Acid-Fast Properties and Pyridine Extraction of *M. leprae* [1,2]

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The nature and varied characteristics of the acid-fast quality of mycobacteria are not well understood. This is particularly true as related to the stages in the life cycle of the leprosy bacillus.

Rogers and Muir noted that while lepra bacilli are more easily decolorized by dilute acids than tubercle bacilli, the latter are more easily decolorized by alcohol ([10]). They also reported that lepra bacilli can be distinguished from saprophytic acid-fast rods, such as the smegma bacillus, by their greater retention of basic dyes, especially when alcohol is used as the decolorizing agent. Murohashi and Yoshida ([7]) found that with the utilization of 4% HNO_3-70% EtOH for 30 seconds the percentage of acid-fast bacilli for a small spectrum of mycobacteria from saprophytic to leprosy bacilli, was about the same. If, however, 0.1% HNO_3-EtOH was used over a time range from 0.5 to 20 minutes, there was a striking range of difference in retention of acid-fast quality. Combining these studies with the addition of the effect of ultraviolet irradiation on acid-fast retention, they concluded that *M. leprae* and *M. lepraemurium* had the strongest acid-fast qualities, the saprophites were weakest and tuberculosis bacilli were intermediate.

Convit and Pinardi ([1]) treated six species of acid-fast bacilli, including *M. leprae*, *M. lepraemurium*, *M. smegmatis* and BCG, with fresh pyridine for two hours, first having fixed the specimens in Bouin's fixative for one hour and then fixing in formol-calcium for one hour after pyridine treatment. They stained the bacilli thereafter with the Ziehl-Neelsen stain, Baker's method for phospholipids and the Truant fluorescent stain for acid-fast bacilli. *M. leprae* was re-ported as being the only organism of those tested that "completely lost its staining capacity with the three methods used." Later ([2]), the same authors stated in reference to the same procedure, "Of all other known mycobacteria, only *M. leprae* completely loses its ability to be stained by the above three methods after 2-hour treatment with pyridine." On this basis they utilized this method as one of three to prove that acid-fast organisms isolated from armadillos after experimental inoculation with *M. leprae* were identical to *M. leprae* isolated directly from human lepromatous patients. Fisher and Barksdale ([4,5]) reported that of bacilli from 54 leprosy patients, 95% showed this pyridine extraction characteristic, the others did not. They also recorded that response to pyridine extraction was not characteristic for an additional ten mycobacterial species including *M. fortuitum*, *M. intracellulare* and *M. phlei*. They stated, "A most important point seems to be the fact that the pyridine-extractable acid-fastness of leprosy bacilli represents the one tinctoral property discovered to date which is unique for 95% of these bacteria" ([5]).

If this be a true identification test for *M. leprae* it is of great importance in leprosy work. Accordingly, it seemed well worth possible confirmatory and extended study. The present presentation concerns its application to *M. leprae*, *M. lepraemurium*, *M. smegmatis* and an additional seven acid-fast organisms, some of which have not been reported on by the above authors as far as we have been able to determine. It also includes consideration of the effect of bacterial aging which seems not to have concerned the previous studies.

MATERIALS AND METHODS

Bacillary specimens. *M. leprae* were obtained in suspension from biopsied lepromas of untreated lepromatous leprosy cases. *M. lepraemurium* were derived from lepromas of Swiss mice infected with the Hawaiian strain of this pathogen.

The other mycobacteria were obtained from stock cultures available and identified in the laboratories of Leahi Hospital, the Hawaii State tuberculosis center. These laboratories have had extensive experience in the isolation and identification of the recognized as well as the atypical cultivable mycobacteria. The strains studied in addition to *M. leprae* and *M. lepraemurium* were: *M. tuberculosis,* var *hominis, M. avium, M. phlei, M. smegmatis, M. kansasii,* a scotochromogen, Group 3, *M. intracellularis,* and *M. fortuitum.* They were freshly cultivated in Dubos medium.

Mycobacterial stains. At the time of initiation of this study, fluorescent facilities were not readily available to this laboratory so the Truant fluorescent technic was not utilized. Baker's method for phospholipids and the standard Ziehl-Neelsen stain for acid-fastness were employed after pyridine extraction following the methods described by Convit and Pinardi ([1]). Cryostat cut skin tissues as well as bacterial smears were used for *M. leprae* and *M. lepraemurium* while only bacterial smears were utilized for the other mycobacteria.

Additional procedures and variations of procedures were utilized consisting, in the main, of pyridine extraction with and without agitation for two hours and pyridine extraction for 24 hours. Following pyridine extraction, restoration of acid-fastness by treatment with periodic acid was attempted ([8]) and another set of slides were immersed in turpentine for two changes of 30 minutes each following the restoration method advocated by Wade ([12]). The procedural schema is summarized in Table 1.

Additional standard staining procedures were employed, these being: Fischler's stain for fatty acids, Grocott's methenamine-silver, and the Gram stain. The total battery of stains was applied to sections and/or bacillary smears from each mycobacterial strain as noted.

A subjective evaluation of degree of acid-fastness from 0 to 6+ was employed, taking into account both the degree of acid-fastness and the relative number of acid-fast bacilli. The values were individually plotted for each procedure as related to each microorganism.

Aged cultures. In addition to the fresh cultures noted above, available aged cultures of *M. fortuitum, M. intracellulare, M. scrofulaceum, M. phlei, M. bovis* (BCG), *M. kansasii, M. smegmatis,* and *M. tuberculosis* var *hominis* were studied for the effect of two hour fresh pyridine extraction on their acid-fast staining. These bacilli were obtained from Wallenstein, Tarshish or Ogawa (whole egg) plate media kept in a refrigerator (2°C) for 4.5 years. They were stained without recultivation. It was thought that these mycobacteria might give a closer comparison to tissue extracted bacilli which, to a considerable extent, are perhaps old or dead organisms. The viability of these old cultures was determined by culturing them again on fresh media.

TABLE 1. *Procedural schema.*

FIXATION AND EXTRACTION PROCEDURES		With pyridine extraction						Without pyridine extraction Formol-Ca
		Bouin's + formol-Ca			Formol-Ca only			
		SET 1A	SET 1B	SET 1C	SET 2A	SET 2B	SET 2C	SET 3
Bouin's	1 hr	X	X	X				
70% EtOH	5 min	X	X	X				
50% EtOH	5 min	X	X	X				
H₂O	2 min	X	X	X				
Formol-Ca	1 hr				X	X	X	X
H₂O	10 min				X	X	X	
Pyridine	2 hr	X			X			
Pyridine with agitation	2 hr		X			X		
Pyridine	24 hr			X			X	
H₂O	10 min	X	X	X				
Formol-Ca	1 hr	X	X	X				
H₂O	10 min	X	X	X	X	X	X	X

RESULTS

Control: without extraction or oxidation.
All bacilli stained 4+ to 5+ with Ziehl-Neel-
sen stain except for *M. fortuitum* which
showed a relatively low degree of acid-
fastness.

With the Baker phospholipid stain only
M. leprae and *M. lepraemurium* stained
moderately well and the scotochromogen
gave a slight reaction.

Fischler's fatty acid stain was moderately
positive for *M. leprae* and *M. lepraemurium*
but negative for all the others.

Grocott's methenamine-silver stained all
bacilli though *M. phlei* stained relatively
weakly and *M. fortuitum* stained strongly.
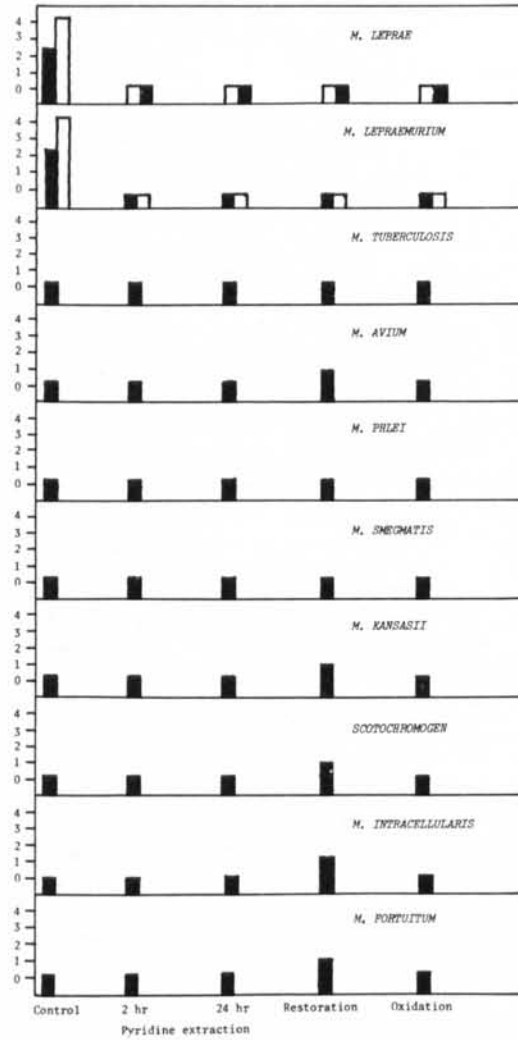
Grocott methenamine-silver and Gram

stains. Neither of these stain results were
affected by any method of pyridine extrac-
tion, or by the restorative or oxidation pro-
cedures.

**Fischler fatty acid and Baker phospholip-
id stains.** *M. leprae* and *M. lepraemurium*
both lost their staining capacity with pyri-
dine extraction (Fig. 1). These stain capaci-

FIG. 1. Baker's phospholipid staining. Solid
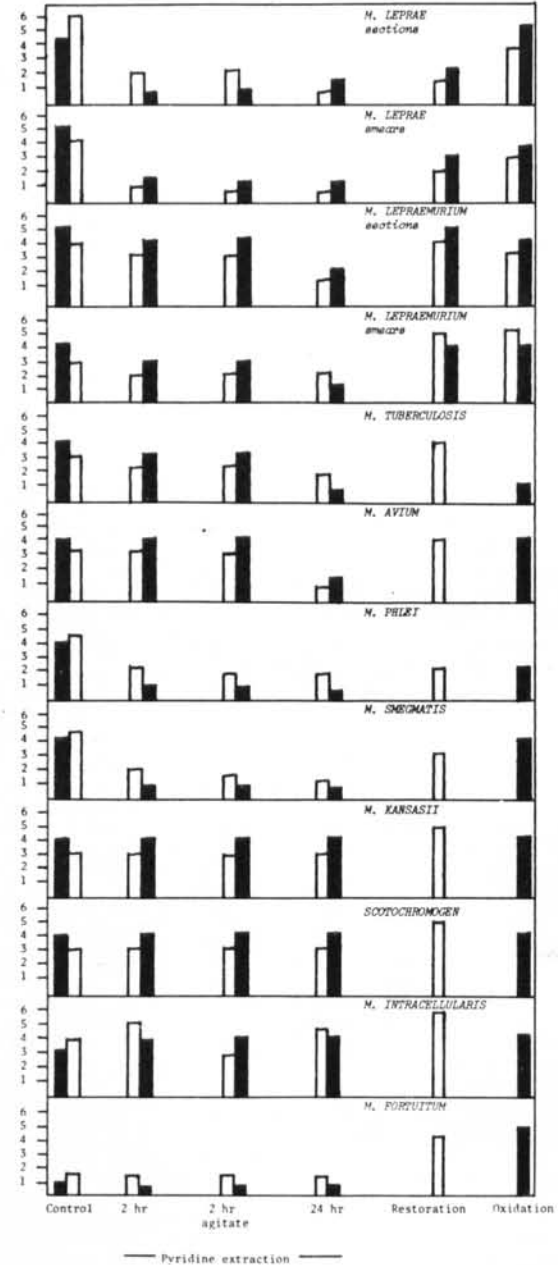bar, bacillary smears. Open bars, tissue section.

FIG. 2. Acid-fast staining. Solid bars, single
fixation with formol-Ca. Open bars, double fixa-
tion: first in Bouin's solution and after pyridine
extraction in formol-Ca.

ties were not restored by either Wade's restoration or by periodic acid treatment.

Acid-fast stain. The findings are summarized in Figure 2. *M. leprae, M. smegmatis,* and *M. phlei* all lost most or all of their acid-fast staining capacity with all three pyridine extractions save that, for some reason not readily evident, *M. phlei* retained more staining capacity when extraction was applied with as compared to without agitation. The other strains did not lose this capacity on extraction save that with 24 hour extraction *M. avium* and *M. tuberculosis* to some degree also lost acid-fastness.

Whether or not the specimens were fixed only in formol-calcium or in Bouin's solution followed by formol-calcium seemed to make no essential difference.

After 24 hour pyridine extraction all strains which had lost acid-fastness or which were very weakly acid-fast in control *(M. fortuitum)* showed restoration of acid-fastness with turpentine or with periodic acid treatment.

Restoration and oxidation. As noted in Figure 2, there was variability in the reestablishment of acid-fast quality by both Wade's restoration method and by periodic acid, though for most bacilli acid-fastness was significantly restored by both procedures. Periodic acid was more effective than Wade's technic for *M. leprae* and also *M. lepraemurium.*

Viability and staining of mycobacteria refrigerated 4.5 years. The bacilli were inoculated in Dubos medium and LA-3 medium

([11]) and observed for a period of 16 days. At this time *M. fortuitum, M. intracellulare, M. bovis* (BCG) and *M. kansasii* showed 6+, 4+, 6+ and 6+ growth respectively on the LA-3 medium while the other cultures showed no growth. On Dubos medium only *M. kansasii* showed 1+ growth, while *M. fortuitum, M. intracellulare* and *M. smegmatis* presented ± growth and the remaining mycobacterial strains showed no growth (Table 2). At six weeks there was no change in viability status. There was no essential difference in acid-fast extractability by pyridine between these aged cultures and the fresh cultures studied.

Effect of second pyridine extraction. The results of a second pyridine extraction following acid-fast restoration by oxidation after a previous, initial pyridine extraction are presented in Table 3. The restoration procedure consisted of oxidation with 10% periodic acid. *M. leprae* in the "old bacilli" columns were either biopsy sections or smears of bacilli from such sections, whereas *M. leprae* in the "fresh culture" columns consisted of bacilli from strain HI-75 grown in LA-3 medium as previously reported ([11]). The cultures were all harvested at 21 days.

The results were quite variable between the various bacillary types. Fresh cultures of *M. tuberculosis, M. phlei, M. smegmatis* and *M. fortuitum* demonstrated periodic acid-fastness which was susceptible to pyridine extraction but *M. avium, M. kansasii, M. scrofulaceum* (scotochromogen) readily lost their restored acid-fastness. Alleged *M. lep-*

TABLE 2. *Viability and pyridine acid-fast extractability in aged cultures.*

	Refrigerator age[a]	Culture viability[b]		After pyridine	
		Dubos	LA-3	Acid-fast	Blue
M. tuberculosis var *hominis*	4.5 years	−	−	4+	−
M. avium	4.5 years			4+	+
M. phlei	4.5 years	−	−	±	4+
M. smegmatis	4.5 years	−	−	±	4+
M. kansasii	4.5 years	+	6+	4+	−
M. scrofulaceum	4.5 years	−	−	4+	−
M. intracellularis	4.5 years	±	4+	4+	+
M. fortuitum	4.5 years	−	6+	−	4+
M. bovis (BCG)	19 months	−	6+	2+	2+

[a] 0°C.

[b] 16 days culture at 37°C.

rae cultures on LA-3 medium demonstrated retention of the acid-fastness induced by oxidation though this acid-fastness was not strong at this stage of cultivation in this particular culture. The amount of aeration is directly related to the development of acid-fastness. In general there seemed to be no significant difference between the 4.5 year old cultures and the 21 day old nonleprosy cultures with respect to their acid-fastness and the pyridine extractability of this acid-fastness after periodic acid treatment.

Pyridine acid-fast extractability of LA-3

cultured M. leprae. A culture of *M. leprae*, strain HI-75 grown in LA-3 medium ([11]) was examined 14 times during a growth period of 60 days for its acid-fast property and pyridine extractability of its acid-fast quality. The culture was grown in 50 ml of LA-3 medium in a 125 ml Erlenmeyer flask and was regularly aerated by shaking to a froth. Staining was by standard Ziehl-Neelsen technic. The technic of pyridine extraction was that reported by Convit and Pinardi ([1]). The results are listed in Table 4.

Acid-fastness of the culture was well es-

TABLE 3. *Repeat pyridine extraction after periodic acid treatment.*

Mycobacteria	Old Bacilli[c]				Fresh Culture Bacilli[d]			
	Oxidation following pyridine		Second pyridine following oxidation		Oxidation following pyridine		Second pyridine after oxidation	
	Acid-fast	Blue	Acid-fast	Blue	Acid-fast	Blue	Acid-fast	Blue
M. leprae, section[a]	4+		1+					
M. leprae, smear[a]	4+		4+					
M. tuberculosis	3+		3+		4+		1+	3+
M. avium	4+		3+	1+	4+		4+	
M. phlei	3+	1+	±	4+	2+	2+		4+
M. smegmatis	1+	3+	1+	3+	3+	1+	1+	4+
M. kansasii	4+		4+		4+		4+	
M. scrofulaceum	4+		4+		4+		4+	
M. intracellularis	4+		4+		4+		2+	2+
M. fortuitum	2+	2+		4+	3+	1+		4+
M. bovis (BCG)					3+		1+	2+
M. leprae[b]					2+		2+	2+

[a] From fresh skin biopsy.
[b] From LA-3 culture.
[c] Plate cultures refrigerated 4.5 years, as in Table 2.
[d] 21 day cultures.

TABLE 4. *Pyridine extractability of acid-fastness of cultured* M. leprae.

Age of culture (days)	Acid-fast staining control	Acid-fast stain after pyridine
3	2+	2+
5	2+	2+
10	3+	3+
13	4+	4+
17	3+	3+
20	3+	2+
24	3+	2+
27	4+	2+
31	4+	2+
35	4+	+
40	4+	+
46	3+	+
53	3+	±
60	3+	±

tablished by day 10 and continued throughout though with slight diminution by day 46. This is part of the growth cycle which will be separately reported in detail. The acid-fastness resisted pyridine extraction during the early phase of the culture but became partially extractable by day 20. By day 27, bacillary granularity and the appearance of short forms became evident and by day 46 there was slight tinctorial loss in the stained bacilli. Pyridine extractability of the acid-fast quality became pronounced by the 35th day and was virtually complete by days 53 and 60. Possible associated changes in viability are under study.

DISCUSSION

These studies may help to clarify the use of pyridine extraction of the acid-fast characteristic in the identification of *M. leprae*. Since at least a few other mycobacteria, in this study our strains of *M. smegmatis* and *M. phlei,* present a similar characteristic it seems premature to assume that only *M. leprae* of all known mycobacteria completely loses its acid-fast staining capacity after pyridine extraction. We have been unable to find reported studies of this nature covering all known mycobacteria. Most particularly, attempted isolation of *M. leprae* from human tissues has been fraught with the hazard of isolation of previously unrecognized mycobacterial strains which have eventually been found not to be *M. leprae*. To accept the pyridine extraction test as proof positive of the presence of *M. leprae* would seem chancy. It would seem that the most that can be claimed on the basis of experience thus far is the consideration that if a given mycobacterial strain does not show such loss of staining capacity after pyridine extraction, then it probably is not *M. leprae*. Here also there seems to be some reservation, in that Fisher and Barksdale ([4, 5]) found this not to be true in 5% of bacillary strains isolated from leprosy patients and, as noted, we have had dissident experience with some mycobacteria.

It is evident that there are many variable factors in the property of acid-fastness and its staining. The dominant trend in the employment of the acid-fast stain in leprosy work has been successive and successful attempts to enhance acid-fast staining. In this sense, some acid-fast staining may be arti-

factual. Less attention has been paid to the possibility that subtle variations in mycobacterial life forms may be significant. Thus, chromophobe organisms can be rendered acid-fast on methylene blue staining by manipulation of the pH and other factors. Bacilli may be "chromophobe" simply because conditions for their staining are not right and this could be related to the life and maturation of the bacilli, and not necessarily to poor staining technic. Bacilli are not ultrastructural "logs" that periodically break in half to achieve reproduction. They are responsive to their environment and may have an adaptive cycle.

These considerations are open to question when related to the identification of possible *in vitro* cultivated *M. leprae*. Mori and associates ([3, 6]) found that the total lipids of mycobacteria may be varied by the composition of the culture media used and by the conditions of cultivation, i.e., whether by surface culture or shake culture. It is not beyond reason to anticipate the possibility that the nature and quality of lipids may also be varied to some degree by the conditions of cultivation, particularly by the composition of the medium.

It is also quite possible that cultivated *M. leprae* may show variations in the quality of acid-fastness related to the length of cultivation, i.e., to the age of the bacilli. The determination of acid-fastness is a variable procedure which can be greatly influenced just by altering the pH of the reagents and by other manipulations. The application of periodic acid will either enhance or restore conventional acid-fast staining. Whether or not this be "acid-fastness" in the usual sense is questionable since it is probably based on a different chemical reactivity than that involved in conventional acid-fast staining. While the latter is probably related to a fuchsin-retaining material ([4]), periodic acid apparently oxidizes cell wall polysaccharides, and possibly lipids, to yield aldehyde linkages which accept the stain ([9]). Though regular acid-fastness has long been associated with the presence of mycolic acid, it is not unlikely that it may also relate to other lipids. In our experience, it seems that the oxidation of unsaturated lipids to a saturated state may also be a mechanism of developing acid-fastness. The presence of a variety of oxidants in skin and other tissues ([13, 14]) may
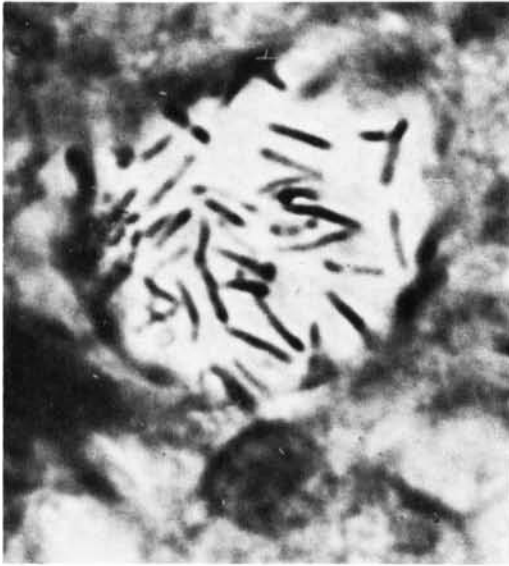
FIG. 3. Acid-fast stain of globus from skin biopsy. Black bacilli are acid-fast. Lighter bacilli stained blue.

thus play a role in maturation in the life cycle of *M. leprae.*

There is a strong probability that there is such a cycle with *M. leprae.* Careful differential Ziehl-Neelsen staining of lepromatous skin biopsies with Loeffler's methylene blue at pH 10 readily reveals distinct tinctorial differences among bacilli within a single globus (Fig. 3). Some bacilli stain a brilliant acid-fast and others stain blue only. This need not be regarded as a failure in proper application of the acid-fast stain, rather it may be a more revealing use of stain procedure. These differences are not dependent on beading or granularity, the latter characteristics seeming to be manifestations predominantly in the acid-fast forms as contrasted with the blue staining forms as they are observed in untreated LL cases. Other observations, to be reported separately, indicate that the blue staining bacilli are the younger viable forms, whereas the acid-fast rods are older and even though solid staining, may often be nonviable. If it be assumed that the generation time in the human host is in the general range of the 12–13 days reported for bacilli in the mouse foot pad, then most of the acid-fast bacilli found in tissues of untreated cases may be relatively old and possibly nonviable organisms, even though solid staining. Their ready loss of acid-fast-

ness, as compared to bacilli actively growing in culture, may also be at least in part a function of age and even of lost viability. In any case, the studies thus far reported seem not to have considered the possible incongruity of comparing characteristics of actively growing *in vitro* mycobacterial cultures with leprosy bacilli isolated from tissues, particularly in as chronic and slowly developing disease as leprosy. It is recognized that the studies of aged cultures herein reported are not strongly supportive of this concept with respect to the mycobacteria studied. On the other hand, the studies employing an alleged culture of *M. leprae* supported the concept.

On the basis of considerations such as these, the utilization of pyridine extraction of acid-fastness from cultivated mycobacteria as a criterion of their identity as *M. leprae* must be regarded with caution. This is particularly so if the cultivated bacilli should be found to grow more rapidly on a suitable medium than they grow in human or susceptible animal tissues. This is certainly true of the recently alleged cultures of *M. leprae* ([11]). The lack of pyridine extraction sensitivity of *M. lepraemurium,* also derived from prolonged presence in host tissues, suggests however a measure of counter-caution to this interpretation. In this instance, nevertheless, the argument may still hold since bacilli obtained from infected mice are participant in a relatively progressive disease phase as compared to the large number of very old bacilli in the tissues of longer duration human disease. The murine lesions are usually harvested within months whereas human biopsied disease may have been present for years. The finding ([2]) that *M. leprae* from growth in the armadillo yield pyridine extractable bacilli may be related to the fact that the bacilli used were from 31 and 26 month old infections, apart from the possibility still remaining that there actually is some, as yet unidentified, responsible structural or chemical acid-fast related difference between *M. leprae* and other mycobacteria.

SUMMARY

The reportedly unique pyridine extractability of acid-fastness as an identifying characteristic for *M. leprae* was examined in the leprosy bacilli and in eight other strains

of mycobacteria. The initial findings were, in general, in accord with previous reports except that *M. smegmatis* and *M. phlei* likewise demonstrated two hour pyridine extractability of acid-fastness. Perhaps, more significantly, it was found that this characteristic in *M. leprae* is related to aged, probably nonviable bacilli. Some other strains of mycobacteria when tested in aged cultures showed the same phenomenon while *M. leprae* cultivated *in vitro* in a recently developed medium resisted pyridine extraction up to three weeks of growth, but thereafter as the culture aged pyridine extractability became characteristic. It is concluded that this pyridine extractability of acid-fastness is a characteristic of aging or nonviable bacilli. As such it is not definitive in the determination of whether or not *in vitro* cultivation of *M. leprae* has been achieved.

Les premiers resultats étaient, d'une manière générale, en accord avec les travaux antérieurs à l'exception des *M. smegmatis* et *M. phlei* qui, tous les deux, montraient un épuisement de deux heures par la pyridine. Un point peut-être plus significatif est que ce trait caractéristique du *M. leprae* était trouvé lié aux bacilles vieillis, non viables. Quand on étude d'autres souches de mycobacteria dans des cultures vieillies, elles montraient le même phénomène tandis que *M. leprae*, cultivé *in vitro* dans un mileu récemment mis au point, résistait à l'épuisement par la pyridine jusqu'à trois semaines de culture, mais après cette période, à mesure que la culture vieillit, l'épuisement par la pyridine devint caractéristique. On conclut donc que l'épuisement de la résistance à l'acide par la pyridine est une caractéristique des bacilles non viables ou de viellissement. C'est pourquoi on ne peut compter sur cette caractéristique pour déterminer de façon conclusive si, oui ou non, la culture *in vitro* du *M. leprae* a été réussie.

RESUMEN

La extractabilidad con piridina de la ácido-resistencia se ha tomado como una característica peculiar que permite la identificación del *M. leprae*. En este trabajo se ha examinado esta propiedad de la piridina sobre la ácido-resistencia del *M. leprae* y de otras ocho cepas micobacterianas. En general, los hallazgos iniciales estuvieron de acuerdo con publicaciones anteriores excepto que también pudo ser extraída la ácido-resistencia del *M. smegmatis* y del *M. phlei* por tratamiento con piridina durante dos horas. Quizá el hallazgo más importante fue que la capacidad de la piridina para extraer la ácido-resistencia del *M. leprae* estuvo relacionada c'n la edad del bacilo y probablemente sea una propiedad de los bacilos no viables. Este mismo fenómeno se encontró en cultivos viejos de algunas otras cepas de micobacterias. El *M. leprae* cultivado *in vitro* en un medio recién desarrollado mostró resistencia a la extracción con piridina durante las primeras tres semanas de crecimiento pero después de ese tiempo, conforme el cultivo envejecía, la extractabilidad con piridina llegó a ser característica. Se concluye que la extractabilidad de la ácido-resistencia con piridina es una característica de los bacilos viejos no viables y como tal, no es definitiva para determinar si se ha logrado o no el cultivo *in vitro* del *M. leprae*.

RÉSUMÉ

On a étudié l'épuisement par la pyridine de la résistance à l'acide chez le bacille lépreux et chez huit autres souches de mycobacteria; le caractère d'épuisement était considéré comme unique dans l'identification du *M. leprae*.

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