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Cytochemical Reactions of Human Leprosy Bacilli and Mycobacteria: Ultrastructural Implications

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Leprosy bacilli harvested from freshly biopsied tissue from cases of lepromatous, borderline and histoid leprosy were, in conjunction with *Mycobacterium lepraemurium* and representative mycobacteria, examined cytochemically with and without their pyridine-extractable acid-fastness. Unlike the mycobacteria, unextracted leprosy bacilli failed to give a positive response to the periodic acid Schiff test or to take up Sudan black B, toluidine blue O, alkaline methylene blue or safranin O. Once their acid-fastness was removed with pyridine, leprosy bacilli were stained by all of the foregoing dyes except Sudan black B, under this condition they remained gram positive. While permanent loss of acid-fastness from leprosy bacilli always resulted in a loss of acid hematein-fixing material (Smith-Dietrich-Baker tests), the reverse was not true. Mild aqueous saponification, bromination, or sequential treatment with lipase and phospholipase D resulted in a loss of acid hematein-positivity but not acid-fastness. After pyridine extraction, bromination, or aqueous saponification, true mycobacteria lost neither their acid hematein-positivity nor their acid-fastness. The acid hematein-positive material and the acid-fastness of both leprosy bacilli and mycobacteria were lost after treatment with alkaline ethanol. These cytochemical findings are discussed in the light of what is known of the ultrastructure of leprosy bacilli and mycobacteria, and of the occurrence of a DL-3,4-dihydroxyphenylalanine oxidase in leprosy bacilli but not in mycobacteria. An effort is made to explain the rather unique cytochemical properties of leprosy bacilli. Since pyridine-extractable acid-fastness (and acid hematein-positivity) serve to distinguish human leprosy bacilli from *M. lepraemurium*, one or the other, or both, are suggested as bases for differentiating these two organisms in animal experiments designed to show the in vivo propagation of human leprosy bacilli.

In the last century, since G. A. Hansen established the presence of rodlike bodies in lesions of human leprosy (32), two positive attributes have been reported for the characterization of leprosy bacilli: (i) acid-fastness (74) and (ii) the presence of a phenolase which converts DL-3,4-dihydroxyphenylalanine to a product having an absorption peak at 540 nm (58).

The phenolase activity was first described, and its specificity was defined with bacilli harvested from lepromatous tissue (58, 59). Comparable activity has been described in propionibacteria cultivated from cases of lepromatous and tuberculoid leprosy and in anaerobic corynebacteria (7). Activity has never been demonstrated in mycobacteria, nocardias, or aerobic corynebacteria (7, 59).

Since the pioneer work of Thin (74) and Neisser (50, 51) with leprosy bacilli, and Ehrlich (21) and Ziehl (79, 80) with tubercle bacilli, acid-fastness has been considered an invariant character of these organisms and is inferred to be a constitutive property of mycobacteria (78). The first methods for demonstration of acid-fastness relied upon decolorization with aqueous solutions of mineral acids. It was not until the work of Neelsen (49) that acidic ethanol was used. While neither the nature of the acid-fastness of leprosy bacilli nor the mycobacterial properties of these bacilli have often been called into question, the diversity of methods described for staining them and the varied criteria used in enumerating

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them indicate that leprosy bacilli possess peculiarities of staining not encountered in ordinary mycobacteria (46, 47, 61, 63).

The first evidence that the staining properties of leprosy bacilli differ from mycobacteria, in this case *Mycobacteria tuberculosis*, was noted by Robert Koch in 1882 (40) and was not mentioned again until now. Some 64 years later, Kenneth Burdon, using Sudan black B stains of dried smears, showed a marked difference in staining between bacilli from bacteriological positive leprous lesions, mycobacteria in caseous material, and cultured mycobacteria. The mycobacteria consistently showed Sudan black B-stained intracellular material. In all leprous preparations examined, Burdon found "no intracellular stainable fatty material" in any acid-fast bacillus (12). This disparity was apparently not an artifact arising from use of lesion-derived material since he did demonstrate intracellular fatty deposits in tubercle bacilli obtained directly from tuberculous lesions. His observations were independently confirmed by Rosemarie Bermann (9) and were confirmed further and extended by Chaussinand (16).

Twenty-two years after the report by Burdon, Campo-Aasen and Convit made the important observation that human leprosy bacilli differed from *M. lepraemurium* as to reactivity in the acid hematein test of Baker (5). Although both organisms were stained blue by the technique of Baker, only the acid hematein-positive material of human leprosy bacilli was extractable with pyridine (13). This implies that the stained material in human leprosy bacilli is phospholipid while that in *M. lepraemurium* is not. Campo-Aasen and Convit gave no information on the reactions of cultivable mycobacteria in the Baker acid hematein test since their concern was with a means of distinguishing noncultivable mycobacteria from one another (i.e., leprosy bacilli from *M. lepraemurium*).

A follow up, in this laboratory, of the finding of Campo-Aasen and Convit lead to the discovery that the acid-fastness of leprosy bacilli was extractable with pyridine, whereas that of mycobacteria such as *M. tuberculosis* strain $H_{37}Rv$ was not (22). This work has been confirmed by Chang and Andersen (personal communication). The fact that the acid-fastness of leprosy bacilli involves a material exhibiting chemical properties different from those of the cellular components responsible for the acid-fastness of mycobacteria raises the question of whether human leprosy bacilli are indeed mycobacteria. The contradiction between experimental observations and the official designation of human leprosy bacilli as members of the genus *Mycobacterium* requires further examination. Any full-scale investigation is, unfortunately, hampered by the lack of a pure culture isolate which can unequivocally be considered as etiological agent of human leprosy. One is left, therefore, with the need to extract as much information as possible from the study of leprosy bacilli freshly harvested from patients. In this report we extend our examination of pyridine-extractable acid-fastness and some of the cytochemical observations of previous investigators in an attempt to more clearly define the staining properties of human leprosy bacilli. It should be clear from the results contained herein that tests for pyridine-extractable acid-fastness and acid hematein-positivity should be of great value in animal experimentation where the hazard of mistaking *M. lepraemurium* for injected human leprosy bacilli can be a matter for serious concern (15, 52, 53, 55).

MATERIALS AND METHODS

Tissues. Fresh tissues biopsied from lepromatous, borderline and histoid cases of leprosy were provided through the generous cooperation of Leandro V. Uyguanco (Director, Bureau of Disease Control, Manilla, Philippines) by arrangement with Chapman H. Binford, Special Mycobacterial Diseases Branch, Armed Forces Institute of Pathology, Washington, D.C., and Jacinto Convit (Director, Instituto Nacional de Dermatologia, Caracas, Venezuela).

M. lepraemurium was graciously provided by T. Imaeda, Department of Microbiology, New Jersey State College of Medicine and Dentistry, Newark.

Bacteria. Cultivable bacteria used in this study were maintained on butter agar (24) or, in the case *M. tuberculosis* $H_{37}Rv$, on Lowenstein-Jensen slants (BBL). The strains employed and their sources include bacterium 22M (38), *Corynebacterium diphtheriae* C7 (6), *Corynebacterium* sp. L.B. (24), *Escherichia coli* B (3), *M. Fortuitum* ATCC 23052, *M. intracellulare* ATCC 13950, *M. phlei* ATCC 11758, and *Mycobacterium* sp. ATCC 607 *Mycobacterium* sp. 1081 and 1285 were kindly supplied by J. Convit and M. E. de Pinardi. *Mycobacterium* sp. 1217 was kindly supplied by G. Lanéelle, and *Mycobacterium* sp. NQ was supplied by J. Wiersma (11, 77). *Mycobacterium* sp. ICRC was supplied by C. V. Bapat through arrangement with Kamal J. Ranadive (60); *M. tuberculosis* strain $H_{37}Rv$ was obtained from the Trudeau Society (no. 102) and *Propionibacterium* sp. LT (24).

Smears were prepared from tissue homogenates as previously described (22) or directly from lipoidal material dissected from fresh tissue with the aid of a stereoscopic dissecting microscope (Bausch and Lomb). Smears were fixed by heat, Baker calcium Formalin, or Bouin fluid as demanded by each staining protocol. To preserve labile material,

stained smears were routinely mounted in glycerine jelly (56).

The following conditions were used for enzymatic hydrolyses: trypsin (Fisher Scientific Co.), 1.0 mg/ml in 0.05 M potassium phosphate buffer (pH 7.5) at 37 C (41); twice crystallized pepsin (Schwarz/Mann), 1.0 mg/ml in glycine hydrochloride buffer (pH 2.0) at 37 C (34); Pronase (*Streptomyces griseus* protease; Calbiochem), 500 μg/ml in 0.05 M potassium phosphate buffer (pH 7.5) at 37 C (27); crude hog pancreas lipase (Sigma Chemical Co.), 1.0 mg/ml in NH_4Cl-NH_4OH buffer (pH 9.0), containing 0.05% $CaCl_2$, at 30 C (10); phospholipase D (Sigma Chemical Co.), 1.0 mg/ml in citric acid-potassium phosphate buffer (pH 5.6) at 30 C (33); ribonuclease (Worthington Biochemical Corp.), 20 μg/ml in 0.05 M potassium phosphate buffer (pH 7.5) at 60 C (43); deoxyribonuclease, crystallized once (Worthington Biochemical Corp.), 20 μg/ml in Earle balanced salt solution, brought to pH 6.5 with gaseous CO_2, at 37 C (44). All enzyme preparations were sterilized by filtration through a membrane filter (0.45 μm pore diameter; Millipore Corp.) prior to use.

RESULTS

Extraction of acid-fastness by pyridine. Pyridine extraction and the acid-fast reaction were carried out as previously described (22). Since the publication of our previous report (22), we have examined further 43 bacteriologically positive leprous smears for pyridine extractability of their bacterial acid-fastness. Of these, 41 became nonacid-fast following pyridine extraction. Of those two smears not fully susceptible to the effects of pyridine, one was derived from a patient from whom a provisionally acid-fast bacillus had been isolated. The second smear (from patient 108) proved to be exceptional in its staining properties and is discussed more fully in subsequent sections. All pyridine-extracted, acid-fast negative bacilli remained gram-positive.

The following summarizes our experience with pyridine extraction of human biopsy specimens. Material from a total of 54 leprosy patients has been examined, including 39 lepromatous cases, 13 borderline cases, and 2 cases described as histoid. The acid-fastness of the bacilli from 51 of these patients was lost after extraction with pyridine. Two patients, from one of whom a provisionally acid-fast bacillus has been isolated, yielded small but consistent numbers of acid-fast bacilli resistant to pyridine extraction; however, the majority of acid-fast bacilli in smears from these two patients were susceptible to the extraction. Bacilli from one patient, 108, were found to have an acid-fastness completely resistant to extraction by pyridine.

As reported previously (22), *M. tuberculosis* $H_{37}Rv$, *M. intracellulare* ATCC 13950, and acid-fast bacilli from tuberculous sputum remain acid-fast after pyridine extraction. This observation has now been extended to include *M. lepraemurium*, *M. phlei* ATCC 11758, *M. fortuitum* ATCC 23052, and *Mycobacterium* sp. 607, 1081, 1285, NQ, and ICRC. We have maintained $H_{37}Rv$ in suspension in pyridine for 48 h at 37 C without loss of acid-fastness, and Chang and Andersen (personal communication) have reported that *M. lepraemurium* did not lose its acid-fastness after 24 h in pyridine at 60 C.

Thus, a clear distinction has been shown to exist between leprosy bacilli and true mycobacteria with respect to the susceptibility of their acid-fastness to extraction by pyridine. Leprosy bacilli consistently become nonacid-fast within 2 h at room temperature, whereas mycobacteria remain acid-fast even after prolonged extraction at elevated temperatures. This differential effect of pyridine upon the acid-fastness of leprosy bacilli and true mycobacteria led us to search for other methods by which differences or similarities in acid-fastness might be demonstrated (Table 1). Two of these methods, extraction with alkaline ethanol and ether extraction of chloroform homogenates, are described more fully below since these or similar procedures of delipidation have been implicated in the loss of acid-fastness from mycobacteria (47) and leprosy bacilli (73).

Treatment with alkaline ethanol. Cummins and Harris (17) described a method for delipidation of mycobacterial cell walls using 0.5% KOH in absolute ethanol. This method resulted in marked alteration of the ultrastructural features of bacterial cell walls without diminishing the cell wall content of diaminopimelic acid or other constituents of the mucopeptide (8, 17, 69, 70). We applied alkaline ethanol to leprosy bacilli or to intact mycobacteria, either by immersion of heat-fixed smears or by suspension of the bacilli in the alkali. When incubated at 37 C, the acid-fastness of most bacilli began to diminish in intensity within a few hours, and both leprosy bacilli and mycobacteria were nonacid-fast by 24 h. Such acid-fast negative bacilli, even after 48 h in alkaline ethanol, were gram positive.

Extraction with chloroform and ether. The procedure of Dharmendra for the preparation of delipidated lepromin involves homogenization of autoclaved leprous tissue in chloroform followed by extraction with ether (18). It has been reported that leprosy bacilli after this preparative procedure lose most of their acid-fastness (73). We also have found that leprosy

TABLE 1. *Extractability or destruction of acid-fastness*

Treatment	Leprosy bacilli	Mycobacteria	Comments
Chloroform	Variable	No	Bacilli not extractable in dry state
Chloroform-ether	Yes	No	
Aqueous saponification	Yes	No	Requires 24 to 48 h in 3 N NaOH
Alcoholic saponification	Yes	Yes	
Sodium-lauryl sulfate	No	No	
Acid	No	No	
Xylene	No	No	
Acid-xylene	Variable	No	Must be less than pH 1.0 to extract
Various enzymes[a]	No	No	

[a] Trypsin, pepsin, lipase, phospholipase D, lysozyme, Pronase, ribonuclease, deoxyribonuclease (for conditions of enzymatic treatment, see Materials and Methods).

bacilli harvested from autoclaved tissue with chloroform, then washed with ether, become nonacid-fast. Such extracted bacilli retained their gram positivity. *M. tuberculosis* $H_{37}Rv$ grown on Lowenstein-Jensen medium and subjected to the same preparative steps remained acid-fast.

The following method for extraction of acid-fastness from leprosy bacilli using treatment with chloroform and ether has been modified from methods of Folch and Lees (23). Fresh biopsy tissue, stripped of epidermis, was minced in 10 volumes of chloroform-methanol (2:1). This mixture was homogenized in a Virtis "45" homogenizer, and the solid debris was discarded. Distilled water was added to 20% of the final volume. Following thorough mixing, the phases were separated by centrifugation, and the chloroform (bottom) phase was recovered and evaporated to dryness. The resulting residue was suspended in ether, and the bacilli recovered by centrifugation. This ether extraction was repeated once, and after the second centrifugation the residual ether was allowed to evaporate. The dried bacilli were suspended in 10^{-3} M potassium phosphate buffer (pH 6.8) containing 1:20,000 Merthiolate. Leprosy bacilli recovered in this manner, while no longer acid-fast, were still gram positive. These bacilli did not regain their acid-fastness after prolonged standing in the buffer. It should be noted here that Taylor (73) reported that acid-fastness was regained during storage of delipidated lepromin.

Periodate oxidation. It has been reported that oxidation with periodic acid enhances the fuchsinophilia of acid-fast bacteria including human leprosy bacilli (4, 54). We have found this to be true especially when the prolonged oxidation in 10% periodic acid employed by Nyka (54) was used. Whether the smears are prepared from aqueous homogenates or from the subcutaneous fat of leprous tissue, the number of acid-fast bacilli increases following periodate oxidation, and the number of bacilli showing a beaded appearance decreases.

When nonacid-fast leprosy bacilli, obtained by pyridine or by chloroform-ether extraction, were oxidized in 10% periodic acid for 24 h, they gained a fuchsinophilia insensitive to acid-alcohol extraction. In this connection, it should be noted that a number of nonacid-fast bacteria, including *Corynebacterium* sp. LB; *C. diphtheriae; Propionibacterium* sp. LT; *E. coli* B; and bacterium 22M, acquired the same acid-stable fuchsinophilia after 24 h in 10% periodic acid and this, of course, without prior extraction with pyridine. Such acid-fastness could be demonstrated after staining in carbol fuchsin, acidified basic fuchsin, or aqueous pararosaniline, but not with other triphenylmethane dyes even when used in carbolized staining media. This chemical conversion to acid-fastness is most probably attributable to the formation of Schiff bases between the basic dyes (primary amines) and aldehydes resulting from periodate oxidation of cell wall or plasma membrane components. However, because of the prolonged nature of the oxidation, it is doubtful that such reaction products could all be accounted for by oxidation of only polysaccharides (see discussion of periodic acid Schiff reaction by Pearse [56]).

Human leprosy bacilli, stained by this pyridine extraction-periodate oxidation-hot carbol fuchsin-acid alcohol rinse method, showed much greater cytological detail than by any other staining technique. The cells appeared as medium-to-long, curved rods of uniform width. Longer cells often showed more than one curva-

ture and some exhibited a central swelling. An occasional grouping of cells gave the appearance of rudimentary branching. A notable feature of these cells was the presence in them of numerous unstained inclusions which varied from none to five or six per cell; there was little uniformity in their size or shape. These inclusions did not stain with Sudan black B, toluidine blue O, alkaline methylene blue, or acidic methylene blue (pH 3.0).

Staining of lipid and lipoidal inclusions with Sudan black B. To demonstrate Sudan black B-stained lipids, air-dried unfixed smears were flooded with a 0.3% solution of the dye in 70% ethanol. After 15 min at room temperature the slides were washed with water, followed by dropwise rinsing with 70% ethanol, and then drained dry. Aqueous homogenates of biopsy specimens showed little Sudan black B-stained material, and the bacilli present could only be found with consistency by employing a counterstain containing basic fuchsin. The fuchsin-stained bacilli in such homogenates never contained Sudan black B-stained material, nor could any Sudan black B-stained bacilli be found in smears not treated with a counterstain.

Smears prepared from excised subcutaneous lipid exhibited homogeneous lakes of Sudan black B-stained lipid within which could be seen clear bacillary bodies. These lipid lakes were susceptible to digestion by crude pancreatic lipase and were readily washed away by a few drops of xylene. After a very brief xylene wash followed by exposure to Sudan black B, leprosy bacilli became surrounded by a capsule of black stain (lipid) but the bacillary body remained clear. Prior washing with a few drops (0.5 ml) of xylene resulted in loss of virtually all Sudan black B-stainable lipid. The matrices of globi did not take up Sudan black B nor did the bacilli within them.

Bacilli from patient 108, already shown to have pyridine-stable acid-fastness, were found also to be a unique exception with respect to staining by Sudan black B. After a brief xylene wash of smears from subcutaneous lipid, the bacilli of patient 108 failed to retain a lipid capsule but showed large numbers of inclusions stainable by Sudan black B. These Sudan black B-stainable bodies were unaffected by prior extraction with pyridine.

Staining for polar lipids. A principal histochemical test for polar lipids is the Baker acid hematein test which, when used subsequent to pyridine extraction, is presumed to distinguish phosphatides from other acid hematein-positive materials (56). This method evolved from the work of J. Lorraine Smith (68) through modifications by Dietrich (19) and Baker (5). It is based upon the binding of the dichromate ion by tissue or cellular components followed by reaction of the bound chromium with acidic hematoxylin. Of the materials which are acid hematein-positive (blue), only phospholipids have been considered to be extractable by pyridine.

To leprosy bacilli and several strains of mycobacteria, we applied three variations of the Smith-Dietrich-Baker reaction. The Smith-Dietrich lipoidal stain as described by Levinson and MacFate (42) was modified by increasing to 72 h (from 48 h) the length of time of mordanting in saturated potassium dichromate at 55 C. No saffranin counterstain was used. Two methods for the Baker acid hematein stain were used, the standard method as described by Pearse (56) and the modification described by Campo-Aasen and Convit (13). The three modified methods are shown in Table 2. The principle variables encountered were derived from the conditions used for chromation. When duplicate smears of any Smith-Dietrich-Baker-positive bacterial population are stained by the three methods, the intensity of color of the acid hematein-positive bacilli increases with increasing severity of the conditions of mordanting in dichromate.

All leprosy bacilli, including those within globi, and most mycobacterial strains tested stained by all three modifications, but only the acid hematein-positive material of human leprosy bacilli was extractable by pyridine or chloroform-ether. The acid hematein-positive material of *M. lepraemurium*, *M. tuberculosis* $H_{37}Rv$, and *Mycobacterium* sp. 607 resisted extraction by pyridine. $H_{37}Rv$, prepared by the chloroform-ether method, also retained its acid hematein-positive material. $H_{37}Rv$ could be kept in pyridine for 48 h at 37 C without loss of reactivity with the Baker-Pearse method. Both leprosy bacilli and mycobacteria lost their acid hematein-positive material when treated with alkaline ethanol.

The bacilli of patient 108 were the single exception found among leprosy bacilli with respect to their Smith-Dietrich-Baker reactivity. These bacilli retained their acid hematein-positive material after treatment with pyridine.

The state of saturation of lipoidal residues is not supposed to affect responses to the acid hematein test even though hexavalent chromium (in the Baker test) is bound by ethylenic bonds (64). Further, in the case of brain lipids, saturation of double bonds following bromina-

TABLE 2. *Smith-Dietrich-Baker staining methods*

Name	Concn of $K_2Cr_2O_8$	Time and temp	Stain	Time and temp	Differentiation	Ref
Smith-Dietrich	Saturated	72 h 55 C	Kulchitsky's hematoxylin	5 h 60 C	Borax ferricyanide Overnight room temp	(42)
Baker-Pearse	5% with 1% $CaCl_2$	Overnight room temp + 24 h 60 C	Acetic hematoxylin	5 hr 60 C	Borax ferricyanide Overnight 37 C	(56)
Baker-Convit	5% with 1% $CaCl_2$	Overnight room temp + 1 h 60 C	Acetic hematoxylin	1 h 60 C	Borax ferricyanide 1.5 h room temp	(13)

tion does not interfere with the reaction that gives rise to acid hematein-positive material (1). However, we have found that immersion of leprous smears in fresh bromine water for 6 h blocks reactivity toward the Smith-Dietrich-Baker tests. Such blockage did not occur when *M. tuberculosis* $H_{37}Rv$ or *Mycobacterium* sp. ICRC or 1217 were treated in the same manner. The acid-fastness of neither human leprosy bacilli nor mycobacteria was affected by bromination.

The acid hematein-positive material of human leprosy bacilli was found to be susceptible to digestion by certain enzymes although acid-fastness and Gram reactivity remained unaffected. Aqueous homogenates of biopsy specimens were treated sequentially for 24 h each with trypsin, pepsin, crude pancreatic lipase, and two changes of phospholipase D. The acid-fast and Gram reactions remained unchanged throughout the entire 120 h of incubation. The Smith-Dietrich-reacting material was unaffected by trypsin or pepsin but was lost after 24 h in lipase. The Baker-Convit reaction remained unchanged until the second 24-h period in phospholipase D, when it too became negative. Phospholipase D when used, without prior digestion of leprosy bacilli by lipase, has little effect upon the acid hematein-positive material on the bacterial cell but causes the dispersal of cells from globus-like clumps and the loss of much, presumably nonbacterial, Smith-Dietrich-positive lipoidal material.

Other cytochemical reactions for lipid. Since the results of bromination indicate that unsaturation may be largely responsible for the positive reaction in the acid hematein tests, we felt it necessary to test for phosphoglycerides by methods which did not rely upon mordanting with metallic ions. Those tests used were the ferric hydroxamate method of Adams and Davison (2), which relies on hydroxylaminolysis of fatty acid esters, and the enclosure method of Feyrter (56), which detects phosphoglycerides by metachromasia of thionin. By neither method were we able to demonstrate phospholipid on the surface of leprosy bacilli. Thus the identity of the generic class of the pyridine-extractable, acid hematein-positive material on the surface of leprosy bacilli has not been determined.

When tested for lipids with unsaturated bonds by the performic acid-Schiff reaction (56), direct smears of leprous material were found to contain large amounts of unsaturated lipids, none of which was specifically localized on leprosy bacilli. Tests for free fatty acids in smears of aqueous homogenates, using the deposition of lead sulfide as an indicator (75, 76), showed abundant black deposits, none of which was localized on leprosy bacilli.

Gram reaction. Throughout, we have stressed that the Gram reactivity of all the organisms tested was retained during the various procedures. With respect to human leprosy bacilli, *M. lepraemurium* and *Mycobacterium* sp. 1217, the Gram reactivity actually increased after pyridine extraction. When two smears from the same homogenate of human biopsy material are tested for Gram reactivity, with or without prior extraction with pyridine, the unextracted smear shows a prevalence of beaded forms and apparently few intact bacilli. This is typical of the Gram reactivity of most bacilli harvested from leprous material. After 2

h in pyridine, the duplicate smear has numerous long curved bacilli which are more uniformly stained by the crystal violet-iodine complex. Similar conversions from beaded to more uniform Gram staining occurred with *M. lepraemurium* and *Mycobacterium* sp. 1217 when they were extracted with pyridine.

Ceroid. Ceroid is an acid-fast material derived, by oxidation, from lipid (26). It occurs under a wide variety of conditions in vivo (57) and is readily produced in vitro (14). Thus, it is potentially a model of material whose acquisition by bacteria could endow them with a lipid-derived pseudo-acid-fastness. Ceroid was produced in vitro by mixing freshly grown and washed bacteria into 5 to 10 times their volume of cod liver oil with the aid of a drop or two of 1% sodium lauryl sulfate. This mixture was allowed to stand at room temperature.

Propionibacterium sp. LT, normally non-acid-fast and Smith-Dietrich negative, became, when maintained in cod liver oil for 4 to 6 months, acid-fast and Smith-Dietrich positive. Apparently, this acid-fastness was due to a xylene-insoluble, pyridine-soluble, external coating of ceroid. *M. tuberculosis* $H_{37}Rv$, when maintained in cod liver oil, retained its native staining properties and did not appear to acquire an extracellular capsule of ceroid. In both cases the cod liver oil became resinous, attained a dark orange color, and was acid-fast. Thus, it had undergone a conversion to ceroid.

DISCUSSION

In their native states, either from tissue or, in the case of mycobacteria, from culture media as well, mycobacteria differ from leprosy bacilli in the manner in which they respond to certain dyes. Whether from in vivo or in vitro sources, populations of mycobacteria contain cells which are readily stained with Sudan black B, revealing fatty inclusions or fat bodies. In addition, mycobacteria take up the so-called (fat stains) Sudan IV and oil red O. Furthermore, mycobacteria are penetrated by toluidine blue and safranin O. To each of these foregoing stains, native leprosy bacilli give a negative response (see ref. 29, 30, 31 regarding capsules and the staining of leprosy bacilli).

In smears prepared from subcutaneous fatty material from leprous lesions, the number of bacilli revealed by the Gram stain is always far less than those seen with the carbol fuchsin stain. However, if the smears are first given a rapid rinse in xylol, the numbers of bacilli revealed by the Gram stain more nearly equal the numbers seen with the acid-fast stain. Such xylol-soluble fatty material does not seem to be of consequence in the case of in vivo grown *M. lepraemurium* or *M. tuberculosis*. There seems, then, to be no parallel between the fatty menstruum in which harvested leprosy bacilli occur and the menstrua accompanying tubercle bacilli and *M. lepraemurium*.

The fatty material in leprous lesions which is dispersed by xylol is also dissipated by pancreatic lipase. When stained with Sudan black B it appears as lakes of lipid. The fact that the acid-fast bacteria of patient 108 gave typical mycobacterial responses to a variety of stains suggests that the lipid material of leprous lesions is not itself responsible for the peculiar staining properties of leprosy bacilli.

Both leprosy bacilli and mycobacteria yield a positive response to the Smith-Dietrich and Baker test for acid hematein-fixing material, presumably phospholipid. In the case of *M. lepraemurium*, this acid hematein-positive material was shown by Campo-Aasen and Convit (13) to resist extraction with pyridine. Prior extraction of leprosy bacilli with pyridine, however, was shown by these authors to eliminate the acid hematein-positive reaction. Fisher and Barksdale (22), working with smears made from leprous material extracted in aqueous buffer, noted that pyridine also extracted the acid-fastness of leprosy bacilli. It has not been possible to decide whether the pyridine-extractable Baker-positive substances and the acid-fastness of leprosy bacilli are one and the same material. It also remains to be determined whether this acid-fastness of leprosy bacilli, which seems to act as a barrier layer to these bacteria, is in fact the same material which enabled Robert Kock in 1882 to note an obvious difference in the response of leprosy bacilli (positive) and tubercle bacilli (negative) when stained, in tissues, with the nuclear stain of Weigert (40). A most important point seems to be the fact that the pyridine-extractable acid-fastness of leprosy bacilli represents the one tinctorial property, discovered to date which is unique for 95% of these bacteria.

Once the pyridine-extractable barrier has been removed from leprosy bacilli, they are stained by toluidine blue and safranin O, dyes excluded from them prior to extraction. Presumably, they now can be penetrated by Sudan black B. If so, however, fat bodies fail to be revealed in them. Thus the original observations of Burdon (12), confirmed and extended by Bermann (9) and by Chaussinand (16), appear to be true: leprosy bacilli contain no Sudan black B-stainable fat bodies. Whether or not such bodies are present but covered with some impenetrable coating remains a possibil-

ity.

In an earlier communication we indicated that leprosy bacilli which had lost their acid-fastness following extraction with pyridine were still gram positive, and we took that to mean that the integrities of their cell walls were intact. The gram positivity we reported could be equated with the capacity to retain the crystal violet-iodine complex. Such retention, however, may indicate no more than that for the majority of leprosy bacilli, the shell of the bacterium is intact. The state of the organelles once contained in the cytoplasm of these bacteria is another matter.

Among the leprosy bacilli we have examined, those from one case, 108, were exceptional: their acid-fastness was not extracted by pyridine, they behaved like mycobacteria in response to bacterial stains, and they contained fat bodies. They were, then, by the cytochemical tests employed, true mycobacteria. The question with regard to patient 108 would appear to be: are those mycobacteria true leprosy bacilli?

Against the preceding cytochemical data which suggests that most leprosy bacilli may well not be mycobacteria, there exist observations on the ultrastructure of leprosy bacilli which indicate that some of them share certain morphological components suggestive of mycobacteria. Studies of Imaeda and Convit (36) of the ultrastructure of leprosy bacilli in sectioned biopsies indicate that, at best (lesions from borderline leprosy), the bacilli are not actively growing as indicated by an absence of polysomes, of dense fibrillar nuclear material, and by the presence of an accumulation of intracytoplasmic membranous structures as vesicular mesosomes and that, at worst (lesions from lepromatous leprosy), the above subcellular components are broken down or absent (see also Imaeda et al. concerning ultrastructural indices of viability in mycobacteria). The pattern of ultrastructure commonly seen in good sections of leprosy bacilli are those found in a number of bacteria and cannot be said to be uniquely suggestive of mycobacteria. For an appreciation of this point the reader is referred to the electron micrographs to be found in each of the references just cited as well as the pictures published by Edwards (20), Klingmuller and Orfanos (39), McFadzean and Valentine (45), Sato and Okura (66), and Rees et al. (61, 62). On the other hand, ropelike structures found about the cell envelope of mycobacteria, superficial to the rigid layer, were observed as early as 1958 by Takeya et al. (71). These structures were further characterized in 1961 (72) and shown to be removable by alkaline-ethanol treatment in 1963 (69, 70). It is now generally appreciated that the peptidolipid superficial to the rigid layers of the walls of such genera of the *Actinomycetales* as *Corynebacterium*, *Nocardia*, and *Mycobacterium* may be removed with alkaline ethanol and that this outer envelope material assumes a different pattern for each genus (8). Only in mycobacteria are the patterns those of ramified ropelike structures. In 1965, Imaeda called attention to a suggestion of such structures on leprosy bacilli (35) and in 1968 Sato and Imi published pictures of negatively stained leprosy bacilli which had been partially freed of a "peripheral transparent zone" thus revealing ropelike structures similar to those found on mycobacteria (65). More recently ropelike "peptido-glycolipid filaments" have been discovered by Gordon and White on leprosy bacilli harvested from a case of lepromatous leprosy (25). Thus, one ultrastructural characteristic of mycobacteria appears to be found on some leprosy bacilli.

While the cytochemical data presented herein suggest that leprosy bacilli on a number of counts do not behave at all like mycobacteria, there is, nevertheless, the established presence of a mycobacterial surface pattern on some leprosy bacilli. If it is assumed that all leprosy bacilli are derived from mycobacteria, how did they come to lose their essentially mycobacterial properties: fat bodies, pyridine-resistant acid-fastness, affinity for the dyes safranin O, alkaline methylene blue, toluidine blue O, and the fat stains, oil red O and Sudan IV? The absence of fat bodies is difficult to explain. In ultrathin sections of aging cultures of mycobacteria, the space once occupied by these fat deposits is readily demonstrable. To date, pictures showing such inclusions in leprosy bacilli have not been published.

The results reported herein clearly indicate that, if the leprosy bacilli we have examined were ever mycobacteria, they have long since been chemically altered in such a way that they show none of the singular mycobacterial properties found in *M. lepraemurium* and *M. tuberculosis* replicating in vivo. Hypothetically, this alteration has mummified the bacilli and in the process destroyed their pyridine-stable acid-fastness. It is clear that in lepromatous leprosy most leprosy bacilli are dead (61, 67), even though one gram of lepromatous tissue may contain up to 10^{10} bacilli (28). The more economic assumption would be that, in the process of mummification, interaction between host material and the bacterial outer envelope (where, in mycobacteria, resides that mycobacterial acid-fastness removable only with alka-

line ethanol) completely modifies the surface chemistry of the bacteria so that they are impermeable to the dyes so often mentioned in this paper.

If the foregoing superficially explains the origin of bacterial mummies in lepromatous leprosy, it does not account for the behavior of the bacteria found in the tissues of case 108. Nor does it account for the fact that leprosy bacilli synthesize a phenolase (58, 59) not yet found in any mycobacteria, corynebacteria, or nocardias but which is known to be produced by certain propionibacteria isolated from cases of tuberculoid and lepromatous leprosy and from infections of the bone marrow (7). These propionibacteria are not normally acid-fast, and their surface patterns do not include ropelike structures. However, as we have shown, in the presence of lipids which give rise to ceroid (26), these propionibacteria do become acid-fast. As pointed out above, most leprosy bacilli exhibit an acid-fastness not found among mycobacteria, and most leprosy bacilli appear to be fossiloid or mummified remains of once viable bacteria. Aside from an absence in most of them of the subcellular components indicative of viable cells, they exhibit a hallmark of their mummified state: pyridine-extractable acid-fastness. Presumably, this property could be acquired by bacteria other than mycobacteria. To account, then, for (i) the presence of ropelike structures, which for purposes of this discussion are assumed to be uniquely mycobacterial on some leprosy bacilli, and (ii) the presence of a nonmycobacterial phenolase (in others), one is forced to question the idea that only one kind of microorganism is involved in the etiology of leprosy. However, in the absence of cultivation of either of these seeming two kinds of bacteria, very little more can be said about their suggested presence in the lesions of leprosy.

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LITERATURE CITED

1. Adams, C. W. M., Y. H. Abdulla, O. B. Baylis, and R. O. Weller. 1965. The reaction of Baker's chromate reagent with lipids. J. Histochem. Cytochem. **13**:410-411.
2. Adams, C. W. M., and A. N. Davison. 1959. The histochemical identification of myelin phosphoglycerides by their ferric hydroxamates. J. Neurochem. **3**:347-351.
3. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
4. Andersen, R. N., and Y. T. Chang. 1970. Morphological changes produced by various Ziehl-Neelsen staining techniques on *Mycobacterium leprae* and *M. lepraemurium*. Int. J. Lepr. **38**:344-346.
5. Baker, J. R. 1946. The histochemical recognition of lipine. Quart. J. Microsc. Sci. **87**:441-469.
6. Barksdale, W. L., and A. M. Pappenheimer, Jr. 1954. Phage-host relationships in nontoxinogenic and toxinogenic diphtheria bacilli. J. Bacteriol. **67**:220-232.
7. Beaman, L., and L. Barksdale. 1970. Phenoloxidase activity in organisms isolated from lepromatous and tuberculoid leprosy. J. Bacteriol. **104**:1406-1408.
8. Beaman, B. L., K. S. Kim, M. R. J. Salton, and L. Barksdale. 1971. Amino acids of the cell wall of *Nocardia rubra*. J. Bacteriol. **108**:941-943.
9. Bermann, R. 1953. Essais de coloration du bacille de Stefansky et due bacille de Hansen par le noir soudan B.N. Bull. Acad. Nat. Med., Paris **137**:295-298.
10. Bier, M. 1955. Lipases, p. 627-642. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
11. Binford, C. H. 1962. Studies on a mycobacterium obtained from the golden hamster (*Cricetus auratus*) after inoculation with lepromatous tissue. Lab. Invest. **11**:942-955.
12. Burdon, K. L. 1946. Disparity in appearance of true Hansen's bacilli and cultured "leprosy bacilli" when stained for fat. J. Bacteriol. **52**:679-680.
13. Campo-Aasen, I., and J. Convit. 1968. Identification of the noncultivable pathogenic mycobacteria *M. leprae* and *M. lepraemurium*. Int. J. Lepr. **36**:166-170.
14. Casselman, W. G. B. 1951. The *in vitro* preparation and histochemical properties of substances resembling ceroid. J. Exp. Med. **94**:549-562.
15. Chatterjee, K. R. 1958. Experimental transmission of human leprosy infection to a selected, laboratory-bred hybrid black mouse. Int. J. Lepr. **26**:195-204.
16. Chaussinand, R., and M. Viette. 1955. Étude de la coloration des bacilles acido-alcoolo-resistants par le noir soudan. Ann. Inst. Pasteur, Paris **89**:280-289.
17. Cummins, C. S., and H. Harris. 1958. Studies on the cell wall composition and taxonomy of *Actinomycetales* and related groups. J. Gen. Microbiol. **18**:173-189.
18. Dharmendra. 1942. Studies of the lepromin test. 9. A bacillary antigen standardized by weight. Lepr. India **14**:122-129.
19. Dietrich, A. 1910. Zur Differenzialdiagnose der Fettsubstanzen. Verh. Deut. Path. Ges. **14**:263-268.
20. Edwards, R. P. 1970. Electron-microscope illustrations of division in *Mycobacterium leprae* (Plates XLIX-L11). J. Med. Microbiol. **3**:493-499.
21. Ehrlich, P. 1882. Neue Methode zum entdechung des Tuberkelbacillus. Deut. Med. Wochenschr. **8**:269-270.
22. Fisher, C. A., and L. Barksdale. 1971. Elimination of the acid fastness but not the gram positivity of leprosy bacilli after extraction with pyridine. J. Bacteriol. **106**:707-708.
23. Folch, J., and M. Lees. 1951. Proteolipides, a new type of tissue lipoprotein. Their isolation from brain. J. Biol. Chem. **191**:807-817.
24. Freer, J., K. S. Kim, M. R. Krauss, L. Beaman, and L. Barksdale. 1969. Ultrastructural changes in bacteria isolated from cases of leprosy. J. Bacteriol. **100**:1062-1075.
25. Gordon, J., and R. G. White. 1971. Surface peptidoglycolipid filaments on *Mycobacterium leprae*. Clin. Exp. Immunol. **9**:539-547.
26. Haas, G. M. 1939. Membrane formation at lipoid-aque-

ous interfaces in tissues. Arch. Pathol. **27**:15-24.
27. Hagihara, B. 1960. Bacterial and mold proteases, p. 206-207. *In* P. D. Boyer, H. Lardy, and K. Myrback (ed.), The enzymes, vol. 4, 2nd ed. Academic Press Inc., New York.
28. Hanks, J. H. 1945. A note on the numbers of leprosy bacilli which may occur in leprous nodules. Int. J. Lepr. **13**:25-26.
29. Hanks, J. H. 1961. Significance of capsular components of *Mycobacterium leprae* and other mycobacteria. Int. J. Lepr. **29**:74-83.
30. Hanks, J. H. 1961. The origin of the capsules on *Mycobacterium leprae* and other tissue-grown mycobacteria. Int. J. Lepr. **29**:172-174.
31. Hanks, J. H. 1961. Demonstration of capsules on M. leprae during carbol-fuchsin staining mechanism of the Ziehl-Neelsen stain. Int. J. Lepr. **29**:179-182.
32. Hansen, G. A. 1955. Spedalskhadens ársager (causes of leprosy), reprinted in translation from Norsk Magazin for Laegevidenskaben 4 (1874). Int. J. Lepr. **23**:307-309.
33. Hayaishi, O. 1955. Phospholipases, p. 660-672. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
34. Herriott, R. M. 1955. Swine pepsin and pepsinogen, p. 3-7. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
35. Imaeda, T. 1965. Electron microscopy approach to leprosy research. Int. J. Lepr. **33**:669-683.
36. Imaeda, T., and J. Convit. 1962. Electron microscope study of *Mycobacterium leprae* and its environment in a vesicular leprous lesion. J. Bacteriol. **83**:43-52.
37. Imaeda, T., F. Kanetsuna, M. Rieber, B. Galindo, and J. M. Cesari. 1969. Ultrastructural characteristics of mycobacterial growth. J. Med. Microbiol. **2**:181-186.
38. Kim, K. S., and L. Barksdale, 1969. Crystalline inclusions of bacterium 22M. J. Bacteriol. **98**:1390-1394.
39. Klingmuller, G., and C. Orfanos. 1966. Der Electronmikroskopische aufbau des *Mycobacterium leprae*. Arch. Klin. Exp. Dermatolo. **224**:373-384.
40. Koch, R. 1882. Die Aetiologie der Tuberculose. Berlin. Klin. Wochenschr. **15**:221-230.
41. Laskowski, M. 1955. Trypsinogen and trypsin, p. 26-36. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
42. Levinson, S. A., and R. P. MacFate. 1956. Clinical laboratory diagnosis, 5th ed. Lea & Febiger, Philadelphia.
43. McDonald, M. R. 1955. Ribonucleases, p. 427-436. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
44. McDonald, M. R. 1955. Deoxyribonucleases, p. 437-447. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
45. McFadzean, J. A., and R. C. Valentine. 1958. An attempt to determine the morphology of living and dead mycobacteria by electron microscopy, p. 89-90. Trans. VII Int. Congr. Leprol. Tokyo.
46. McRae, D. H., and C. C. Shepard. 1971. Relationship between the staining quality of *Mycobacterium leprae* and infectivity for mice. Infect. Immunity **3**:116-120.
47. Murohashi, T., E. Kondo, and K. Yoshida. 1968. Role of lipids in acid-fastness of mycobacteria. Amer. Rev. Resp. Dis. **99**:794-798.
48. Nakamura, M., T. Tsuchiya, T. Nagamatsu, Y. Aono, and M. Ishida. 1968. Staining conditions influencing morphological index of acid-fast bacilli. Kurume Med. J. **15**:39-41.
49. Neelsen, F., cited by H. A. Johne. 1885. Ein zweifelloser Fall von congenitaler Tuberculose. Fortschr. Med. **3**:198-202.
50. Neisser, A. 1889. Ueber die Structur der Lepra-und Tuberkelbacillen mit specieller Berucksichtigung der Rosanilin und Pararosanilin-Farbstoffe, und über Leprazellen. Verh. Deut. Dermat. Ges. **1**:29-56.
51. Neisser, A. 1889. Ueber die tinctoriellen Verhältnisse der Leprabacillen; Erwiderung an P.G. Unna. Fortschr. Med. **7**:816-820.
52. Nishimura, S. 1966. Investigation for acid fast bacilli in normal mice with special reference to normal pregnant mice and their fetuses. Lepro **35**:179-187.
53. Nishimura, S., Y. Kawaguchi, K. Kohsaka, and T. Mori. 1964. Contamination of healthy mice with murine like acid fast bacillus. Lepro **33**:245-256.
54. Nyka, W. 1971. Influence of oxidation and reduction on the acid-fastness of mycobacteria. Infect. Immunity **4**:513-515.
55. Pattyn, S. R., and G. V-V. Loo. 1969. Isolation of a strain of *Mycobacterium lepraemurium* from normal laboratory mice. Ann. Soc. Belg. Med. Trop. **49**:465-468.
56. Pearse, A. G. E. 1968. Histochemistry, theoretical and applied, vol. 1, 3rd ed. Little, Brown and Co., Boston.
57. Porta, E. A., and W. S. Hartroft. 1964. Lipid pigments in relationship to aging and dietary factors (lipofuscins), p. 191-235. *In* M. Wolman (ed.), Pigments in pathology. Academic Press Inc., New York.
58. Prabhakaran, K., and W. F. Kirchheimer. 1966. Use of 3,4-dihydrophenylalanine oxidation in the identification of *Mycobacterium leprae*. J. Bacteriol. **92**:1267-1268.
59. Prabhakaran, K., W. F. Kirchheimer, and E. B. Harris. 1968. Oxidation of phenolic compounds by *Mycobacterium leprae* and inhibition of phenolase by substrate analogues and metal chelators. J. Bacteriol. **95**:2051-2053.
60. Ranadive, Kamal J. 1963. Experimental studies on human leprosy, p. 61-79. *In* G. E. W. Wolstenholme and Maeve O'Connor (ed.), The pathogenesis of leprosy. Little, Brown and Company, Boston.
61. Rees, R. J. W., and R. C. Valentine. 1962. The appearance of dead leprosy bacilli by light and electron microscopy. Int. J. Lepr. **30**:1-9.
62. Rees, R. J. W., R. C. Valentine, and P. C. Wong. 1960. Application of quantitative electron microscopy to the study of *Mycobacterium lepraemurium* and *M. leprae*. J. Gen. Microbiol. **22**:443-457.
63. Ridley, M. J., and D. A. Ridley. 1971. Stain techniques and the morphology of *Mycobacterium leprae*. Lepr. Rev. **42**:88-95.
64. Roozemond, R. C. 1971. The staining and chromium binding of rat brain tissue and of lipids in model systems subjected to Baker's acid hematein technique. J. Histochem. Cytochem. **19**:244-251.
65. Sato, S., and M. Imi. 1968. The surface structure of *M. leprae*. Int. J. Lepr. **36**:303-308.
66. Sato, S., and E. Okura. 1967. Electron microscopical study of cell division in *Mycobacterium leprae* and *Mycobacterium lepraemurium*. Sci. Rep. Res. Inst. Tohoku Univ. **14**:122-129.
67. Shepard, C. C., and D. H. McRae. 1965. *Mycobacterium leprae* in mice: minimal infectious dose, relationship between staining quality and infectivity, and effect of cortisone. J. Bacteriol. **89**:365-372.
68. Smith, J. L., W. Mair, and J. F. Thorpe. 1909. An investigation of the principles underlying Weigert's method of staining medullated nerve. J. Pathol. Bacteriol. **13**:14-27.
69. Takeya, K., and K. Hisatsune. 1963. Mycobacterial cell walls. I. Methods of preparation and treatment with various chemicals. J. Bacteriol. **85**:16-23.
70. Takeya, K., K. Hisatsune, and Y. Inoue. 1963. Mycobacterial cell. II. Chemical composition of the "basal layer." J. Bacteriol. **85**:24-30.

71. Takeya, K., R. Mori, M. Koike, and T. Toda. 1958. Paired fibrous structures in mycobacteria. Acta Biochim. Biophys. **30**:197-198.
72. Takeya, K., R. Mori, T. Takunaga, M. Koike, and K. Hisatsune. 1961. Further studies on the paired fibrous structure of mycobacterial cell wall. J. Biophys. Biochem. Cytol. **9**:496-501.
73. Taylor, C. E. 1962. Specificity of the Dharmendra antigen as tested in guinea pigs. Int. J. Lep. **30**:175-190.
74. Thin, G. 1883. On the bacillus of leprosy. Brit. Med. J. **1**:1178-1179.
75. Tuqan, N. A., and C. W. M. Adams. 1961. Histochemistry of myelin. I. Protein and lipid-protein complexes in the normal sheath. J. Neurochem. **6**:327-333.
76. Wachstein, M., and M. Pisano. 1950. A new staining technique for polar bodies. J. Bacteriol. **59**:357-360.
77. Wiersma, J. P., C. H. Binford, and Y. T. Chang. 1965. Nerve involvement. Comparison of experimental infections by *Mycobacterium leprae* and *Mycobacterium lepraemurium*. Int. J. Lepr. **33**:(Suppl.)617-633.
78. Wilson, G. S., and A. A. Miles. 1964. Topley and Wilson's principles of bacteriology and immunity, 5th ed., vol. 1. Williams and Wilkins Co., Baltimore.
79. Ziehl, F. 1882. Zur Farbung des Tuberkelbacillus. Deut. Med. Wochenschr. **8**:451.
80. Ziehl, F. 1883. Ueber die Farbung des Tuberkelbacillus. Deut. Med. Wochenschr. **9**:247-249.