MASS CULTIVATION OF MYCOBACTERIUM LEPRAE[1]

By EARL B. MCKINLEY, M. D.
Washington, D. C.

AND WALFRIDO DE LEON, M. D.
Manila, P. I.

In June, 1931, McKinley and Soule (9) described the cultivation of *Mycobacterium leprae* before the American Medical Association in Philadelphia. By June, 1937, this organism had been carried through approximately sixty culture generations and showed no tendency to grow any more luxuriantly than when originally isolated over six years before. In this respect and in view of the conditions under which it is cultivable, this acid-fast bacterium, isolated from cases of leprosy, apparently differs radically from all other so-called strains of *M. leprae* described in medical literature.

Subsequent progress reports dealing with the cultivation of this organism were published by Soule and McKinley (16, 17), McKinley and Verder (10, 11), Soule (15) and McKinley (8). The general method of cultivation of *M. leprae* employed by us was based upon the attempts of Wherry (18) to cultivate this organism in a gaseous atmosphere of oxygen and carbon dioxide in 1930. In Wherry's experiments he apparently employed a very small inoculum since he used only a loopful of blood expressed from a leprosy nodule. While he states that bacilli were "numerous" in the loopful, still the inoculum must be regarded as exceedingly small as compared with most cultivation experiments of this nature. Even so, although Wherry's methods of producing his various gaseous tensions were rather crude, he reported definite proliferation of *M. leprae* in cultures from three cases, at the end of four to six weeks incubation. He further stated that subculture of a loopful of material containing several dozen colonies resulted in the appearance of a large number of sub-colonies, isolated masses and scattered acid-fasts upon his special medium (glycerinized-ovomucoid-yolk-oleic acid-dextrose-agar). Working with this general principle of cul-

[1]This report comes from the Department of Bacteriology, Hygiene and Preventive Medicine, George Washington University, Washington, D. C., and the Department of Sanitary Bacteriology and Immunology, School of Hygiene and Public Health, University of the Philippines, Manila, P. I. This work is supported by a grant from the American Leprosy Foundation (The Leonard Wood Memorial), New York City.

tivation under gaseous tension, but having in mind the work of previous investigators on this subject such as Hesse (5), in 1893; Moore and Williams (7), in 1909; Corper, Gauss and Rensch (3), in 1921; and Novy, Roehm and Soule (13), in 1925, we found that various common laboratory culture media would support growth of this peculiar acid-fast organism with definite colonial formation. The most successful medium, however, was found to be hormone-glycerol-agar. Later in this paper the method of preparing this media will be given.

Our immediate purpose in this report is to present the foregoing brief statement of our culture work with reference to the original papers which contain detailed descriptions of the methods of cultivation employed and to describe a gaseous tension incubator, designed by one of us (E. B. McK.), which is now being used for mass culture work at the School of Hygiene and Public Health in Manila.

Since 1931 few attempts have apparently been made to repeat our culture experiments. We are informed that one laboratory has succeeded in repeating the work but has not published a report concerning it. Another laboratory (The Henry Phipps Institute of Philadelphia), having large and extended experience with the acid-fast group of organisms, has carried our culture of *M. leprae* in subculture for nearly two years. In 1934 Soule repeated the culture work, originally carried out in Puerto Rico, at Culion in the Philippines. Identical strains were isolated on opposite sides of the world. Meanwhile further experiments in Washington have failed to produce a satisfactory laboratory animal and, indeed, it seems probable that no experimental animal for leprosy may exist. If this be true then the opportunity of establishing our organism, or any other for that matter, as the true germ of leprosy by the usual methods of Koch's postulates may not be possible. Because of this situation the problem is now being attacked from another angle.

In recent years new chemical methods have been evolved for the isolation of various chemical fractions (protein, polysaccharide, phosphatide, and lipoid fractions) from acid-fast organisms by Anderson (1, 12, 2, 4) and his colleagues at Yale University and by Long and Campbell (6) and by Seibert and Munday (14), to mention the leading workers only. The protein of *M. tuberculosis* has been employed successfully as an antigen in tuberculosis. It occurred to some of us that an immunological approach in leprosy with various chemical fractions prepared from known so-called cultures of *M. leprae* might succeed in bringing light to the problem of etiology

in the absence of Koch's postulates. It seemed quite possible that one or more of these fractions might be useful in early diagnosis of the disease when applied as an intradermal test. With this thought in mind the available chemical fractions of many so-called strains of *M. leprae* and other acid-fast organisms are being tested in the Philippines and these will be the subject of an extended report in the near future. These have been supplied by Anderson at Yale University and by Long of the Henry Phipps Institute in Philadelphia.

At the same time it seemed advisable to attempt to procure enough of our own organism, grown under gaseous tension, so that similar chemical fractions might be prepared. These could also be tested in leprosy and control groups. Should any fraction act specifically it would be strong evidence that the organism from whence the fraction came is related etiologically to the disease. The problem then was to design an incubator which would permit of mass culture work and in which the proper gaseous atmosphere could be maintained. Such an apparatus was designed and built for the American Leprosy Foundation (The Leonard Wood Memorial) and is briefly described as follows (see illustration):

The main shell of the incubator, as will be seen, is merely one of the modern types of steel disinfectors which has been adapted to our purpose. With such strong walls and with rounded edges it permits one to produce a considerable negative pressure inside. The size of this apparatus is 52 x 27 x 27 inches, interior dimensions. The shell is mounted on pipe legs, giving it a total height of 55 inches. The interior is insulated with celotex covered with a light metal sheeting. The door is also insulated and fits snugly against a gasket when closed and the flange tightened. The apparatus is absolutely air proof as tested by vacuum. There is an opening at the top in which a pressure gauge is attached by means of a short pipe which is screwed into the shell wall. A pet cock at the lower end of this pipe has a nipple to which pressure rubber tubing is attached and leads to the gas tank. Back of the incubator on the floor rests a vacuum pump (Ingersoll Rand Co., Type 30, with moisture trap arrangement) which is operated by a three-quarter horsepower motor. This motor connects, as seen in the illustration, with a switch mounted on the left side of the incubator shell with the other control instruments. On the right inner wall just above the second shelf one sees where the pipe leading to the vacuum pump connects. We have thought it best to cover this opening with a thin brass wire gauze so that debris of any kind may not enter the pump during operation.

The heating elements are twelve in number and are calculated to use not more than 2,500 watts of electricity. They rest in a frame on short metal legs on the bottom of the incubator shell and this is easily movable. Wires carrying the current are connected at the back and pass through insulators fitted to openings in the shell wall and cemented in tightly. The wires then connect on the rear end of the shell with a thermo-regulator (Central Scientific Co.) and pass in turn, as seen on the left side of the shell, through a conduit to the switch and relay box. The current from the power line enters the top of this box (not shown in the illustration). There is a pilot lamp which shows through the red glass window directly above the switch. A temperature recording device (Taylor Instrument Co.) is also mounted on the left side of the incubator shell and the tubing passes out the bottom and enters the chamber through the rear wall near the top and is wound around the top inside of the shell, attached to it by several small metal clips. Finally there is a valve in the pipe connection from the vacuum pump to the shell which may be opened or closed as desired, and one sees also a pipe connection with valve entering the bottom of the shell for the purpose of permitting a rapid release of negative pressure if desired. Another small opening, closed with a nipple, is seen on the top wall of the shell near the front which permits placing a thermometer through the wall to compare periodically the temperature within with the recorded temperature on the chart. Before the pump is started this opening is closed tightly. The inside of the shell is fitted with three shelves, and special test tube racks holding 80 culture tubes each are employed. The incubator holds 36 of these racks and therefore has a capacity of 2,880 cultures.

The method of cultivation is very simple. First the leprosy nodules are excised under the strictest aseptic technique. The skin over the nodules is cleaned thoroughly with tincture of green soap with a stiff hand brush. Then it is washed with water, then with alcohol. Tincture of iodine is then used and this is followed again with alcohol. Incision is made in the skin and the instruments discarded. The nodule is dissected free with fresh instruments and removed. It is dipped in alcohol for only a moment and placed in a sterile dish. Later it is minced finely with sterile scissors, then placed in a sterile agate mortar and ground with sterile sand. We have found it advantageous to carry out the mincing and grinding of the tissue inside a glass chamber designed by one of us (W. de L.) which greatly lessens any chances of air contamination. Sterile physiological saline is then added and the tissue suspension made

by continued grinding and mixing. This material is then allowed to stand covered for a short time to permit particles of sand to settle out, after which it is poured through a fine brass wire mesh, shaped by bending like a small funnel, previously sterilized in a bunsen burner to a red heat, into sterile test tubes. Some further tissue debris settles to the bottom and the supernatant is decanted off with fine pipettes and this material, after stained smear preparations prove satisfactory by microscopic examination, is employed for making the cultures. One loopful of this suspension is spread over the entire surface of each tube of slanted hormone-glycerol-agar. The cultures having been seeded, the 36 racks are placed in the incubator. The door is closed and tightened. With all openings closed except the valve in the pipe line connecting the interior of the chamber with the vacuum pump, the switch to the motor is turned on, the pet cock to the pressure gauge opened, and 20 to 25 inches of negative pressure is produced. The valve to the pump is then closed and the gauge is watched for a few moments to test for any possible leaks. If none (and we have had none) the gas mixture (10 percent carbon dioxide, 40 percent oxygen and 50 percent nitrogen) is allowed to flow through the pet cock at the top of the shell and the needle on the gauge gradually falls to zero. The pet cock is turned off, the door tightened more if possible, and the incubator is started and regulated at 37.5 degrees C. Incubation continues for six weeks, but at two week intervals the cultures are removed and examined, any contaminations removed, and if moulds appear on the cotton plugs they are flamed with a bunsen burner. To provide some additional moisture we have found it of advantage to place a small bottle containing water on the middle shelf of the incubator. Apparently very little of it is consumed.

We have referred to the culture media used in this work. The hormone-glycerol-agar is prepared as follows: (1) Use fresh lean ground beef. (2) Add 1 liter distilled water plus 10 grams of peptone and 5 grams of sodium chloride per pound of beef. (3) Make an infusion and leave in the icebox for 24 hours. (4) Remove meat tissue by straining through washed cheesecloth into large glass beakers. (5) Add to filtrate 15 cc. N/1 NaOH per liter. (6) Mix thoroughly. Place in boiling water bath for 20 minutes, or until the protein is coagulated. (7) Cool. Decant clear supernatant broth into large beakers or glass dishes. (8) Add 50 cc. glycerol per liter of decanted solution. (9) Adjust pH to 7.4. (10) Add 15 grams of agar per liter of decanter fluid. Dissolve by heat. (11) While melted, distribute to plugged sterile test tubes. (12) Autoclave at 110 degrees C. for 20 minutes. (13) Slant. Soule believes that the absence of

metal and filter paper in the preparation of the media and the early addition of peptone and salt is of distinct value.

While we have presented here the methods now being employed for the mass cultivation of *M. leprae*, the same principles apply for experimental work on a smaller scale where Novy jars or similar anaerobic jars are used as gas chambers and the whole is placed in an ordinary incubator. The only advantage here, which permits cultures by the thousands rather than a limited number, is the principle of incubating directly in a gaseous environment in the incubator itself. Cultivation work with leprosy material according to these methods yields small colonies averaging about 1 mm. in diameter, and heaped up, with a distinct mucoid appearance and a loose filamentous border. The colonies are made up of characteristic bacilli which stain solidly with carbolfuchsin and are not decolorized with 10 percent sulphuric acid. No chromogenic acid-fast organisms are found in such carefully prepared cultures; nor have we met with any other bacillus, coccus or actinomyces types, excepting air contaminants (to date in our mass culture work some 20 or more varieties) which usually appear within the first few days of incubation. A special note will be published later dealing with these air contaminants which have been of small number in our hands, considering the large numbers of cultures, but are of interest particularly in this disease where so many such organisms have apparently been erroneously assigned important significance.

It is our hope later to have enough culture material for chemical studies, after which the significance of the various antigens may be tested in patients and controls.

REFERENCE

(1) Anderson, R. J. and Nao Uyei. Jour. Biol. Chem. **97** (1932) 617-637.
(2) Anderson, R. J., Crowder, J. A., Newman, M. S., and Stodala, F. H. Jour. Biol. Chem. **113** (1936) 637-647.
(3) Corper, H. J., Gauss, H., and Rensch, O. B. American Rev. Tuber. **5** (1921) 562-587.
(4) Crowder, J. A., Stodala, F. H., and Anderson, R. J. Jour. Biol. Chem. **114** (1936) 431-439.
(5) Hesse, H. Zeits. f. Hyg. & Infekt. **15** (1893) 17-37.
(6) Long, E. R. and Campbell, L. K. American Rev. Tuber. **6** (1922-23) 636.
(7) Moore and Williams: Quoted from Novy, F. G. and Soule, M. H. Jour. Infect. Dis. **36** (1925) 168-232.
(8) McKinley, E. B. Medicine **13** (1934) 377-504. Monograph.
(9) McKinley, E. B. and Soule, M. H. Jour. American Med. Assoc. **98** (1932) 361-367.
(10) McKinley, E. B. and Verder, E. Proc. Soc. Exper. Biol. & Med. **30** (1933) 659-661.

(11) McKinley, E. B. and Verder, E. Proc. Soc. Exper. Biol. & Med. **31** (1933) 295–296.
(12) Nao Uyei and Anderson, R. J. Jour. Biol. Chem. **94** (1932) 653–660.
(13) Novy, F. G., Roehm, H. R., and Soule, M. H. Jour. Infect. Dis. **36** (1925) 109–167.
(14) Seibert, F. B. and Munday, B. American Rev. Tuber. **25** (1932) 724.
(15) Soule, M. H. Proc. Soc. Exper. Biol. & Med. **31** (1934) 1197.
(16) Soule, M. H. and McKinley, E. B. American Jour. Trop. Med. **12** (1932) 1–36.
(17) Soule, M. H. and McKinley, E. B. American Jour. Trop. Med. **12** (1932) 441–452.
(18) Wherry, W. B. Jour. Infect. Dis. **46** (1930) 263–266.

DESCRIPTION OF PLATES

PLATE 24

The caseous-tension incubator described.

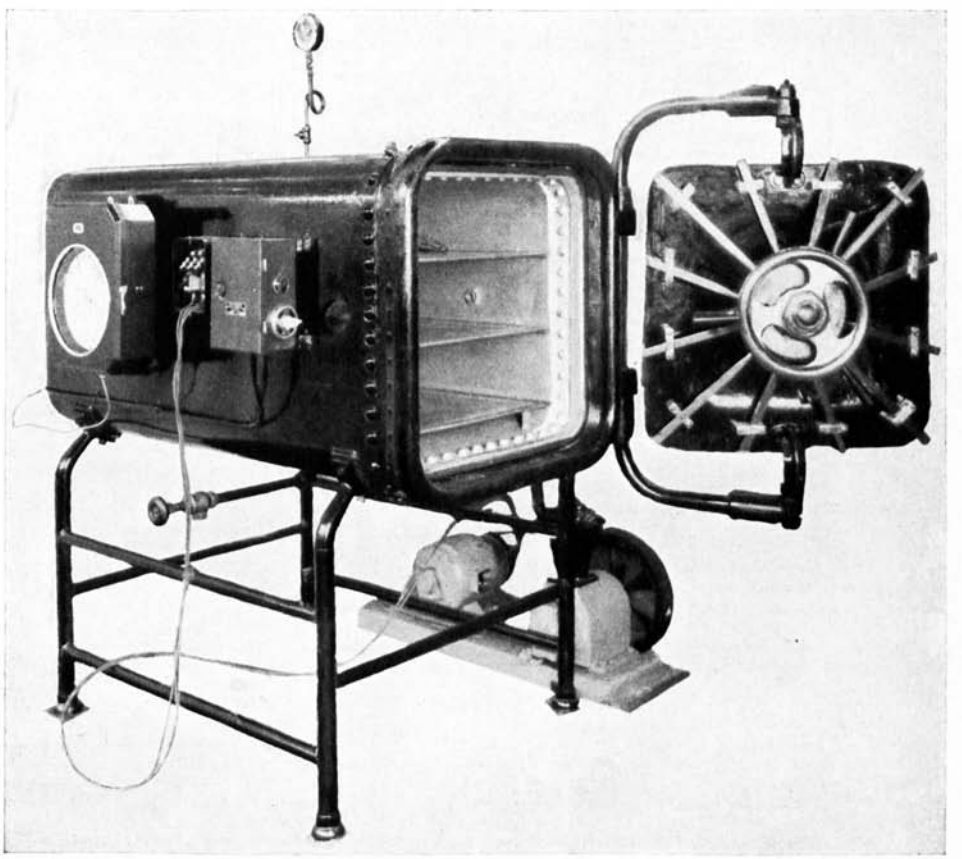

Plate 24

PLATE 25

The inoculation chamber (devised by W. de L.) used in the work described. It consists of an all-glass rectangular chamber, the front corners replaced by oblique surfaces in each of which is a small rectangular window which allows convenient insertion of the arm to the elbow into the chamber. The arrangement permits comfortable movement of the arms, with clear and complete visibility, without danger of contaminating the material inside the chamber from the nose or mouth. This chamber can be used for inoculating or autopsying small animals, for transferring cultures, macerating sterile materials, and for tissue culture work.

It is constructed of clear, 1/8-inch glass pieces, joined by lead strips. It measures 80 cm. high, 50 cm. in depth and 65 cm. in width at the back, the front face being 39 cm. wide. The bottom is of glass, to facilitate clearing the chamber and sterilizing it in case of spilling of infectious materials. The whole is supported on a one-piece wooden base two inches thick which is provided with three ball rollers to facilitate moving it on the work table. Memoranda or notes may be inserted for reference between the bottom glass and the wooden support.

A small hole is provided at the lower rear corner to admit a small-sized rubber tubing for a microburner. The small windows, which measure 20 by 28 cm., are closed by glass doors opening backward; they will admit a small-sized pan of disinfectant when this is necessary for work.

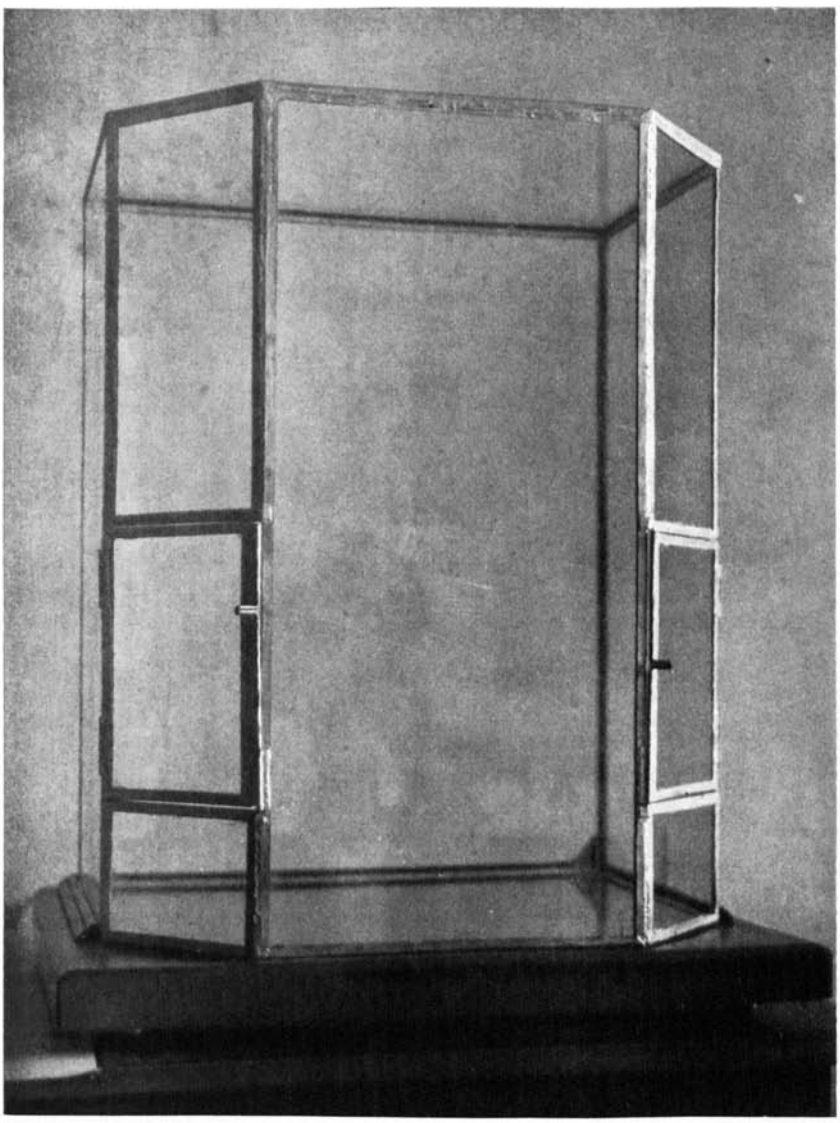

Plate 25