Advances in the Microbiology of *M. leprae* in the Past Century

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Facts of profound interest, not merely with respect to the pathogenesis of leprosy, but for the general history of bacteriology are evident in an attempt to trace the segment of history related to investigations in leprosy following the discovery of the leprosy bacillus to the establishment of its role as the pathogen causative of the disease.

In Europe, the most widespread epidemics of leprosy occurred about the thirteenth and fourteenth centuries, followed by a gradual decline. The disease was drastically diminished by the middle of the nineteenth century, with the exception of a few limited areas in Europe. On the other hand, an extensive epidemic of leprosy developed in Norway in about the middle of the nineteenth century.

In 1847, D. C. Danielssen, the then chief physician at the leprosy hospital in Bergen, and C. W. Boeck, the then professor of dermatology at Christiania Medical School published from Christiania the book Om Spedalskhed (A Study of Leprosy), which represents an epochal achievement of clinico-pathologic research on leprosy based on their wide experience and profound knowledge of the morbid anatomy of the disease. The classification of leprosy into two principal types, the tubercle (nodular) type and the anesthetic type, promulgated by them, was indeed a foresight that deserves the highest admiration. The contributions made by these great men in the classification of leprosy into two principal forms are still shedding light on the medical science of leprology.

From his early investigative days, Danielssen had recognized the presence of small brown or yellowish, grossly discernible "granular masses" or "brown elements" demonstrable on histopathologic preparations from leprosy nodules and had been convinced of their peculiarity to leprosy. However, perhaps he did not think that the "masses" had any vital bearing on the etiology of leprosy. In 1859 Danielssen asked the opinion of R. Virchow, who was visiting him, of the "brown masses." Virchow was not particularly impressed with Danielssen's discovery and interpreted the bodies as representing mere clumps of degenerated fat and, therefore, having nothing to do with leprosy. Years later, Virchow altered this initial view and designated the brown masses as "lepra cells" (⁸).

The discovery of the leprosy bacillus. In 1868, Gerhard Henrik Armauer Hansen, as a young physician, began to attend his duties at the Nursing Home for Lepers No. 1 and subsequently, in the same year, filled the post of assistant physician at Lungegaarden Hospital. In 1870, Hansen, having returned from a twelve month sojourn at Bonn and Vienna to acquire basic knowledge and skill in pathologic anatomy and microscopy, pursued investigations on leprosy in an area of western Norway having a high incidence of the disease, as well as Lungegaarden Hospital.

He summarized the results of these investigations in a paper which he submitted in 1873 to the Norweigian Medical Association in Christiania, the body by which he had been awarded his research scholarship. This work, which he entitled "A Report to the Norwegian Medical Association in Christiania on a Journey Undertaken with the Backing of the Association to Investigate the Causes of Leprosy," was printed in 1874 as a supplement to NORSK MAGAZIN FOR LAEGEVIDENSKABEN (The Norwegian Journal of Medical Science) (³²).

This paper contains the first description of the causative agent of leprosy: "There are to be found in every leprous tubercle extirpated from a living individual—and I have examined a great number of them—

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small, staff-like bodies, much resembling bacteria, lying within the cells; not in all. but in many of them" (36, 13). Furthermore, he emphasized in an indirect yet presumptive fashion, evidence corroborating the concept that leprosy is a chronic communicable disease caused by a specific agent. Hansen, therefore, eminently deserves credit for the conception of the pathogen he demonstrated as being responsible for leprosy since at that time the prevailing view was that leprosy was not a contagious but a humoral or hereditarily constituted disease.

In what year did Hansen first discover the leprosy bacillus? His discovery of the leprosy bacillus was in 1873 according to one article while another claims the year of discovery to be 1874.

In an editorial in this JOURNAL in 1964, Wade (³⁶) noted that 1874 was certainly the year of publication by Hansen of his discovery of the leprosy bacillus. Therefore that year is the recognized date of the discovery according to modern practice.

As a comment on this matter, H. P. Lie, who had been Hansen's assistant and succeeded him, wrote that the observations published in 1874 had been made in the previous year and that consequently Hansen himself had maintained that the discovery of the bacillus must be reckoned as from 1873. Vogelsang stated that if we credit the work to the time it was done rather than to the time of publication of the report, then 1873 is the year in which Armauer Hansen discovered the leprosy bacillus.

In his popular book *The Fight Against Leprosy* (1964), Patrick Feeny gives what purports to be the actual date of the finding that the brown bodies were masses of individual rods as February 28, 1873, and even notes the name of the patient concerned. Certain other cases in the same period are also cited.

A brief description of the medical and microbiological background of the days of Hansen's discovery is of interest as related to this discovery. Rudolf Virchow published in 1858 Die Zellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebelehre. Diseases had generally been believed to exist in the organs at the time of Giovanni Battista Morgagni (1761), the founder of modern pathologic anatomy, and to exist in the tissues at the time of Marie Francois Zavier Bichat (1771-1802). With further development, the contemporary cellular pathologic concept that diseases have cellular bases was shown by Virchow and his school. Meanwhile, advances in microscopy not only contributed much to the development of cellular pathology but resulted in a great deal of progress in the microbiology of pathogenic organisms. Beginning with the microscope of Antony van Leeuwenhoek (1632-1723) up to the development of apochromat objectives with extremely low aberration by Ernst Karl Abbe (1887), prodigious progress was made in microscopy and, in consequence, bacteriology as a genuine branch of science was born late in the nineteenth century, and thereafter made great strides. In the records of the observation of a great variety of things in the natural world, which Leeuwenhoek saw with the microscope he invented, it is said that there is a description of a group of microorganisms which we nowadays should recognize as bacteria. However, no further progress could be made along this line at his time because science in general was not well-developed enough to permit utilization of his observation. Thus, Leeuwenhoek, as the father of microscopy, introduced the concept of microbes but did not become the father of bacteriology.

The first development of contemporary bacteriology was brought about, as is widely known, by the two distinguished scientists Louis Pasteur (1822-1895) and Robert Koch (1843-1910). They were the first to demonstrate and describe various activities of microorganisms as pathogenic agents. Thus, great strides were not made until after the lapse of more than a century following mere recognition of the occurrence of microbes. With a profound interest in the phenomenon of fermentation, Pasteur as a chemist demonstrated through experiments that various forms of fermentation are brought about by actions of specific microorganisms. This dazzling scientific achievement exerted a great influence upon the subsequent development of pathogenic bacteriology. Three years following the discovery of the leprosy bacillus by Hansen, Koch succeeded in cultivating the anthrax bacillus in 1876. In 1881 he devised a solid medium which permitted the formation of colonies as pure bacterial cultures, and in 1882 he identified the tubercle bacillus as the etiologic agent of tuberculosis.

Koch was a student of the pathologist Jacob Henle (1809-1885), who drew up a statement in 1840 of the conditions required to provide acceptable proof of a casual relationship between an infectious agent and a given disease. These conditions undoubtedly represented his own concepts though they cannot be found in his writings. They were succinctly formulated by Koch and are widely known as "Koch's postulates." These postulates came to constitute the recognized basis for establishing an agent as having specific etiologic significance. Their promulgation contributed greatly to the development of pathogenic bacteriology. Bacteriology marked rapid progress on the basis of methods of laboratory studies found by Koch, and there followed in rapid succession the identification of the typhoid bacillus in 1883, the diphtheria bacillus and cholera vibrio in 1884, the tetanus bacillus in 1886, and in increasing tempo, many others.

The time of Hansen's discovery of the leprosy bacillus, about 1873, was the pre-Pasteur and pre-Koch era when the concept of pathogenic bacteriology as yet was scarcely established. There was no proof that leprosy was caused by infection with a living germ, nor was the microscope, as then available, completely adequate.

Five years following Hansen's report on the observations of microscopic bacillary objects in leprous tissue, Albert Neisser, a 24 year old microbiologist, visited Norway in 1879 where he made clinical observations of numerous patients at a leprosy hospital and received various leprous tissues from Hansen. He returned to Breslau where, with the aid of Koch, Weigert and Ehrlich, he made detailed microscopic examinations of the specimens with remarkable skill in staining technics. As a result, Neisser published "Uber die Ätiologie des Aussatzes" in 1879 in which he stated: "There were revealed everywhere bacilli in large numbers, in all 14 pieces of skin and nodules." Similar bacilli were demonstrable, according to him, in the liver, spleen, lymph nodes, cornea and most abundantly in the testis (²⁶).

In- 1879 Hansen further published a drawing of leprosy bacilli as he observed them under the microscope. Neisser continued bacteriologic examination of additional leprous material which he had collected in 1881 from Spain, Dutch Guyana, Brazil, Rumania, the East Indies, Palestine and other areas. In all these tissues he demonstrated the same bacilli as those seen in the earlier studies, thus confirming that the bacilli were the etiologic agent of the disease.

Subsequently, at the First International Leprosy Congress held in Berlin in 1897, it was generally admitted that Hansen was the discoverer of the leprosy bacillus and that this bacillus was the organism causative of leprosy.

Meanwhile, Weigert in 1875, was the first to devise staining of microorganisms with solutions of dyes, and discoveries of new dyes and mordants as well as advances in the technics of staining followed. In 1882 Ehrlich discovered the peculiar straining reactions of tubercle bacilli, which are difficult to stain but when once stained with gentian violet and saturated anilin solution in water they resist decolorization by mineral acids; hence, the name acid-fast staining. This peculiarity of Mycobacteriaceae became the principal method of differentiating them from other microorganisms. This acid-fast staining technic was subsequently improved and came to be generally designated as the Ziehl-Neelsen stain. In 1879 Hansen called the leprosy organism Bacillus leprae, but in 1896 the term Mycobacterium leprae was proposed by Lehmann and Neumann, and this term is generally used at present.

Bacterial cytology of *M. leprae*. In the hundred years since the discovery of the leprosy bacillus great progress has been made in increasing the resolving power and magnifying capacity of the light microscope, and the advent of the electron microscope, which made a rapid progress after the introduction by B. Borries and E. Ruska in 1938 of a transmission electron microscope, heightened these capacities. Additionally, a variety of excellent staining technics have been devised as the result of the increase in and refinement of useful dyes as well as advances in the theoretical aspects of staining. There resulted a rapid progress in the morphologic observation and description of bacteria and the development of knowledge of bacterial cytology.

As for the morphology of *M. leprae*, it has been recognized since the beginning of this century that *M. leprae* varies in morphology with the phases of leprosy. Numerous leprosy bacilli have been noted to undergo various transformations during lepra reactions. Thus, diphtheroid, beaded, spore-like and granular forms, as well as poorly acid-fast organisms have been found to appear.

Promin (glucosulfone sodium) was first tried by Faget et al in 1943, in the treatment of leprosy (7), with results so gratifying as to be called a miracle at Carville. This orginated the antileprotic chemotherapy of the present day. In association with this development, methods for examination of the quantitative and qualitative changes in leprosy bacilli in smears from the skin and nasal mucosa came to assume considerable clinical importance in the evaluation of therapeutic effects. The Technical Panel on Bacteriology and Immunology of the Eighth International Congress of Leprology in 1963, provided advice for standardization of these technics calling for: 1) uniform methods for obtaining samples and preparing smears; 2) a logarithmic or arithmetical scale for expressing the numbers of concentration of bacilli seen; and 3) classification and interpretation of the following morphologic features in M. leprae: solid form, which are thought to be viable; fragmented or disorganized forms, which are thought to be damaged or dead; coccoid or granular forms, which may or may not be viable; acid-fast debris, which likely indicates rapid destruction of bacilli within the recent past.

As a result of comparative observation of

the appearance of dead and degenerated leprosy bacilli by light and electron microscopy, evidence was obtained that the nonsolid form demonstrable with the Ziehl-Neelsen stain was suggestive of degenerate and dead organisms. Accordingly, a hypothesis developed that the solidly and uniformly Ziehl-Neelsen stained *M. leprae* were viable whereas those which were nonsolid represented nonviable cells (30).

Meanwhile, experimental studies on infectivity as related to the average doubling time of solid bacilli of *M. leprae* was pursued by the technic of inoculation into foot pads of mice. A close relationship was shown to exist between the number of solid staining bacilli and the infectiousness of these bacilli for the mouse foot pad (³⁴). However, there exists a widespread opposition to the concept that all nonsolid *M. leprae* cells are dead, because in practice the judgment of nonsolidness of the bacillus is not always made.

On the occasion of the WHO meeting of investigators from research centers working on M. leprae in 1968, the technics for the preparation of smears, fixation and staining were standardized. Ridley's Bacteriological Index (BI) was recommended as standard for quantitization. In addition, it was recommended that the Morphologic Index (MI) should not be included in routine bacteriological examination because the results obtained were too variable to be of value. Furthermore, the Expert Committee on Leprosy of the World Health Organization in 1970, reported on the Morphologic Index (solid ratio) as follows: "It is the most convenient laboratory method available for following the therapeutic response of patients in short-term clinical trials." Although considerable evidence has been provided that the Morphological Index reflects the viability of M. leprae, "because of its limits of sensitivity, it is not a suitable procedure for distinguishing the infectious from the noninfectious patient, even when performed under optimal conditions by highly experienced investigators."

Since 1948, when Bishop *et al* $(^{3})$ first applied electron microscopy to the study of the morphology of *M. leprae*, this technic has been utilized widely in leprosy research.

Studies have been performed on the ultrastructure of the M. leprae cell (4), on ultrastructure of various types of leprosy lesions (37), cytoplasmic reactions to the leprosy bacillus and intracytoplasmic enzymes of the host cell (15, 16). Nevertheless, there is still inadequate experimental evidence to clarify the relationship between the phagocytic membrane and the hostmycobacterial interaction. Recently, some clarification has been made as to the interrelationship of lysosomal substance and intracellular bacilli by cytochemical electron microscopic examination of the lepra cell. It seems highly probable that the relationship between the structure and the function or physiologic states of M. leprae will be clarified through cytochemical and enzymatic studies combined with electron microscopy.

Various investigations have pursued studies on the chemical properties of cellular components of M. leprae since the studies made by Unna and his son in 1910. Nevertheless, present knowledge in this area is still quite sparse. Anderson et al conducted a series of subtle chemical analyses on the constitutents of the tubercle bacilli and other mycobacteria and thereby demonstrated the presence of lipoid (phospholipid, lipid, wax, bound lipoid), protein, carbohydrate and pigments (phthiocol, cartinoid) as the components of mycobacterial cells and designated neutral wax isolated from M. leprae under the name of "leprosin" (2). However, it is not generally accepted that the cultures analyzed were actually those of M. leprae.

While it is evident from a number of studies that M. *leprae* shares common serologically active antigens with M. *tuberculosis* and other mycobacteria, it is almost impracticable to conduct antigenic analyses of M. *leprae* unless sufficiently large amounts of pure M. *leprae* cells are available. Nevertheless, it was revealed recently by immunoserological studies, immunodiffusion and immunofluorescence tests that M. *leprae* contains a protein antigen showing an entirely different specificity from other species of mycobacteria (¹). A recent electron microscopic study (¹⁰) revealed that M. *leprae* possesses, like all other mycobacteria, a superficial network of filaments fundamentally identical with adjuvant active peptido-glycolipid the filaments. The immunological phenomena common to leprosy and tuberculosis are largely dependent upon the biological activity of the mycobacterial peptido-glycolipid and its ability to resist the action of host enzyme and is thought to be of importance for its functions as an immunological adjuvant (10). Moreover, in recent chemical analyses and electron microscopic studies, the cell wall of M. lepraemurium was found to bear ultrastructural and chemical resemblance to those of other mycobacteria and to contain such components as mucopeptide, arabino-galactan and ester-linked lipid (6, 17).

Advances in animal transmission of *M*. *leprae*. Insomuch as this subject will be dealt with in detail by another contributor, it will be alluded to only briefly. Despite various attempts at *M*. *leprae* inoculation into laboratory animals and sometimes into humans by a number of investigators, including Hansen and Danielssen, no single case of success has been certainly recorded.

In 1960 Shepard inoculated leprosy bacilli isolated from nasal washings and lesions of lepromatous patients into the foot pads of CFW mice which were maintained at a room temperature of 20° C. The bacilli were noted to show local multiplication when the inoculum was diluted appropriately. Thus, when 103 to 104 bacilli were injected, five to ten months were required to yield a harvest of 10⁵ to 10⁷ bacilli (³³). Subsequently, the mouse foot pad infection was adopted in laboratories in many countries and, at present, is employed as a reliable screening procedure for drugs for leprosy. Although the mouse foot pad inoculation technic has failed to produce experimental leprosy in mice, this distinguished contribution opened the way anew for experimental animal transmission of the leprosy bacillus which had long been looked forward to by leprologists.

Ensuing foot pad studies have disclosed similar results in other rodent species including rats, hamsters and mystromys, though varying among species to some extent. In normal mice the logarithmic phase of multiplication of *M. leprae* is maintained for six to eight months, followed by a plateau and then a regression phase. The generation time is 12 to 13 days in the logarithmic phase of multiplication. More recently it has been shown that this limited phase of logarithmic multiplication can be extended by reducing the immunologic capacity of the mice by thymectomy followed by total body irradiation (900 r) before infection, necessitating a small, life-saving injection of syngeneic bone marrow cells (29).

Advances in cultivation of M. leprae. In 1882, after a lapse of several years following his discovery of the leprosy bacillus, Hansen tried to cultivate the leprosy bacillus. Studies representing an attempt to cultivate the bacillus have been continued by an extremely great number of investigators in the past one hundred years. These elaborate attempts encompassed trials with practically all conceivable methods, such as varying conditions of cultivation (temperature, aerobiasis, anaerobiasis, O2 and CO2 gas environment, etc.), investigation of composition of culture media including growth promoting substances, special cultivation methods such as symbiotic culture with other bacteria, cultivation by the use of special double tube apparatus and diffusion chambers, cultivation in embryonated chicken eggs, tissue culture and many other technics. Nevertheless, the organism has never been accepted generally as having been cultured with certainty.

Mycobacterium lepraemurium, the agent causative of leprosy in rats and mice with lesions in the skin and lymph nodes that resemble those of human leprosy, was discovered in 1903 by Stefansky. Since this organism also is noncultivatable, *M. lep*raemurium has been extensively employed as a model in studies of cultivation of *M. leprae.*

The strains of acid-fast bacilli which have been isolated in attempts to obtain microbial growth from leprous materials are innumerable. All constitute a history full of value despite the failures associated with them. The following is only a brief description of such attempts in accord with the classification of these cultivated strains by Rogers and Muir (1925) (³¹).

Strains belonging to diptheroids or Streptothrix. Representative of this group is the bacillus isolated by Kedrowsky (1901) with human placental extract-agar, horse serum agar and so forth. Many of the strains in the diptheroid group are similar in many respects. They are labile with respect to their acid-fast properties, pleomorphic, and grow readily on the ordinary culture media. Their acid-fastness varies with the culture medium; it diminishes in fate-free media and is enhanced in fat-containing media.

Chromogenic acid-fast bacilli. These have been isolated with the greatest frequency and most organisms grown and assumed to be *M. leprae* frequently fall in this category. Their biologic characteristics seem to be entirely comparable to those of the so-called spontaneously occurring nonpathogenic mycobacteria. They are easily grown, their acid-fastness varies from strain to strain, and they are mostly pleomorphic.

Nonchromogenic dcid-fast bacilli. Organisms of this group are generally difficult to subculture. Those isolated by Emile-Weil, Karlinski, Marchoux, Twort, Duval and Wellmann, etc., belong to this group. The organism isolated by Twort from nasal mucus grew only in a Dorset's egg medium containing 1% tubercle bacilli and could not be subcultured. However, the "Dalva" strain which Souza-Araujo (1952) isolated in Löwenstein's medium from nasal mucus was subcultivable.

Anaerobic bacilli. Miscellaneous. Among the numerous cultivation attempts that have been made in the past, there have been several experimental findings reported to be convincing as probable true growth of *M. leprae* though only to a microscopically demonstrable extent. Nevertheless, evident formation of colonies which might be successively cultivable has never been accomplished.

With regard to relation of these isolated strains and *M. leprae*, it was reported by the Technical Committee on Bacteriology, the Seventh International Leprosy Congress, 1958, that the various mycobacteria isolated theretofore from lepromatous tissues were all incidental or passenger strains and none could be regarded as the causative agent of leprosy. Furthermore, according to a presentation made by Kirchheimer and Prabhakaran at the Ninth International Leprosy Congress, 1968, a series of 15 strains listed under Mycobacterial "Species" in the "Catalogue of Cultures," 7th edition, 1964, of the American Type Culture Collection (ATCC), as believed at one time to be leprosy bacillus, and some mycobacteria listed in the catalogue as isolated originally from ticks experimentally infected with lepromatous material, were examined for dopa-oxidation activity characteristic of M. leprae and by mouse foot pad tests. The results showed that all the 15 isolates examined had little resemblance to M. leprae (18).

Studies on the cultivation of M. leprae were carried forth actively during the period of about 50 years after Hansen's discovery of the leprosy bacillus, yielding a number of reports on isolates from leprous materials. The study along this line, however, remained temporarily in a state of extreme depression under the influence of World War II. On the occasion of the Seventh International Leprosy Congress, the Committee on Bacteriology and Pathology presented the following two objectives as the basic approach to the cultivation of M. leprae to be achieved in the future: first, to conduct biochemical studies on deficits and impediments of the enzyme system of noncultivated mycobacteria; and, second, to look for tissue cell systems or other biologic systems which may permit substitution for the natural hosts.

Meanwhile, bacteriologic studies of the biologic characteristics of microorganisms, especially biochemical physiology, were actively pursued after World War II with consequent rapid progress in the understanding of the basic physiologic aspects of bacteria such as structure, mechanism of respiration, nutrition and genetics. Thanks to the advances in general microbiology, a rational way of approach to the microbiology of *M. leprae*, especially to its cultivation, has emerged on the basis of the newly gained physiologic knowledge and new concepts. At the symposium on research in leprosy held at Baltimore in 1961, at the Eighth International Leprosy Congress, and at the LWM-AFIP Conference on research problems in leprosy, held at Washington in 1965, the necessity of assessment of the problem of cultivation of *M. leprae* and *M. lepraemurium* from a new point of view on the basis of new microbiologic knowledge was generally admitted to be imperative.

Tissue culture. Tissue culture of small pieces of lepromatous nodule was first tried about 20 years following the introduction of the tissue culture method by Harrison (1907) and Carrel (1910). With the subsequent immense progress in tissue culture technics, numerous human and mammalian cell strains were isolated and maintained and, by utilizing them, active investigations were carried out on cultivation of M. leprae and M. lepraemurium. Garbutt et al (⁹) reported multiplication of rat leprosy bacilli in cultures of rat fibroblasts. Chang (5) achieved successful cultivation of M. lepraemurium for 20 to 110 days within mouse peritoneal macrophages maintained in vitro, thereby demonstrating a definite intracellular multiplication of the bacilli associated with elongation.

Outweighing any other consideration in the cultivation of *M. leprae* in cell cultures is the search for a host cell system suitable for parasitization by this organism. Several types of cultures have been employed. Among these have been: 1) explant cultures made from human tissues; 2) cell lines of animal origin such as L cells, rat fibroblasts and monkey kidney cells; 3) cell lines of human origin such as KB, HeLa, amnion, embryonic lung, etc.; 4) cell strains derived from human tissues, chiefly normal skin fibroblasts, fibroblasts from lepromatous skin and fetal spinal ganglion fibroblasts; and 5) mouse peritoneal macrophages.

Although extensive work has been done along these lines, very little success has been achieved. Bacillary elongation associated with a limited degree of multiplication was observed in some culture systems, as for example, in Schwannoma cells, fibroblasts derived from lepromatous skin and in mouse macrophages.

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Biochemical studies. Biochemists having special interests in bacteriology have become more and more involved in the problem of bacterial growth and, as a result, there have been significant contributions both theoretical and practical. It has become clear that the particular culture media which bacteriologists use for cultivation of pathogenic bacteria which are otherwise difficult to cultivate require supplements of substances which the parasites under study are incapable of synthesizing for their own growth requirements. A substantially marked advance has thus been made in the knowledge of microbiology in the last 40 years. With this newly acquired knowledge and advanced technics, the study of cultivation of *M. leprae* has undergone a drastic change, both with respect to theoretical and practical aspects.

In the study of the cultivation of M. leprae in cell-free media, it has been an object of prime importance in the past 20 years to investigate the biochemical system involved in the supply of energy required by the leprosy bacillus for growth in vitro. Investigations of culture media, cofactor and growth factor supplements, and environmental conditions for cultivation of the leprosy bacillus have been increasingly initiated on the basis of biochemical understanding of the metabolic capabilities and deficiencies of the organism. The pressing practical necessity arose for an ample supply of fresh bacilli at any given time, and definite amounts of pure bacilli uncontaminated with tissue or tissue debris were needed to facilitate bacteriologic and biochemical studies of M. leprae. These, not being generally available, studies with M. lepraemurium as the model system for M. leprae came to be performed in many laboratories.

Experimentation on oxygen consumption by M. lepraemurium by the manometric method of Warburg with the various substances that have been used for the cultivation of mycobacteria revealed that the organism is destitute of the capacity to utilize any of the substances usually employed as a source of energy (¹¹). Subsequently, evidence has been obtained of a correlation between mycobacterial oxidative metabolism and the ease with which they can be cultivated *in vitro* (1^2) .

In recent years isotope tracer technics became available for the study of bacterial metabolism and it became practicable to detect and measure the extremely minute amounts of radioactive substrates oxidized by or incorporated into bacteria. These investigations by isotope tracer technics demonstrated M. lepraemurium capability in oxidizing and assimilating exogenous substrates, and that the rate for the utilization of the exogenous substrates is remarkably low, being lower even than that shown by noncultivated Rickettsiae and Chlamydiae (35). Recently, studies of in vitro fatty acid uptake by M. lepraemurium have been conducted by the use of 14Clabeled fatty acids. Incorporations of radioactivity from 14C-decanoate into M. lepraemurium were observed, and these incorporations were demonstrated to be due to a biological function of this organism (19).

Energy yielding pathways of mycobacteria depend mainly on respiratory systems because of their absence of anaerobic glycolysis. The noncultivable state of leprosy bacilli seems to depend on minimal capacity for respiration. It has been observed that M. lepraemurium separated from infected tissues does not exhibit detectable cytochrome adsorption bands which are readily ascertainable in culturable mycobacteria. This seems to indicate that leprosy bacilli have genetically lost the adaptive capacity of a respiratory system. If a clearer understanding of the defect in respiratory systems of mycobacteria can be obtained it may be possible to find some approach to the puzzling problem of cultivating leprosy bacilli.

It has been found that as the difficulty in cultivation *in vitro* increases from saprophytic to attenuated and finally to parasitic strains of mycobacteria, their respiratory activities diminish sharply. Both *M. lepraemurium* cells obtained from leprous nodules of infected mice and *in vivo* grown BCG cells exhibit extremely faint activity or are even devoid of enzymes of the cytochrome series. Of particular note is the finding that *in vitro* cultivated BCG cells become cytochrome deficient, like M. lepraemurium, when grown in vivo (²⁰). Clarification of the mechanism whereby BCG cells lose the ability to synthesize cytochromes may throw light on the basic biochemical features of leprosy bacilli.

M. lepraemurium has been shown to be incapable of initiating the TCA cycle; hence, an incomplete TCA cycle enzyme system exists in this bacillus $(^{23})$. Experimental evidence has also been secured for the pattern of TCA cycle impediment seen with strongly respiratory-impaired mutants of *M. smegmatis* which resemble that of the defect in TCA cycle observed with *M. lepraemurium* $(^{21})$.

It is reasonable to think that an understanding of the changes in the carbon, nitrogen and oxidative metabolism that must occur when mycobacteria undergo successful transference would provide a logical basis for analyzing the particular steps which host-dependent organisms are unable to accomplish (²⁴).

Cultivation trials in cell-free media. Extensive studies on the cultivation of *M*. *leprae* in cell-free media have been undertaken in many laboratories throughout the world. Successful growth has been claimed by several groups of investigators during the last decade. However, none of these are as yet accepted as successful growth. Most of the isolated organisms have been identified as strains of saprophytic mycobacteria or Runyon group III of atypical mycobacteria and, with some organisms, no sufficient identification tests for *M. leprae* have been performed.

With respect to cultivation trials with *M*. *lepraemurium*, on the other hand, there are a few recent reports of considerable interest.

Formation of slow growing, dull yellow, rough colonies was evident following a heavy inoculation of M. lepraemurium onto egg yolk medium and 16 serial subcultivations have been successful. The rate of positive serial subcultivation has been 46/469 with the Hawaiian strain and 25/63with the Keishicho strain, respectively. These bacilli were demonstrated to produce characteristic murine leprosy in mice (27). A growth of *M. lepraemurium* could be seen in the cell-free medium, NC-5 medium, containing *a*-ketoglutaric acid, cytochrome C, hemin, 1-cysteine, and goat serum in Kirchner medium. The bacilli gradually elongated and began to multiply approximately ten days after incubation. Thereafter, the number of bacilli increased and reached about 50 times the inoculum size in a period of 60 days. These bacilli maintained their pathogenicity for mice and produced characteristic murine leprosy (2^5) .

Although an attempt at cultivation of *M*. *leprae* was made under the same condition as employed in *M*. *lepraemurim*, the results obtained showed no morphological changes in the bacilli and no demonstration of multiplication by *M*. *leprae*.

Advances in identification of *M. leprae*. The WHO Expert Committee on Leprosy, 1970, discussed the identification of *M. leprae* and stated: "In all cultivation work it is important to prove the viability of the purported growth and to identify it by the methods now available (the inoculation of mouse foot pads, lepromin tests, enzymatic studies of DOPA oxidation, and serological identification of leprosy nodular extract antigen)."

These points may be elaborated to indicate in greater detail present data available for the identification of M. leprae. Characteristic lesions, particularly involvement of peripheral nerves, should be found on inoculation into normal or thymectomizedirradiated mice. The skin reactivity elicited in lepromatous and tuberculoid patients by antigen prepared from the culture in question, should conform with their reaction to lepromin. Assay of the bacteria for DOPA oxidase activity is specific to M. leprae. It has been recently demonstrated that M. leprae, unlike other species of mycobacteria, can oxidize 3,4-dihydroxyphenylalanine (DOPA) and that the DOPA oxidase of *M. leprae* differs from that present in mammalian tissues (28). Additionally, it has recently been shown by immunodifussion and immunofluorescence studies that M. leprae contains a protein antigen showing an entirely different specificity from other species of mycobacteria. It was

found that leprosy nodule extact (NE) contained at least two antigens which are easily differentiated from human serum proteins by immunodiffusion. One of these antigens is a heat stable polysaccharide, and the other is a heat labile protein which gave a single precipitation line with anti-NE serum absorbed with human serum. Insomuch as this antigen is highly specific for *M. leprae*, serological identification of *M. leprae* became practicable through the use of antiserum prepared against the antigen (1).

Lepromin reaction. The Mitsuda reaction described by Mitsuda in 1919 (22), and Hayashi in 1933 (14), was interpreted as expressing the resistance of individual hosts to the leprosy bacillus, by the Immunology Committee of the Sixth International Leprosy Congress in 1953. Standards for the preparation of lepromin and criteria for evaluation of the result of the test were stipulated. Investigations were carried out over the ensuing years to examine the relationship between Mitsuda reactivity and the resistance to leprosy and, under the auspices of WHO, standardization of bacillary concentrations in lepromin was investigated. An antigen containing 160 million bacilli per milliliter was initially recommended as the standard by the Eighth International Congress of Leprology and the Third WHO Expert Committee on Leprosy.

Subsequent studies of the preservation of lepromin showed that the bacterial count of lepromin preserved in a refrigerator is reduced to about 74% of the original bacterial count after three years and about 70% after five years, while the lepromins preserved by lyophilization showed no decrease in their bacterial count. Lepromin can be preserved for three years in a refrigerator without appreciable loss of potency. Lyophilization of lepromin should be recommended for a prolonged preservation. Studies in recent years have been pursued with diluted lepromin in attempts to determine more specifically what bacterial concentration of lepromin is required for maximum potency and efficacy of the lepromin test.

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