultures, but not in others, the appearance changed after a time and the organisms became smoother and longer, or shorter. The factors which were responsible for this change were not determined. In some very old cultures, and in cultures made on slightly acid media, the mycobacteria became fragmented and appeared as cocci or acid-fast debris. A few sometimes showed curved forms, making almost a complete ring. It was first thought these ring forms might represent the beginning of globus formation, but it is likely they were degeneration forms, because after a while no acid-fast organisms or debris could be found in these cultures.

The persistence of *M. leprae* on the culture media seemed to depend on at least three factors; first, their number in the inoculum, second, the pH value of the medium, and third, moisture.

Many organisms were seen in smears from cultures on Wherry's, Petroff's, and Loewenstein's media, fish agar, oyster broth or oyster agar, and many other media kept moist at 37° C. for twenty to thirty weeks, both aerobically and under modified conditions. Many of them were observed in smears made from five cultures which were kept for over a year, and from a similar number kept for over two years. The media used in these instances happened to be (a) tryptophane broth with cystine, tyrosine, and leucine; (b) tryptophane broth with the above amino acids and trypsinized egg albumen; (c) glycerol nutrient broth with amino acids and cotton; (d) oyster agar; (e) oyster egg media; and (f) dextrose glycerol meat infusion broth. One of the cultures examined after more than a year, and another examined after two years, had been treated by Wherry's method.

Mycobacteria were found in as many as six transplants from primary cultures made on a number of media at intervals of three weeks, but there was a diminution in their numbers instead of an increase as found by Soule and McKinley. This diminution occurred, whether the cultures were kept aerobically or under the conditions employed by Wherry or by Soule and McKinley. Mycobacteria could also be found, but in decreased numbers, from transplants made after a few days or after a few months.

In cultures in which trypsin was used to decompose the tissue (at the same time forming protein-split products), and in those in which trypsinized egg albumen and various amino acids were used, the organisms could be obtained from both primary cultures and transplants, but there was a decrease in numbers in the transplants. The same was true of cultures on media containing various carbohydrates, glucosides or alcohols, and of the chick-embryo media, oyster or fish media, and those containing ascitic fluid, serum and urine from leprosy patients—in fact, of most of the special media described.

The organisms that were inoculated on cultures of *E. coli* or *E. typhosa*, either living or killed by heat, disappeared in a short time. Enough acid may have been produced by these microorganisms to cause destruction of the mycobacteria. They similarly disappeared in cultures contaminated by spore-bearing bacilli or staphylococci. In smears from cultures made on slightly acid media they appeared coccoid and fragmented after several weeks, and after a time no more were found. Fewer organisms were seen in smears from partially dried cultures than from cultures which had been kept moist.

Commensal or contaminating microorganisms.—From the sample of ascitic fluid used an organism resembling *M. tuberculosis avium*

was cultivated. Later cultures were made from the skin of the same patient, but no mycobacterium grew.

The characteristics of this organism have not been worked out completely. In four days there appeared on Petroff's media small, round, moist, smooth, glistening white colonies which gave an abundant, slimy, white growth on transfer. Some transplants at 37° C. showed a pinkish tinge, and some removed to room temperature assumed an orange color. After a few transplants on Petroff's medium growth occurred on glycerin meat infusion agar and broth. On agar at 37° C. the growth in two or three days was abundant, slimy, mucoid and white. Broth was cloudy and there was a slight surface growth which was easily disturbed and fell to the bottom as a sediment.

Microscopically the bacilli when first isolated were very short, and were smooth-staining or bipolar. In older cultures they tended to be longer, curved, and more of them were granular, usually monopolar or multigranular. Many were nonacid-fast. In broth cultures there were many of still greater length, and in Rosenow's brain broth they were extremely long, almost filamentous, and very curved. This organism resembles one isolated by Kedrowsky from a leprous patient.

Three animals, a guinea pig, a rabbit, and a chicken, were injected with a heavy suspension of a four-day culture grown on Petroff's medium. The guinea pig, injected subcutaneously with 0.5 cc. of the suspension, remained negative for eight weeks and presented no lesions at autopsy. The rabbit, given 1 cc. of the suspension subcutaneously, died six days later. The inguinal gland near the site of inoculation was enlarged, and the liver showed a few whitish lesions from which an organism similar to that injected was isolated. The chicken was injected with 1 cc. intraperitoneally and in eight weeks had lost considerable weight. The liver showed some mottled areas, and the lungs a few small white lesions from which short acid-fast bacilli were seen in direct smear.

Some of the tubes inoculated with leprous material showed ordinary contaminating microorganisms—molds, spore-bearing bacilli, and in a few instances staphylococci. These cultures were discarded. Two other groups of microorganisms were found, actinomycetes and a short nonacid-fast bacillus.

The two most common actinomycetes were aerobic and grew abundantly on ordinary solid media as dry, red, rugose colonies or as moist, mucoid, translucent, rounded colonies. Microscopically they were alike, appearing as long filaments that in old cultures broke up to produce long or short bacillary forms, or even coccoid forms that occurred in pairs or chains. They were nonacid-fast and only partially Gram positive, only gram-negative microorganisms being sometimes found adjoined to positive ones. A more uncommon actinomyces was a scantily growing orange-colored organism that formed small, dry, rough colonies. In smears the filaments were long and branching, and there was less tendency to break up than in the more common types mentioned.

The dry, red, actinomyces was also obtained by exposing media to the air in the laboratory, and it is likely that all of these organisms were contaminants.

The short, nonacid-fast bacillus grew on a variety of special media inoculated with various materials from several cases. In three or four days there appeared small, smooth, moist, translucent, grayish colonies not unlike those of *Corynebacterium diphtheriae*. After a few transfers growth was more abundant and took place on meat infusion agar and broth. On the agar the growth was as described but white, later becoming opaque. The broth became cloudy, with a scanty flocculent sediment. Some strains produced a scanty pellicle.

Microscopically these bacilli when first isolated were extremely small, so small that they could almost be mistaken for micrococci. They occurred in parallel arrangement, and were smooth or showed bipolar granules. In older cultures they were longer and often multigranular, looking very much like *C. diphtheriae*.

Although transplants were made on a number of different media, these organisms did not become acid-fast. In smears from cultures on egg media the granules of a few of the individuals sometimes retained the stain, but this was not constant.⁴ The organisms were pleomorphic, often showing swollen, club-shaped ends and long forms, but no filamentous forms were seen. Gram's stain was retained, the bacilli appearing as solid rods.

When leprosy bacilli were present in cultures together with these organisms, they appeared as they had in the material used for inoculation, as monopolar, bipolar, or multigranular rods, not broken up or fragmented.

Similar nonacid-fast bacilli have been obtained from lepers by a number of workers, but their rôle in leprosy has not been determined. It is very possible that they are commensal organisms present in the leprosy patient, and as has been suggested by others, it is possible that they may in some way promote the growth of *M. leprae* in the patient's body. On the other hand, they may be a nonacid-fast form of *M. leprae*.

SUMMARY

Many media inoculated with different kinds of material from a number of leprosy patients showed no growth of any microorganism that resembling *M. leprae* as it occurs in the tissues.

The mycobacteria of the inoculum persisted on many media for long periods of time, depending on their number in the inoculum, the reaction of the media, and the conditions of moisture. Mycobacteria were found in smears from cultures on several different media kept moist at 37° C. for twenty to thirty weeks, in five cultures kept for over one year, and in five others kept for over two years. On slightly acid media the organisms became coccoid and fragmented after a few weeks, and finally disappeared. Fewer were seen in smears from partially dried cultures than from moist cultures.

Mycobacteria were found on transplants made from the primary cultures, but there was a diminution instead of an increase in their

⁴Smears from a culture of staphylococci grown on egg media sometimes also retained some of the stain.

numbers. As many as six transplants made on several media continued to show organisms.

An organism resembling *M. tuberculosis avium* was isolated from the ascitic fluid of one leprous patient. Cultures made from the skin of the same patient were negative.

Of the nonacid-fast microorganisms that appeared in some of the cultures, there were some (molds, spore-bearing bacilli, and staphylococci) that were considered as ordinary contaminants and were discarded.

Two other groups of microorganisms were also obtained, actinomycetes and small nonacid-fast bacilli. It was not determined whether any of them had any relation to leprosy. One similar actinomyces was obtained on medium exposed to the air in the laboratory. The small nonacid-fast bacillus grew on several media inoculated with material from a number of cases of leprosy.

I wish to express my thanks to Dr. O. E. Denney, Dr. H. E. Hasseltine, Dr. F. A. Johansen, and Dr. S. H. Black for their helpful suggestions, and to Dr. F. A. Johansen, Dr. J. G. Wooley, Dr. S. H. Black and Sister Hilary Ross for obtaining from the patients the material used for all the cultural experiments.

REFERENCES

- (1) CORPER, H. J. A tissue substrate microculture for tubercle bacilli. *Jour. American Med. Assoc.* **99** (1932) 315.
- (2) DUVAL, C. W. [Cited by Wolbach, S. B., and Honeij, J. A. A critical review of the bacteriology of human and rat leprosy. *Jour. Med. Res.* **29** (1914) 367.]
- (3) FREY, C. A. and HAGAN, W. A. The distribution of acid-fast bacteria in soils. *Jour. Infect. Dis.* **49** (1931) 497.
- (4) HENRICI, A. T. *Molds, Yeasts and Actinomycetes*. John Wiley and Sons, Inc. New York, 1930.
- (5) HERROLD, R. D. Egg yolk agar for the growth of tubercle bacilli. *Jour. Infect. Dis.* **46** (1930) 263.
- (6) JORDAN, E. O. *General Bacteriology*. W. B. Saunders Co. Philadelphia and London, 1931.
- (7) KENDALL, A. D. A medium for isolation and cultivation of bacteria in the filterable state. *Northwestern Univ. Bull.* **23** (1931) 8. *Abstr. Jour. Lab. and Clin. Med.* **17** (1931) 286.
- (8) KOLMER, J. A. and BOERNER, F. *Approved Laboratory Technic*. D. Appleton Co. New York and London, 1931.
- (9) KRENN, O. and LOWENSTEIN, E. [Direct culture of *B. tuberculosis* from the blood.] *Klin. Wchnschr.* **10** (1931) 974. *Abstr. Jour. Lab. and Clin. Med.* **17** (1932) 830.
- (10) LONG, E. R. and SEIBERT, F. B. Chemical composition of the active principal of tuberculin. Non-protein medium suitable for the production of tuberculin in a large quantity. *American Rev. Tuber.* **17** (1926) 393.

- (11) MCCOY, G. W. The cultivation of acid-fast bacilli from lepers by the use of symbiotic organisms. U. S. Public Health Bull. No. 66. Studies on leprosy. Washington, 1914.
- (12) MCKINLEY, E. B. The etiology of leprosy. *Medicine*, **13** (1934) 377.
- (13) SHINOHARA, K. and KILPATRICK, M. The stability of cystine in acid solution. *Jour. Biol. Chem.* **105** (1934) 241.
- (14) SOULE, M. H. and MCKINLEY, E. B. Cultivation of *B. leprae*, with experimental lesions in monkeys. *American Jour. Trop. Med.* **12** (1932) 1.
- (15) SOULE, M. H. and MCKINLEY, E. B. Further studies on experimental leprosy and cultivation of *Mycobacterium leprae*. *American Jour. Trop. Med.* **12** (1932) 441.
- (16) TERZANI, A. [Comparison of Petroff's and Petraghani's methods for primary culture of the tubercle bacillus.] *Gior. Clin. Med.* **11** (1930) 7. *Abstr. Jour. Lab. and Clin. Med.* **15** (1930) 697.
- (17) WHERRY, W. B. Cultivation of an acid-fast bacillus from leprosy. *Philippine Jour. Sci.* **43** (1930) 577.
- (18) WHERRY, W. B. Note on the cultivation of an acid-fast bacillus from leprosy. *Jour. Infect. Dis.* **46** (1930) 263.