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Adenosine Triphosphate Content of *Mycobacterium leprae*: Effect of Purification Procedures[1]

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Mycobacterium leprae, the causative agent of human leprosy, has been considered as an "obligate intracellular microbe" since it has not yet been grown in cell cultures or in bacteriological media. If investigations leading to *in vitro* cultivation of *M. leprae* are to be efficient, one should have tissue suspensions containing only *M. leprae*, and these cells should possess originally essentially the same levels of metabolic activity and infectiousness as those present in the cells while they were growing in the host.

Because of an inadequate supply of *M. leprae*, *Mycobacterium lepraemurium*, the causative agent of rat leprosy, have been used as an interim model of *M. leprae*. *M. lepraemurium*, like rickettsia ([11]), have been considered as leaky organisms when transferred from their natural host to an *in vitro* environment ([3,4,6]). In such cases, separation of these intraphagosomal agents from their host by rigorous washing or even by dilution and mild washing causes damage to their cell membranes, resulting in loss of their internal metabolic pools. In order to quantitate the impairments caused by these agents and manipulations, Dhople and Hanks have used adenosine triphosphate (ATP) as a biochemical indicator ([4,6,7,8]) because of the ubiquity of this compound in all living cells.

Since the first report ([10]) on the development of disseminated infection in nine-banded armadillos (*Dasypus novemcinctus*) after inoculation with *M. leprae*, the scientific world has been given an opportunity to obtain adequate quantities of *M. leprae* for sophisticated microbiological investigations. Dhople and Hanks ([8]) reported, for the first time, on the ATP content of *M. leprae* harvested from such armadillos. The present report concerns the effects of various procedures applied to separate *M. leprae* free of host tissue on the ATP content of *M. leprae*.

MATERIALS AND METHODS

Bacilli. *M. leprae* were obtained from three different sources: a) nine-banded armadillos infected with *M. leprae*, b) a nude mouse inoculated into the foot pad with *M. leprae*, and c) a human leprosy patient. Livers, spleens, and lymph nodes from armadillos were obtained from Dr. Kirchheimer (National Hansen's Disease Center, Carville, Louisiana), Dr. Meyers (A.F.I.P., Washington, D.C.), and also from this institute. The foot pad of a nude mouse infected with *M. leprae* was obtained from Dr. Hastings (National Hansen's Disease Center, Carville, Louisiana), while Dr. Nelson (University of Illinois, Chicago, Illinois) provided a skin biopsy specimen from a leprosy patient in Thailand. *M. lepraemurium* were obtained from the livers of mice infected earlier with the Hawaiian strain of *M. lepraemurium*.

The tissues from infected armadillos obtained from Dr. Kirchheimer and Dr. Meyers, as well as those used from this institute, and also the foot pad of a nude mouse from Dr. Hastings had been stored at −76°C. The tissues from outside sources were transported to this institute on dry ice and were again stored at −76°C until use. The biopsy specimen from a leprosy patient from Thailand was fresh; it was shipped to this Institute on dry ice and was stored at −76°C until use. The livers of mice infected with *M. lepraemurium* were also stored at −76°C. All tissues were thawed by immersing the containers in water at room

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temperature for 15 min and processed immediately.

Suspensions of both *M. leprae* and *M. lepraemurium* were prepared by homogenizing the tissue in five times its volume of either 0.05 M phosphate buffer, pH 7.0, or a mixture ($Alb_{10}YS_{10}$, pH 7.0) containing 10% (w/v) bovine serum albumin, fraction V (Alb_{10}) and 10% (v/v) Difco yeast supplement B (YS_{10}) using a Ten Broeck glass homogenizer. The suspension was then centrifuged at 4°C for 6 min at 200 × *g* to sediment large tissue particles. The supernate was then removed and used for all experimental procedures, after appropriate dilutions as described in the results section.

In vitro grown *M. lepraemurium* and *M. marinum* and the livers and spleens of noninfected armadillos were used as controls. The Hawaiian strain of *M. lepraemurium* was grown at 38°C in modified NC-5 medium (5) and maintained in the same medium by serial transfers every 4 weeks. *M. marinum* (TMC 1218) was grown at 37°C in Middlebrook 7H9 broth containing 10% oleic albumin complex. Suspensions of spleens and livers from noninfected armadillos were prepared in the same way as those of their infected counterparts, keeping the weights and volumes identical.

Purification Procedures. 1) Washing: The diluted suspensions (10–20 × 10^6 AFB/ml) were centrifuged at 4°C for 20 min at 3,000 × *g*, and the sediments washed in 0.05 M phosphate buffer, pH 7.0, and centrifuged again similarly. The sediments were then used for ATP assays, microscopic counts, and mouse foot pad inoculations.

2) Decontamination: Dilute suspensions (as above) were subjected to three different decontamination procedures normally adopted to isolate mycobacteria from clinical specimens ([1]). These were a) treatment with 4% w/v NaOH for 20 min at 37°C, neutralizing with 1 N HCl and washing in 0.05 M phosphate buffer, pH 7.0; b) treatment with 5% w/v oxalic acid for 30 min at room temperature, neutralizing and then washing in phosphate buffer; and c) digesting with saturated trisodium phosphate containing 0.03% w/v zephrian for 20 min at room temperature, and then washing with phosphate buffer.

3) Enzyme treatment: The method of Talwar, *et al.* ([16]) of incubating suspensions for 30 min at room temperature in the presence of 0.25% w/v trypsin was followed. This procedure was further modified by using a) 0.1% each of trypsin and chymotrypsin, or b) 0.1% each of trypsin, chymotrypsin, and collagenase.

4) Removal of non-bacterial ATP: This was accomplished by using a non-ionic detergent ([12]). Suspensions (10–20 × 10^6 AFB per ml) were treated first with Triton X-100, in a final concentration of 0.1% for 10 min. The non-bacterial ATP released in this manner was thus made accessible to hydrolysis by ATPase (Sigma Chemical Company, St. Louis, Missouri) at a final concentration of 0.17% containing 0.005 M $CaCl_2$, the latter used as a cofactor usually required for ATP hydrolyzing enzymes. The suspensions were then centrifuged and washed in phosphate buffer before using for any of the assays.

Assay methods. Microscopic counts were performed by the pin head method of Hanks, *et al.* ([9]). The method of Dhople and Hanks ([2]), using chloroform and heat, was adopted to extract the bacterial ATP. The final extract was suspended in 0.4 ml of 0.05 M TRIS buffer, pH 7.7, containing 0.01 M $MgSO_4$. This extract (0.1 ml) was injected with a one ml tuberculin syringe into 0.1 ml of a luciferin-luciferase acceptor system. The peak height of the reactions was measured and compared with that obtained with a standard ATP solution subjected to a similar extraction procedure as applied to the bacterial suspension.

The luciferin-luciferase mixture of DuPont Company (Wilmington, Delaware) was used. The contents of one vial of the lyophilized mixture was dissolved in 3 ml of 0.05 M TRIS buffer, pH 7.7, containing 0.01 M $MgSO_4$. This was further supplemented with pure, crystalline luciferin (Sigma Chemical Co., St. Louis, Missouri) to a final concentration of 0.5 mg per ml. The mixture was stored at 4°C in the dark for 18–24 hr; 0.1 ml of this was then pipetted into 6 × 50 mm disposable glass cuvettes before injecting the ATP extract.

The Chem-Glow Photometer (American Instrument Company, Silver Spring, Maryland), with a 6 × 50 mm cuvette adapter, was used in these studies. The read-out was

TABLE 1. *ATP content of* M. leprae *and* M. lepraemuriam *before and after washing.*[a]

Organism	Source	No. of specimens	Stock suspension in	Before washing		After washing	
				Picograms	%	Picograms	%
M. leprae	Armadillo Nude mouse	4 1	Phosphate buffer[b]	1.40 ± 0.067 (1.30–1.47)[c]	100	1.36 ± 0.078 (1.26–1.46)	97
M. leprae	Armadillo	4	$Alb_{10}YS_{10}$[d]	1.41 ± 0.101 (1.30–1.53)	100	1.37 ± 0.105 (1.26–1.51)	97
M. lepraemurium	Mouse	3	Phosphate buffer	2.37 ± 0.145 (2.22–2.51)	100	1.37 ± 0.148 (1.27–1.54)	58
M. lepraemurium	Mouse	3	$Alb_{10}YS_{10}$	2.80 ± 0.161 (2.62–2.93)	100	2.02 ± 0.183 (1.81–2.16)	72
M. lepraemurium	*In vitro*	3	Phosphate buffer	2.83 ± 0.161 (2.68–3.00)	100	2.77 ± 0.161 2.62–2.94	98
M. marinum	*In vitro*	3	Phosphate buffer	8.22 ± 0.320 (7.91–8.55)	100	8.13 ± 0.355 (7.77–8.48)	99
Non-infected tissue[e]	Armadillo	3	Phosphate buffer	0		0	

[a] Washing with 0.05 M phosphate buffer, pH 7.0, by centrifugation.
[b] Phosphate buffer = 0.05 M phosphate, pH 7.0.
[c] Data presented as mean ± S.D. (range) picograms of ATP per 10^6 cells.
[d] $Alb_{10}YS_{10}$ = 10% w/v bovine serum albumin and 10% v/v Difco yeast supplement, pH 7.0.
[e] Suspensions treated identically as infected tissue suspensions.

monitored by a strip chart recorder (Linear Instrument Corporation, Irwin, California).

The sterility of each suspension was checked on Löwenstein-Jensen medium, Middlebrook 7H11 medium, nutrient broth, trypticase soy broth and thioglycolate medium, both at 30° and 37°C. The viability of *M. leprae* before and after each step was evaluated by inoculating them into foot pads of BALB/c mice according to the method of Shepard ([15]).

For each step, a minimum of three experiments were performed, each with different tissue specimens, and the data were pooled.

RESULTS

Effect of washing. In this set of experiments, tissues with high bacterial counts ($1–3 \times 10^{11}$ AFB per gram) were utilized. Stock suspensions, after low speed centrifugation, were diluted 2000–4000-fold. One set of aliquots (1 ml each, containing $10–20 \times 10^6$ AFB) was used directly for ATP assays and microscopic counts, while another set was subjected to the washing procedures. The results presented in Table 1 suggest the following points:

a) The ATP content of rapidly growing *M. marinum* is highest among the three species tested (8.22 picograms per 10^6 cells).

b) The ATP content of *in vivo* grown *M. lepraemurium* (suspended in $Alb_{10}YS_{10}$) is the same as its *in vitro* grown counterpart.

c) The ATP content of *in vivo* grown *M. lepraemurium* is lower when prepared in phosphate buffer than when prepared in $Alb_{10}YS_{10}$, because of leakiness of the organism.

d) When suspensions from non-infected armadillo tissues were prepared in identical ways as those of infected ones, no ATP can be detected in equivalent aliquots.

e) The ATP content of *M. leprae* is the same whether prepared in phosphate buffer or in $Alb_{10}YS_{10}$ and is approximately 50% that of *M. lepraemurium*.

f) When suspensions are washed, only *M. lepraemurium* loses a substantial amount of its original ATP.

The *M. leprae* suspensions, both before and after the washing procedure, were diluted and 6×10^3 AFB inoculated into each hind foot pad of mice ([15]). Six months later, foot pads were harvested to assess the growth of the inoculated *M. leprae*. Inocula from both the suspensions gave standard growth curves (yields of $\pm 1 \times 10^6$ AFB per foot pad), thus indicating no loss of infec-

TABLE 2. *ATP content of* M. leprae *and* M. lepraemurium *before and after decontamination with 4% w/v NaOH for 20 min at 37°C.*

Organism[a]	Source	No. of specimens	Sterility test[b]		Before		After	
			Before	After	Picograms	%	Picograms	%
M. leprae	Armadillo	4	+[c]	–	1.31 ± 0.055 (1.23–1.35)[d]	100	1.29 ± 0.055 (1.20–1.32)	98
M. lepraemurium	Mouse	3	+	–	2.41 ± 0.130 (2.28–2.54)	100	1.76 ± 0.026 (1.73–1.78)	73

[a] Both *in vivo* grown and prepared in 0.05 M phosphate buffer, pH 7.0.
[b] Sterility test: on nutrient broth, thioglycolate medium, Lowenstein-Jensen medium and Middlebrook 7H11 medium.
[c] No growth on Löwenstein-Jensen and Middlebrook 7H11 medium.
[d] Data presented as mean ± S.D. (range) picograms of ATP per 10^6 cells.

tiousness of *M. leprae* due to the washing procedures.

When livers and spleens of infected armadillos with lower bacterial counts ($1–2 \times 10^{10}$ per gram) were used in similar studies, the ATP content of one million *M. leprae* cells was the same as with armadillo tissues of high bacterial counts.

Decontamination. Tissues with bacterial loads of $2–3 \times 10^{10}$ AFB per gram were used. The suspensions, after low speed centrifugation, were inoculated on nutrient agar at different dilutions. It was determined that for each gram of tissue (or for each $2–3 \times 10^{10}$ AFB) there were approximately 4×10^3 contaminating (non-acid fast) organisms. Thus, after appropriate dilution, an aliquot with about 20×10^6 AFB should contain only four contaminating organisms. The data presented in Table 2 clearly suggest that sodium hydroxide is capable of decontaminating the *M. leprae* suspension and at the same time has no adverse effect on the ATP content of *M. leprae* (98% recovery). On the other hand, under identical conditions, *M. lepraemurium* lost 27% of its original ATP. Both oxalic acid and zephiran were equally successful in decontaminating *M. leprae* suspensions, but the loss of bacterial ATP due to either of the treatments was about 10% (data not shown). After treatment with NaOH *M. leprae* suspensions gave standard growth curves 6 months after inoculation into mouse foot pads.

Enzyme treatment and removal of non-bacterial ATP. For these studies armadillo tissues containing $1–2 \times 10^9$ AFB per gram were selected. Stock suspensions, prepared in phosphate buffer, after low speed centrifugation were diluted to obtain approximately 20×10^6 AFB per aliquot of 1 ml. Aliquots were first decontaminated with NaOH and then incubated with trypsin, with and without the other two enzymes, before being treated with Triton X-100 and ATPase. The results are presented in Table 3. As expected, *M. leprae* suspensions with low bacterial counts had higher ratios of tissue to bacteria, and, therefore, all non-bacterial ATP was not eliminated by merely diluting the suspensions as before. This is reflected in items 1 and 1′ under column A of Table 3. However, when the suspensions were treated with trypsin, approximately 85% of the non-bacterial ATP was eliminated, as seen from items 2′, 3′, and 4′ under column A, and this is further reflected in items 2, 3, and 4 under the same column (using infected tissues). Triton X-100 with ATPase, without pre-treatment with trypsin, was not capable of removing all the non-bacterial ATP (items 1 and 1′ under columns B and C). Again, suspensions after treatment with enzyme and then with Triton X-100 plus ATPase were inoculated into mouse foot pads. Both gave standard growth curves 6 months later.

ATP content of *M. leprae* from human biopsy material. The above procedure was applied to the *M. leprae* suspension prepared from human biopsy material (1×10^9 AFB per gram), and the results are presented in Table 4. Again NaOH was able to decontaminate the suspension. Triton X-100 plus ATPase alone was not able to remove all non-bacterial ATP (Item 1), and even pre-treatment with trypsin and chymotryp-

TABLE 3. *ATP content of* M. leprae *and non-infected tissue suspensions. Effect of purification.*

Treatment	pg ATP/ml suspension[b]			pg ATP/10^6 bacterial cells		
	A[c]	B[d]	B′[e]	A[c]	B[d]	B′[e]
M. leprae suspension[a]						
1) No treatment	46.0 ± 3.523 (41.7–50.1)	29.5 ± 2.602 (25.2–32,2)	28.0 ± 2.546 (23.6–30.2)	2.57 ± 0.174 (2.36–2.77)	1.78 ± 0.188 (1.60–2.00)	1.72 ± 0.201 (1.53–1.94)
2) NaOH + trypsin	24.3 ± 1.616 (22.2–26.0)	19.7 ± 1.839 (16.7–21.7)	19.6 ± 1.751 (16.7–21.4)	1.51 ± 0.090 (1.41–1.64)	1.36 ± 0.071 (1.29–1.46)	1.32 ± 0.060 (1.26–1.40)
3) NaOH + trypsin + chymotrypsin	24.4 ± 1.770 (22.1–25.9)	19.2 ± 1.853 (16.2–21.3)	19.2 ± 1.923 (16.1–21.4)	1.52 ± 0.086 (1.43–1.61)	1.35 ± 0.064 (1.28–1.44)	1.30 ± 0.066 (1.22–1.39)
4) NaOH + trypsin + chymotrypsin + collagenase	23.1 ± 1.630 (21.0–25.0)	19.0 ± 1.802 (16.4–21.2)	19.0 ± 1.751 (16.4–21.1)	1.48 ± 0.071 (1.40–1.55)	1.32 ± 0.058 (1.25–1.40)	1.30 ± 0.054 (1.24–1.37)
Non-infected tissue suspension[f]						
1′) No treatment	22.6 ± 1.530 (21.2–24.7)	7.6 ± 0.483 (7.2–8.3)	7.4 ± 0.465 (7.0–8.0)			
2′) NaOH + trypsin	3.2 ± 0.216 (3.0–3.5)	0	0			
3′) NaOH + trypsin + chymotrypsin	2.8 ± 0.183 (2.6–3.0)	0	0			
4′) NaOH + trypsin + chymotrypsin + collagenase	2.4 ± 0.141 (2.3–2.6)	0	0			

[a] Suspensions from five different infected armadillo liver specimens.
[b] Suspensions from infected tissues were prepared and diluted so that aliquots of 1 ml contained 10–20 × 10^6 *M. leprae*. Suspensions from non-infected tissues were prepared in a similar way so that volumes and weights were identical to those used in preparing suspensions from infected tissues.
[c] A = Direct assays.
[d] B = After treatment once with Triton X-100 + ATPase.
[e] B′ = After treatment twice with Triton X-100 + ATPase.
[f] Suspensions, from four different armadillo specimens, prepared from livers of non-infected armadillos and processed identically as those from infected armadillos above.

sin was not effective (Item 2). Figures from Item 3 demonstrate that, at least for human biopsy material, collagenase is also essential along with trypsin and chymotrypsin to release non-bacterial cells so that Triton X-100 (plus ATPase) will act on these cells to eliminate all the non-bacterial ATP. The suspensions from Item 3 gave standard growth curves when inoculated into mouse foot pads.

DISCUSSION

When intracellular agents are liberated from host cells into the extracellular environment, the *in vitro* environment is not always optimum. In order to define the effects of this artificial environment on such cells by assessing their physiological status, methods for bioluminescent determinations of ATP were developed by Dhople and Hanks ([2]). Using this biochemical indicator, they have demonstrated that *M. lepraemurium*, when removed from the livers of infected mice and placed in an *in vitro* environment, loses its ATP and hence its growth potential ([6]). This has further been confirmed in the present studies. However, *M. leprae*, while still being an "obligate intracellular microbe," is totally different from *M. lepraemurium* in leakiness, and the findings reported here have proved this point.

Shepard ([15]) has treated bacilli from the nasal passages of leprosy patients with NaOH and has shown that the bacilli multiply in mouse foot pads. Similar findings have re-

TABLE 4. *ATP content of* M. leprae *harvested from human biopsy material.*

Treatment	Sterility tests[a]	Picograms ATP/10^6 bacterial cells		
		A[b]	B[c]	B′[d]
1) No treatment	+[e]	7.82	4.48	4.19
2) NaOH + Trypsin + Chymotrypsin	–	1.89	1.54	1.43
3) NaOH + Trypsin + Chymotrypsin + Collagenase	–	1.34	1.18	1.17

[a] Sterility tests on nutrient broth, thioglycolate medium, Löwenstein-Jensen and Middlebrook 7H11 medium.
[b] A = Direct Assays.
[c] B = After treatment once with Triton X-100 + ATPase.
[d] B′ = After treatment twice with Triton X-100 + ATPase.
[e] Growth in nutrient broth and thioglycolate medium. No growth on Löwenstein-Jensen and Middlebrook 7H11 medium.

cently been shown by Prabhakaran and coworkers ([13, 14]). They have further shown that neither NaOH or trypsin diminishes the o-diphenol oxidase activity of *M. leprae*. The present studies, using ATP as an indicator of metabolic activity and growth potential, have again shown the relative lack of harmful effects of these procedures on *M. leprae*.

The ATP content of *M. lepraemurium* harvested from livers of infected mice has been the same as that of its *in vitro* grown counterpart, provided $Alb_{10}YS_{10}$ is used as a suspending medium. This suggests that when a stock suspension is diluted 4000-fold, essentially all the host-originated ATP has been eliminated. The same should also be true for *M. leprae* harvested from livers of infected armadillos showing heavy bacterial loads. Under identical conditions, the suspensions prepared from livers of non-infected armadillos failed to show the presence of ATP. Further, when the bacterial load in infected armadillo tissues was one log lower, the ATP content of *M. leprae* (expressed as ATP per 10^6 bacilli) was the same as that observed when the bacilli were harvested from heavily loaded livers. Indeed, when *M. leprae* were harvested from various armadillo tissues, even though bacterial loads varied from 10^{11} to 10^9 AFB per gram of tissue, the ATP content per 10^6 bacterial cells was between 1.3 and 1.4 picograms. The various steps employed to purify the suspensions free of host tissue and to eliminate all host-derived ATP seem to be adequate for this purpose. Otherwise, one should have seen large variations in the ATP content per 10^6 *M. leprae* when tissues with varying bacterial loads were used.

Similar results were obtained when *M. leprae* suspensions prepared from armadillo livers with low bacterial loads were treated with Triton X-100 plus ATPase. Under identical conditions, suspensions prepared from livers of non-infected armadillos showed no ATP at all. We have found that Triton X-100 has no effect on the electron microscopic structure of *M. leprae* (Dhople and Zeigler, unpublished data).

Even though only one human biopsy specimen was used to derive data on the ATP content of *M. leprae*, the procedure developed for armadillo tissues has applied here, and the ATP content of *M. leprae* has not been significantly different from that obtained from armadillo-derived *M. leprae*.

In short, in our judgment, the procedures used in the present studies have succeeded in eliminating essentially all the ATP of host-origin and the values reported are valid measurements of the ATP content of *M. leprae*.

SUMMARY

Various procedures to decontaminate and purify *M. leprae* free of host tissue material resulted in total retention of their intracellular ATP and also infectiousness. The ATP content of one million *M. leprae* cells, isolated from either livers, spleens, or lymph nodes of infected armadillos, or a nude mouse foot pad or a human biopsy specimen, was in the range of 1.17 to 1.40 picograms. Suspensions could be decontaminated with 4% NaOH and all non-bacterial ATP could be eliminated by the combined action of trypsin, chymotrypsin, and collagenase initially, followed by Triton X-100 plus ATPase. These findings further assure that *M. leprae* are different from *M. lepraemurium* in that they can withstand even the severest purification procedures that are necessary in order for them to be

used for sophisticated biochemical and metabolic studies.

RESUMEN

Varios procedimientos para descontaminar y purificar al *M. leprae* libre de material tisular del huésped, resultaron en una total retención de su ATP intracelular y también de su infecciosidad. El contenido en ATP por millón de células de *M. leprae* aisladas de hígados, bazos o ganglios linfáticos de armadillos infectados, o del cojinete plantar de ratones desnudos, o de una biopsia humana, estuvo en el rango de 1.17 a 1.40 picogramos. Las suspensiones pudieron ser descontaminadas con NaOH al 4%, y todo el ATP no bacteriano pudo eliminarse por la acción combinada de tripsina quimotripsina, y collagenase seguida de un tratamiento con Triton X-100 más ATPasa. Estos hallazgos reforzan la observación de que el *M. leprae* difiere del *M. lepraemurium* en que el primero resiste aún los procedimientos de purificación más severos que son necesarios para obtener bacilos utilizables en sofisticados estudios bioquímicos y metabólicos.

RÉSUMÉ

Divers procédés utilisés pour décontaminer et purifier *M. leprae* en lui enlevant tout matériel tissulaire provenant de l'hôte, ont entraîné une rétention complète de l'ATP intracellulaire; le caractère infectieux a été préservé. Après isolement à partir de foies, de rates, ou de ganglions lymphatiques de tatous infectés, de même qu'à partir de coussinets plantaires de la souris glabre ou d'échantillons de biopsies humaines, le contenu en ATP de un million de cellules de *M. leprae* allait de 1,17 à 1,40 picogrammes. Ces suspensions pouvaient être décontaminées par 4% de NaOH, et tout l'ATP non bactérien pouvait être éliminé par l'action combinée de la trypsine, de la chimotrypsine, de la collagènase et au début, après administration de Triton X-100 avec ATPase. Ces observations indiquent une fois de plus que *M. leprae* est différent de *M. lepraemurium*, en ceci qu'il peut supporter même les procédés de purification les plus poussés qui sont nécessaires dans le cadre d'études biochimiques et métaboliques fort élaborées.

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