Use of Pyridine for Differentiating *Mycobacterium leprae* from Other Mycobacteria in Direct Microscopy [1, 2]

M. Slosarek, L. Sula, S. Theophilus and L. Hruby [3]

Campo-Aasen and Convit ([2]) described a modification of Baker's test with acid hematein as a cytochemical method for differentiating *Mycobacterium leprae* from *M. lepraemurium*. They used two-hour pyridine treatment at room temperature to extract phospholipids from cells. Fisher and Barksdale ([8]) demonstrated that in most instances pyridine extraction deprives *M. leprae* of their acid-fastness. Out of 19 homogenized tissue samples obtained from leprosy patients, positive for acid-fast bacteria microscopically, 15 lost their acid-fastness after treatment with pyridine. In another study, the same authors ([7]) expanded their experimental material by including another 43 tissue samples containing *M. leprae;* pyridine completely deprived 41 of them of acid-fastness. Fisher and Barksdale ([7]) simultaneously demonstrated that pyridine has no effect on the acid-fastness of some cultivable mycobacteria. Essentially the same results were obtained by Convit and Pinardi ([3,4]). We decided to verify the reported specific effect of pyridine in a variety of materials obtained from leprosy patients, as well as on a broader spectrum of mycobacterial cultures. Special attention was paid to specimens prepared from different tissues of a fatal case of BCG granulomatosis which is histologically characterized by massive intracellular proliferation of mycobacteria in macrophages.

MATERIALS AND METHODS

Material containing *M. leprae* was partly provided by one of the authors, Dr. S. Theophilus, and was partly received through the courtesy of Dr. Kyaw Lwin, Leprosy Control, Ministry of Health, Rangoon, Burma. We received direct smears from lepromatous foci—two slides per focus prepared simultaneously, most of them ear secretions or different skin lesions. From some patients in India we also received, in addition to direct smear "doublets," biopsy material for histologic examination.

The smears were heat fixed and then one smear of each "doublet" was extracted with pyridine (2 or 24 hours at room temperature or 24 hours at 60°C), rinsed with water and then both slides were simultaneously stained by a modified Ziehl-Neelsen method. Fresh pyridine (Pyridine p.a., Naftochem Krakow, Poland) was used for extracting every ten slides or biopsy sections. In the first pilot experiments, the procedure of phospholipid extraction was that of Baker according to Campo-Aasen and Convit's ([2]) modification. Subsequently, fixation with Bouin's solution was substituted by heat fixation in direct smears and fixation with a 10% water solution of formalin in biopsy sections. Extraction of phospholipids with hot pyridine for 24 hours was performed by Baker's original method ([5]). The procedure of direct-smear staining by the Ziehl-Neelsen method was as follows: hot carbol fuchsin 5 minutes, water rinse; decolorization with 25% sulphuric acid (1-2 minutes); counterstain in a 1% water solution of malachite green. The same staining procedure was used in smear "doublets" of different strains of the following mycobacterial species, one strain each except as noted: *M. abscessus, M. aquae, M. avium* (2 strains), *M. bovis, M. bovis* BCG, *M. diernhoferi, M. flavescens, M. fortuitum* (4 strains), *M. kansasii* (4 strains), *M. nonchromogenicum, M. phlei, M. scrofulaceum* (6 strains), *M. smegmatis, M. szulgai, M. terrae, M. triviale, M. tuberculosis* (2 strains), *M. vaccae* and *M. xenopi.* All these strains were from the Czechoslovak National Collection of Type Cultures, Institute of Hygiene and Epidemiology, Prague.

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[3] M. Slosarek, R.N.Dr., C.Sc., Research Assistant; L. Sula, M.D., Dr.Sc., Assistant Professor, Chief Research Unit for Mycobacterioses, Institute of Hygiene and Epidemiology, 100 42 Prague, Srobarova 48, CSSR; S. Theophilus, B.Sc., M.B.B.S., Leprologist, Sacred Heart Leprosy Hospital, Sakkottai-612 401, Kumbakonam, Tanjore Dt., India; and L. Hruby, M.D., C.Sc., Assistant Professor, Institute of Hygiene and Epidemiology, Prague.

Biopsy specimens from lepromatous tissues and tissues from a fatal case of BCG granulomatosis in a child were fixed with formalin and then ten consecutive paraffin sections were made per specimen and numbered sequentially. The even-numbered sections were subjected to pyridine extraction (2 hours at room temperature), the odd-numbered ones served as controls. Both groups of histologic sections were stained simultaneously as follows: carbol fuchsin 30 minutes at 37°C in thermostat, a rinse under running water 3 minutes, decolorization with 1% hydrochloric acid to a light pink, again 3 minute rinse under running water, and counterstain in a 0.14% solution of methylene blue. The sections were then dried in the air, cleared in xylene and embedded in Canada balsam.

RESULTS

Seventy-two "doublets" of direct smears from leprous foci (42 of them after 2-hour pyridine extraction, 30 after 24-hour pyridine extraction, 8 of the latter at 60°C), 32 "doublets" of slide preparations of pure mycobacterial cultures and ten biopsy specimens containing acid-fast rods were treated as described above. The results of the experiments showed that, under the conditions used, pyridine extraction leads to a loss of acid-fastness in *M. leprae* in histologic sections only. All control sections were massively positive (Figs. 1, 3), whereas the same sites in adjacent sections after 2-hour exposure to pyridine were completely free of acid-fast bacteria, except that at the places of their greatest concentration there remained slightly violet, poorly distinguishable amorphous spots (Figs. 2, 4). A loss of acid-fastness after pyridine action was not observed in histologic sections from tissue specimens excised post-mortem from the lungs, lymph nodes and intestine of a child who died of BCG vaccine dissemination. Numerous acid-fast rods were found in all of these specimens, even though the intensity of their coloring was somewhat

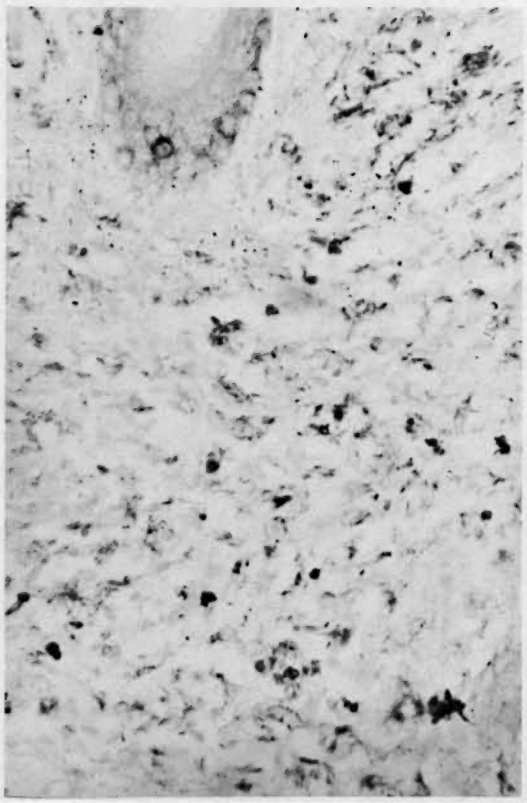

FIG. 1. Section through a biopsy specimen (right pinna, No. 10225) from a lepromatous patient, Ziehl-Neelsen stain, × 125.

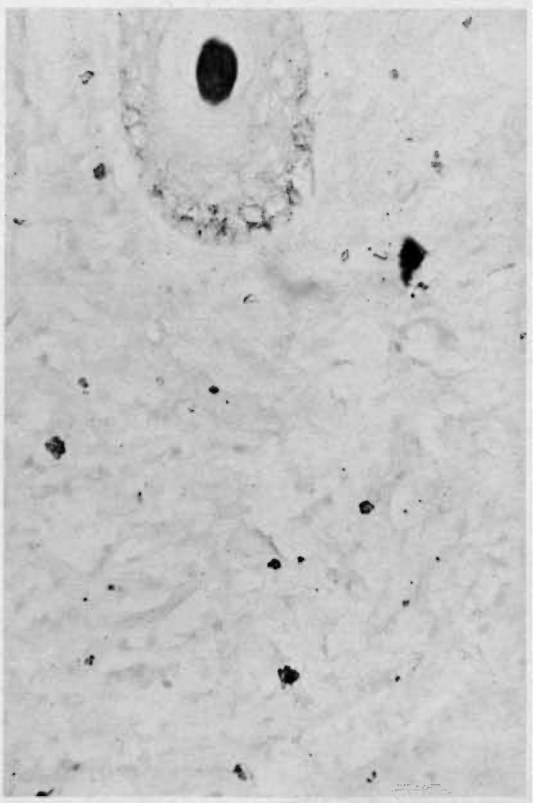

FIG. 2. Section through a biopsy specimen (same as Fig. 1). Ziehl-Neelsen stain after two-hour pyridine extraction, × 125.

weaker after pyridine extraction (Figs. 5, 6).

Smear "doublets" prepared simultaneously either from different parts of the same patients from whom biopsy specimens had been obtained or other patients with established leprous infection, for the most part showed unchanged acid-fastness of mycobacteria after pyridine extraction, except that in rare cases, after 24-hour extraction, the color of some rods turned a weaker red or the number of stained mycobacterial cells was lower. There was no instance of a completely negative stain after pyridine extraction with the control slide being positive.

In 32 cultures of 18 different mycobacterial species, complete loss of acid-fastness after two-hour pyridine extraction was not observed in any instance. Nevertheless, some pyridine-extracted slides (*M. avium, M. diernhoferi, M. fortuitum, M. scrofulaceum, M. vaccae* and especially *M. phlei*) displayed a smaller or larger number of non-acid-fast cells and many other cells with a weaker color than in their respective controls. The culture of the *M. smegmatis* strain employed was only very weakly acid-fast, both in the control and the pyridine-extracted slide.

DISCUSSION

The mechanism of mycobacterial acid-fastness has not yet been sufficiently elucidated, although its association with the content of lipid components in cells seems to be beyond doubt. It is known that different mycobacterial species display acid-fastness of different intensities, often co-dependent on culture medium composition, and that even monospecific cultures of different ages stain differently ([6, 12, 14]). In evaluating the effect of HNO_3-ethanol mixture in different concentrations and of UV light on the acid-fastness of some mycobacteria, Murohashi and Yoshida ([11]) concluded that acid-fastness is weakest in saprophytic species and strongest in the strictly pathogenic ones, i.e., *M. bovis, M. tuberculosis* and *M. leprae.*

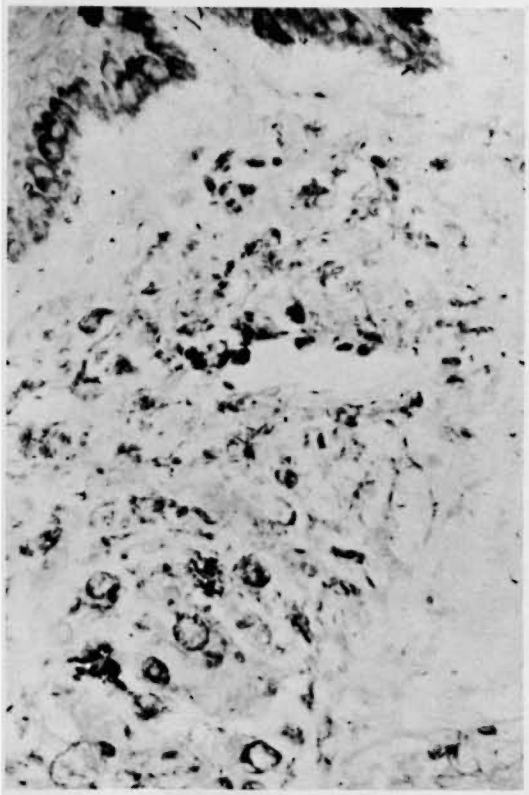

FIG. 3. Section through a biopsy specimen (right thigh, No. 10226) from a lepromatous patient. Ziehl-Neelsen stain, × 125.

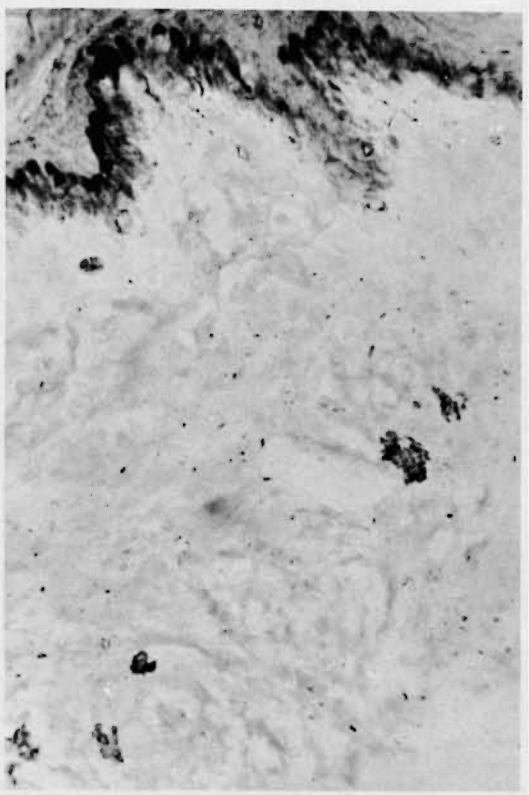

FIG. 4. Section through a biopsy specimen (same as Fig. 3). Ziehl-Neelsen stain after two-hour pyridine extraction, × 125.

That the technic of fluorescent microscopy may be successfully applied not only to *M. tuberculosis* but also *M. leprae* likewise suggests that both of these pathogenic species have similar staining properties ([9]). On the other hand, some studies ([7]) suggested that native leprosy bacilli respond differently to the effect of various stains (Sudan IV, toluidine blue, safranine O, etc.) than do other mycobacteria.

The first data suggesting that pyridine has a weakening effect on the acid-fastness of mycobacteria was presented more than 50 years ago ([6]). Only much later was it suggested that the effect of pyridine might be utilized in differentiating *M. leprae* from *M. lepraemurium* ([2]) or from all other mycobacteria ([3,7,8]). In the present experiments, an attempt was made to verify the reportedly selective effect of two-hour pyridine extraction; a variety of materials obtained from leprosy patients and quite a large number of pure cultures of different mycobacterial species were used for this purpose. The results of these experiments indicate that a specific effect of pyridine extraction for differentiating *M. leprae* from other cultivable mycobacteria cannot as yet be considered established. The most important piece of evidence in this respect is the failure of pyridine to abolish acid-fastness in direct smears from various positive materials and, on the other hand, findings of numerous nonacid-fast cells in some pure cultures of mycobacteria. These observations of ours are essentially in accord with the data of Skinsnes *et al* ([13,14]) who reported loss of acid-fastness after two-hour pyridine action in *M. fortuitum, M. phlei* and *M. smegmatis*. The fact that the cultures in which we demonstrated an effect of pyridine on acid-fastness included all of the six *M. scrofulaceum* strains tested is of additional interest with reference to the hypothesis that there may be a rather close relationship between *M. leprae* and *M. scrofulaceum*, which is sometimes cultivated from leprous foci ([10]).

Another point that should not be over-

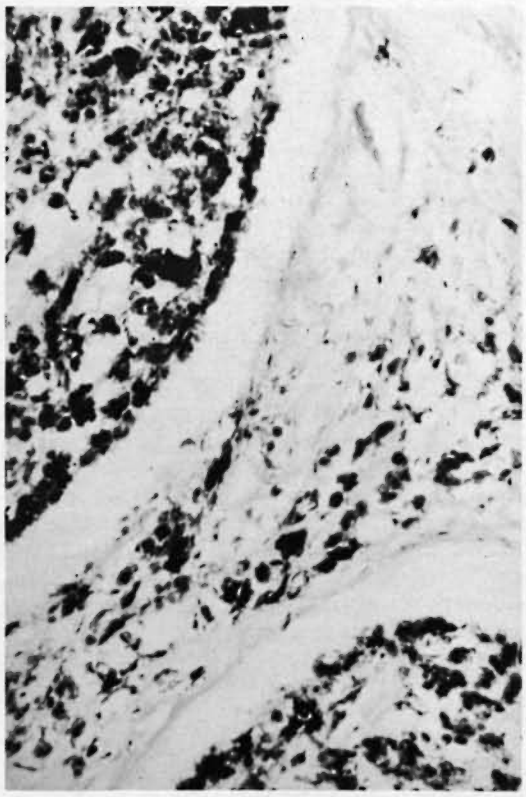

FIG. 5. Section through intestine wall from a fatal case after BCG vaccination. Ziehl-Neelsen stain, × 125.

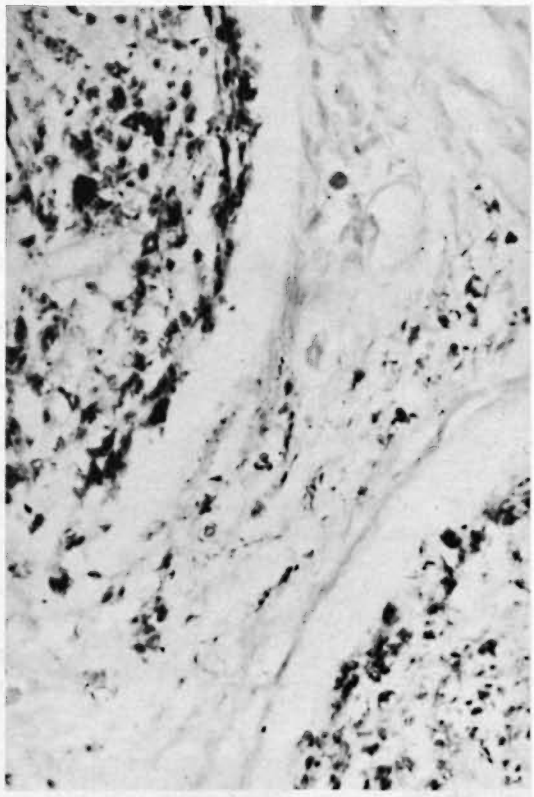

FIG. 6. Section through intestine wall (same as Fig. 5). Ziehl-Neelsen stain after two-hour pyridine extraction, × 125.

looked is that loss of acid-fastness after pyridine extraction has also been demonstrated in nocardias ([1]), both pure cultures and smears from tissues of mice infected with different nocardia strains; filaments of nocardias tend to break up into acid-fast cocci and rods that may easily be mistaken for mycobacteria.

We also tested pyridine resistance in 14 Ziehl-Neelsen stained cultures of nine different mycobacterial species and in ten similarly stained *M. leprae*-positive preparations. In both cases, two-hour action of pyridine completely decolorized previously vividly red mycobacterial cells, but after recolorization of these preparations by the same technic (Ziehl-Neelsen), acid-fast rods approximately equally well or even better stained than before extraction were invariably found. Thus, pyridine only caused decolorization of mycobacteria without depriving them of the acid-fast quality in this case.

The mechanism of pyridine action in histologic examination of biopsy specimens is probably associated with a complex of histochemical reactions that take place in the course of section preparation and staining, which are procedures very different from those used in staining direct smears. Moreover, as Skinsnes *et al* ([14]) point out, the age of *M. leprae* may play some role in the pyridine test or in staining leprosy bacilli. These factors may account for the discrepant results obtained in our direct smears and biopsy materials from the same patients. Preliminary experiments performed with smears from livers of mice infected with *M. lepraemurium* showed that pyridine does not lead to a loss of acid-fastness in this mycobacterial species.

Our present experiments as well as some data in the literature have convinced us that, at the present, the loss of acid-fastness after two-hour exposure to pyridine cannot be considered a sign demonstrably characteristic of *M. leprae* only and distinguishing this species from all other mycobacteria.

SUMMARY

The loss of acid-fastness by *M. leprae* after two-hour pyridine extraction, reportedly a specific test for differentiating *M. leprae* from all other mycobacteria, was verified on different materials obtained from leprosy patients, histologic sections from a fatal post-BCG vaccination case and smears prepared from pure cultures of 32 strains of 18 different mycobacterial species. Under the conditions used, pyridine extraction led to complete loss of acid-fastness in *M. leprae* only in histologic sections of biopsy specimens from leprosy patients, whereas in direct smears from skin lesions containing *M. leprae* the number of acid-fast rods after pyridine extraction was either equal to or only slightly smaller than in control preparations. Moreover, since smears from pure cultures of *M. avium, M. diernhoferi, M. fortuitum, M. scrofulaceum, M. vaccae* and especially *M. phlei* displayed a smaller or greater number of nonacid-fast cells as well (in some instances only 10% to 20% of cells were found stained whereas control slides contained 90% to 100% acid-fast rods), loss of acid-fastness after two-hour pyridine extraction cannot be considered a property typical of *M. leprae* only.

RESUMEN

Se estudió la pérdida de la ácido-resistencia del *M. leprae* después de 2 hr de extracción con piridina (propuesta como una prueba específica para diferenciar al *M. leprae* de las otras micobacterias) en diversos materiales obtenidos de pacientes con lepra, en cortes histológicos preparados a partir de un caso fatal post-vacunación con BCG y en extensiones de los cultivos de 32 cepas de 18 especies micobacterianas. Bajo las condiciones usadas, la extracción con piridina condujo a la pérdida completa de la ácido-resistencia del *M. leprae*, sólo en los cortes histológicos de las biopsias de los pacientes con lepra mientras que en las extensiones directas de las lesiones de piel conteniendo *M. leprae*, el número de bacilos ácido-resistentes post-extracción con piridina fue igual o sólo ligeramente menor que en las preparaciones control. Además, puesto que en los extendidos preparados con los cultivos puros de *M. avium, M. diernhoferi, M. fortuitum, M. scrofulaceum, M. vaccae* y especialmente *M. phlei*, hubieron números variables de bacterias que perdieron su ácido-resistencia (en algunos casos, sólo entre el 10 y el 20% de las células estuvieron teñidas mientras que las preparaciones control contenían entre el 90 y el 100% de ácido-resistentes), la pérdida de la ácido-resistencia después de 2 hr de extracción con piridina no puede considerarse como una propiedad única del *M. leprae*.

RÉSUMÉ

La perte d'acido-résistance de *M. leprae* après deux heures d'extraction par la pyridine, qui a été

signalée comme épreuve spécifique pour différencier *M. leprae* de toutes les autres mycobactéries, a été vérifiée sur différentes substances obtenues à partir de malades de la lèpre, ainsi que sur des coupes histologiques provenant d'issues fatales à la suite d'une vaccination BCG, et sur des frottis préparés à partir de cultures pures de 32 souches de 18 espèces différentes de mycobactéries. Dans les conditions d'étude, l'extration par la pyridine n'a conduit à une perte complète d'acido-résistance de *M. leprae* que dans des coupes histologiques d'échantillons biopsiques provenant de malades de la lèpre, alors que dans des frottis préparés directement à partir de lésions cutanées contenant *M. leprae*, le nombre de bâtonnets acido-résistants après extraction par la pyridine était, soit égal, soit légèrement plus faible que le nombre trouvé dans des préparations témoins. De plus, puisque des frottis préparés à partir de cultures pures de *M. avium*, de *M. diernhoferi*, de *M. fortuitum*, de *M. scrofulaceum*, de *M. vaccae* et spécialement de *M. phlei*, présentaient également des cellules non acido-résistantes, en nombre soit plus faible ou plus élevé (et même dans quelques cas il a été observé que seulement 10 à 20% des cellules pouvaient être colorées, alors que les lames témoins contenaient 90 à 100% de bâtonnets acido-résistants), la perte d'acido-résistance après deux heures d'extraction par la pyridine ne peut être considérée comme une caractéristique typique de *M. leprae*.

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REFERENCES

1. Beaman, B. L. and Burnside, J. Pyridine extraction of nocardial acid-fastness. Appl. Microbiol. **26** (1973) 426-428.
2. Campo-Aasen, I. and Convit, J. Identification of noncultivable pathogenic mycobacteria *M. leprae* and *M. lepraemurium*. Int. J. Lepr. **36** (1968) 166-170.
3. Convit, J. and Pinardi, M. E. A simple method for the differentiation of *Mycobacterium leprae* from other mycobacteria through routine staining techniques. Int. J. Lepr. **40** (1972) 130-132.
4. Convit, J. and Pinardi, M. E. Leprosy: confirmation in the armadillo. Science **184** (1974) 1191-1192.
5. Culling, C. F. A. *Handbook of Histopathological Techniques*, 2nd ed., London: Butterworths, 1963, p 553.
6. Eisenberg, P. Theorie der Bakterienfärbung. *In:* Handbuch der mikrobiologischen Technik, R. Kraus and P. Uhlenhuth, eds., Berlin: Urban and Schwarzenberg, vol. 1, 1923, pp 161-266.
7. Fisher, C. A. and Barksdale, L. Cytochemical reactions of human leprosy bacilli and mycobacteria: ultrastructural implications. J. Bacteriol. **113** (1973) 1389-1399.
8. Fisher, C. A. and Barksdale, L. Elimination of the acid-fastness but not gram positivity of leprosy bacilli after extraction with pyridine. J. Bacteriol. **106** (1971) 707-708.
9. Haebler, T. Von and Murray, J. F. Fluorescence microscopy as a routine method for the detection of *M. tuberculosis* and *M. leprae*. S. Afr. Med. J. **28** (1954) 45-48.
10. Kato, L. *In vitro* grown *Mycobacterium leprae* probably a member of the *Mycobacterium scrofulaceum* species. Int. J. Lepr. **44** (1976) 385-386.
11. Murohashi, T. and Yoshida, K. Biological significance of acid-fastness of mycobacteria. Ann. N.Y. Acad. Sci. **154** (1968) 58-67.
12. Nyka, W. and O'Neill, E. F. A new approach to the study of nonacid-fast mycobacteria. Ann. N.Y. Acad. Sci. **174** (1970) 862-871.
13. Skinsnes, O. K. Problems in identifying *M. leprae*. Int. J. Lepr. **43** (1975) 267-269.
14. Skinsnes, O. K., Chang, P. H. C. and Matsuo, E. Acid-fast properites and pyridine extraction of *M. leprae*. Int. J. Lepr. **43** (1975) 339-347.