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Limited *in vitro* multiplication of *Mycobacterium leprae:* application to screening potential antileprosy compounds

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Inability to cultivate *Mycobacterium leprae in vitro* has been regarded as the bottleneck which obstructs research on modes of transmission, pathogenesis, immunization and the treatment of leprosy. The bottleneck in the cultivation problem always has been that of gaining significant information from an organism that has given no useful response to intensive inquiries by the disciplines of biochemistry, metabolism or biology. The two mycobacterial pathogens have been regarded as 'obligate intracellular parasites', because the efforts of several investigators have failed to induce their growth in bacteriologic media. The work on *M. leprae* has been continuous since shortly after its discovery by Hansen in 1873, a period of 112 years. *Mycobacterium lepraemurium*, agent of murine leprosy, was noncultivable since its discovery by Stefansky in 1903 until the convincing report of Nakamura in 1972, a total period of 69 years.

Table 1 offers a comparison of pertinent properties of *M. leprae* and *M. lepraemurium*. Both species fail to grow in pulmonary tissues of natural and experimental hosts (occasionally, *M. leprae* grows slowly in lungs of infected armadillos). This property is interpreted to mean that the oxygen sensitivity of *M. lepraemurium* will also be encountered in *M. leprae*. The table also reveals why *M. lepraemurium* was used earlier as an interium model for *M. leprae*.

Table 2 describes the significance of adenosine triphosphate (ATP) in such studies. In order to investigate obligate intracellular bacteria one needs a method that measures immediately and directly the physiologic status of unwashed cells. Bioluminescent determinations of ATP qualify for several reasons. ATP measures energy levels that are fundamental to biosynthesis and growth. During growth the increase in ATP per culture should coincide with microscopic counts.[1]

Figure 1 demonstrates the utility of ATP data while cells are growing and, even more importantly, while their metabolic systems are deteriorating. Curves A and M show that during growth of *M. lepraemurium* in Nakamura's medium the ATP per culture and microscopic counts are equivalent. Curve B, with 67% air

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Table 1. Properties of *M. lepraemurium* and *M. leprae*.

Property	*M. lepraemurium*	*M. leprae*
Intracellular habitat	+	+
Growth in pulmonary tissue	−	−
Ratios of single bacterial cells from tissue	4+	1+
Generation time in mice (days)	7	14–21
Dissemination from focal lesions (mice)	4+	1+
Fatal disease in mice	+	−
Growth in cell culture	+	−
Growth in cell-free system	+	+

Table 2. Significance of ATP.

1 ATP pools are extremely labile, either used rapidly for biosynthesis, exchanged with related nucleotides, or in damaged cells, degraded rapidly by ATPase.

2 Under constant conditions (e.g. *in vivo*), the ATP pools within bacterial cells are controlled by the net balance between rates of generating energy and rates of biosynthesis. ATP per aliquot or per culture measures functional biomass or cell numbers.

3 Minimal levels of ATP suffice for energy of maintenance; maximal levels promote maximum growth rates. Thus, ATP per bacterial cell can rank suspension of a given species in terms of growth potential.

space per culture tube, demonstrates the oxygen sensitivity of *M. lepraemurium* and reveals why oxidation-reduction potential must be regulated.[2] Curves C and D show the results when *M. lepraemurium* are incubated in typical mycobacterial media such as Dubos or Middlebrook 7H10. Note that ATP data define 25% air space as superior to 67% air space, even when the organism's metabolic systems are failing rapidly.

In our cultivation studies with *M. leprae* we have adopted two other parameters, besides ATP, to monitor the cultures, and these are uptake of tritiated thymidine (^{3}H-thymidine) by *M. leprae* and intracellular content of deoxyribonucleic acid (DNA).

Recent advances have resulted in an understanding of the process of DNA replication in prokaryotes. DNA is replicated by a multi-enzyme complex containing a relatively large number of different proteins. The replication is both semi-conservative and discontinuous.[3,4]

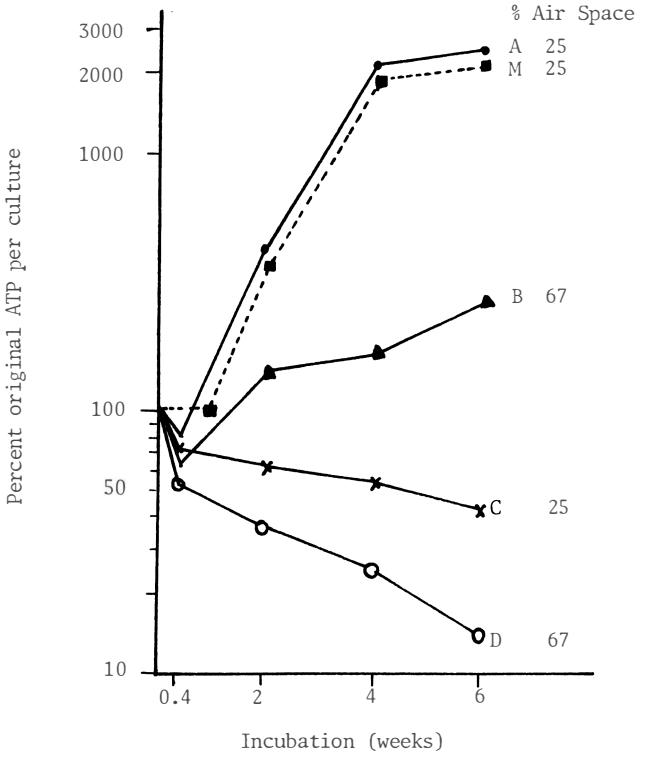

Figure 1. Significance of ATP determining in the cultivation studies of *M. leprae*. Data for *M. lepraemurium* incubated at 30°C.

Drutz & Cline[5] were the first to show that *M. leprae*, slowly metabolizing in human monocytes, are capable of incorporating ^{3}H-thymidine, presumably during synthesis of DNA, and provided an approach to evaluate at least the DNA-synthetic activity of this organism. Later Khanolkar *et al.*[6,7] found that *M. leprae* suspensions freed from mammalian cells would likewise incorporate ^{3}H-thymidine and also ^{3}H-DOPA. Subsequently, Nath *et al.*[8,9] showed that thymidine is incorporated by human-derived *M. leprae* in infected macrophages from mice and humans and used this technique to assess dapsone resistance of *M. leprae*. Since thymidine is a precursor of DNA but not RNA, its incorporation represents a fundamental correlate (DNA synthesis) with viability of *M. leprae*.

M. leprae suspensions were prepared by two different procedures, one by simply treating with trypsin[6] and the other by treating first with DNAase and then using Percoll density gradient.[10] These purified *M. leprae* were incubated at 34°C in presence of ^{3}H-thymidine. At various intervals samples were taken, washed either with saline and TCA or with TCA and methanol and counted in a scintillation counter. It was observed that uptake of ^{3}H-thymidine by *M. leprae*

was optimum after 24 hr. The best activity was obtained when the suspension was prepared by the Percoll gradient method and washed with TCA and methanol. With this the uptake of ^{3}H-thymidine by *M. leprae* was 0·12 pmol per 10 million *M. leprae* in 24 hr. It was interesting to note that under identical conditions uptake of ^{3}H-thymidine by *M. avium* and *M. tuberculosis* was very poor.

Incorporation of [^{3}H] thymidine into DNA: In order to use the [^{3}H] thymidine as an indicator of bacterial viability and DNA synthesis, it is important to determine if the [^{3}H] thymidine gets incorporated into bacterial DNA. For this purpose, three types of experiments were carried out: (a) direct assay of [^{3}H] thymidine into DNA isolated from pre-labelled *M. leprae*, (b) assay of thymidine kinase, and (c) assay of thymidine phosphorylase.

(a) Incorporation of [^{3}H] thymidine into *M. leprae*-DNA: *M. leprae* were labelled with [^{3}H] thymidine for 24 hr. In one set of experiments, uptake of [^{3}H] thymidine by *M. leprae* cells was measured. In second set of experiments, the labelled *M. leprae* (above) were used to isolate nucleic acids by the method of Wayne & Gross,[11] and the isolate was taken in Dimilume for scintillation counting. This gave the incorporation of [^{3}H] thymidine into total nucleic acids. In the third set of experiments, the pre-labelled *M. leprae* were further incubated for 24 hr in the presence of 0·3N KOH before extracting DNA and counting. This gave incorporation of [^{3}H] thymidine into DNA and the difference in the counts between the second and third set of experiments gave incorporation of [^{3}H] thymidine into RNA.

The results are presented in Table 3. Seventeen percent of the total [^{3}H] thymidine uptake by whole cells is attributed to the incorporation into DNA and only one percent into RNA. This is reasonably good evidence to justify using [^{3}H] thymidine uptake by *M. leprae* cells as an indicator of DNA synthesis.

(b) Assay of thymidine kinase in *M. leprae*: Thymidine, which generally has no role other than DNA building blocks, has the advantage of rapid conversion to the nucleotide level. Thymidine kinase (EC 2.7.1.75) is an enzyme of the pyrimidine salvage pathway which catalyzes the phosphorylation of thymidine to

Table 3. Incorporation of ^{3}H thymidine by whole *M. leprae* and *M. leprae* DNA.

	Incorporation of ^{3}H thymidine pmoles/10^{9} cells/24 hr	%
Whole *M. leprae* cells	12·70	100
Total nucleic acid	2·30	18
DNA (after KOH treatment)	2·22	17
RNA	0·10	1

thymidine monophosphate (TMP),[4] and with further phosphorylation, TMP is converted to TDP and then to TTP. The enzyme, DNA polymerase (EC 2.7.7.7) utilizes TTP to incorporate thymine (through precursor thymidine) into DNA. Thus, the incorporation of thymidine into bacterial DNA can be demonstrated by the presence of thymidine kinase (analyzing the reaction product, TMP). The modified method of Lee and Chang was adopted for this assay.[12]

There was a steady increase in DE-81 filter-retained CPM, which represent TMP. The thymidine kinase activity is linear up to 120 min. As seen in Table 4, during this period, the average value of thymidine kinase activity has been calculated to be 12·66 pmol/min/mg protein.

(c) Assay of thymidine phosphorylase in *M. leprae*: Thymidine phosphorylase (EC 2.4.2.4) within a few minutes converts thymidine to thymine and thus shuts off incorporation into thymidylate. This assay, in cell-free extract of *M. leprae* was performed by the method of Scocca, measuring spectrophotometrically free pyrimidine base (thymine) produced.[13] As seen in Table 4, thymidine phosphorylase is absent in *M. leprae*, another indirect piece of evidence which suggests that thymidine is converted to TMP and thus incorporated into *M. leprae*–DNA.

For comparison, assays for thymidine kinase and thymidine phosphorylase were performed with cell-free extracts of *M. lepraemurium* (*in vitro* grown), *M. lufu, M. avium* and *M. tuberculosis*. The results are presented in Table 4. It is interesting to note the relationship of [^{3}H] thymidine uptake by various mycobacterial cells to thymidine kinase and thymidine phosphorylase.

Another important development in the last decade is the successful transmission and growth of *M. leprae*. Yields of up to 10^{10} organisms per gram of liver have been found, thus supplying a constant source of organisms for such work. The armadillos source of *M. leprae* offers lessened metabolic variations in batch-to-batch preparations.

For all our *in vitro* cultivation studies, we used *M. leprae* suspensions purified by the Percoll gradient method. For ATP assays the aliquots of cultures were

Table 4. Thymidine kinase and thymidine phosphorylase in cell-free extracts of *M. leprae* and other mycobacteria.

Organism	Thymidine kinase*	Thymidine phosphorylase*
M. leprae	12·66	nil
M. lepraemurium (*in vitro* grown)	17·42	nil
M. lufu	9·37	4·21
M. avium	2·21	15·65
M. tuberculosis	0·85	22·37

* pmol/mg protein/min.

centrifuged, washed twice and treated with a mixture containing 0·1% each of trypsin, chymotrypsin and collagenase. Finally, cells were exposed to Triton X-100 followed by ATPase before extracting bacterial ATP. Triton X-100 selectively ruptures all the mammalian cells, liberating host ATP which is then destroyed by ATPase.[14]

Purified suspensions of *M. leprae* were inoculated in DH (Dhople), MY (Murohashi-Yoshida), Middlebrook 7H9 and Dubos broth (plus serum) media and incubated at 34°C for up to 20 days. At periodic intervals aliquots were taken and assayed for intracellular ATP. The results presented in Figure 2 suggest that *M. leprae* retain 75 and 54% of their original intracellular ATP in DH and MY medium respectively at the end of 20 days, while the loss of ATP is disastrous when incubated in 7H9 or Dubos medium. During the same period, the uptake of [^{3}H] thymidine by *M. leprae* was 86% and 72% of the original in DH and MY medium respectively. The data suggest that *M. leprae* remain metabolically active in both DH and MY media for at least 20 days. This is an important observation for cultivation studies and could not have been obtained by routine microscopic counts.

Table 5 shows the effect of incubating *M. leprae* in various media on its metabolic activity and viability.[15] As seen in Table 5, both DH and Mahadevan media support the maintenance of growth potential of *M. leprae* for at least 8 weeks. There is 20–30% drop over original in ATP levels and ^{3}H-thymidine uptake at the end of 4 weeks of incubation, which can be considered as the 'lag period'. This may reveal the serious decline in energy production even in the optimized system. However, between 4 and 8 weeks of incubation, the bacilli recover their metabolic integrity in these media. This represents the expansion of energy production and synthesizing useful membranes. The bacilli attain the original levels of ATP and ^{3}H-thymidine uptake by the end of 6 weeks. This can be interpreted as the stimulatory nature of these two media for maintaining the original growth potential of *M. leprae* and also the viability of *M. leprae* as evidenced by their ability to grow at the normal rates in the footpads of mice even from 4-week old cultures.

On the other hand, the effects of incubating *M. leprae* in either Murohashi–Yoshida, Dubos or Middlebrook 7H11 were disastrous. In Murohashi–Yoshida medium, by the end of 4 weeks ATP levels and capacity for ^{3}H-thymidine uptake had dropped to 50% of the original and declined steadily thereafter. The cells were removed at 4 weeks and later failed to multiply in the footpads of mice indicating that they had lost their viability also. The situation was still worse in the remaining two media.

Thus, the results suggest an excellent correlation between metabolic activity (ATP levels and capacity for ^{3}H-thymidine uptake) and viability of *M. leprae*.

There have been several reports of so-called successful *in vitro* growth of *M. leprae* in various media, but these findings could not be reproduced by other investigators in this field. We undertook studies to assess the fate of *M. leprae* in

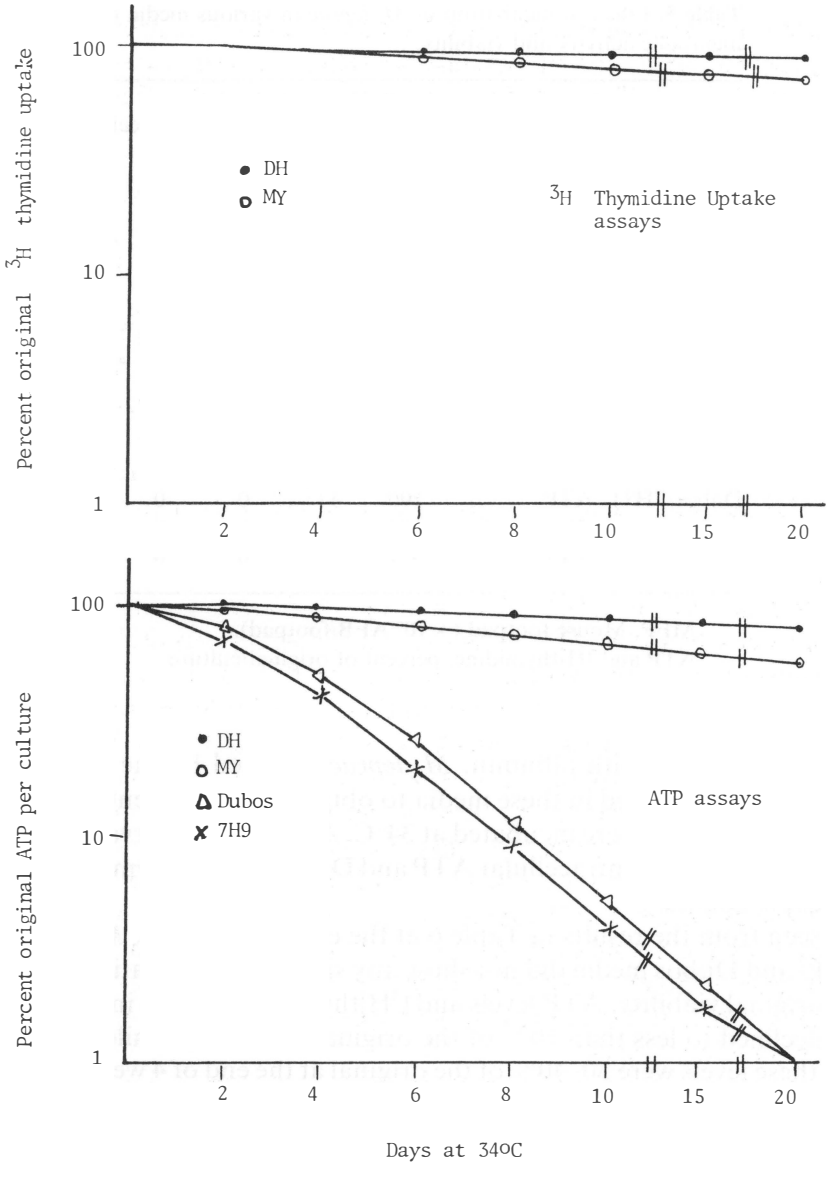

Figure 2. Stability of *M. leprae* in various media incubated at 34°C.

these six media; these included Dhople–Hanks medium (DH) that gave continuous *in vitro* growth of *M. lepraemurium*, Mahadevan's conditioned medium containing supernates from dorsal root ganglian cultures, Skinsness' hyaluronic acid medium, Murohashi–Yoshida medium (MY), Middlebrook 7H9

Table 5. Effect of incubation of *M. leprae* in various media on its metabolic activity and viability.

Medium	Activity	Incubation at 34°C for weeks				
		0	2	4	6	8
DH	ATP	100	80	72	95	138
	^{3}H-thymidine	100	82	70	102	149
	MFP	4·8		1·8	3·4	2·8
Mahadevan	ATP	100	83	77	102	149
	^{3}H-thymidine	100	86	78	105	132
	MFP	4·8		2·1	4·1	3·6
Murohashi–Yoshida	ATP	100	65	48	37	11
	^{3}H-thymidine	100	72	51	30	0
	MFP	4·8		0	0	0
Dubos/7H11	ATP	100	11	0	0	0
	^{3}H-thymidine	100	9	0	0	0
	MFP	4·8		0	0	0

MFP, Mouse footpad ($\times 10^6$ AFB/footpad).
ATP and ^{3}H-thymidine: percent of original/culture.

and Dubos medium with albumin. *M. leprae*, purified by the Percoll gradient method, were inoculated in these media to obtain initial concentration of 1×10^7 cells/ml, and cultures were incubated at 34°C. At periodic intervals, aliquots were taken for the assays of intracellular ATP and DNA, for [^{3}H]thymidine uptake and microscopic counts.

As seen from the results in Table 6 at the end of 16 weeks, *M. leprae* in MY, SK, 7H9 and Dubos media did not show any sign of multiplication and retention of any original viability. ATP levels and [^{3}H]thymidine uptake in 7H9 and Dubos media declined to less than 10% of the original 2–3 weeks while in MY and SK media, these levels were 30–40% of the original at the end of 4 weeks and less than 10% of the original at the end of 6 weeks. Cells recovered at the end of 16 weeks did not show any DOPA-oxidase activity and also failed to multiply in the footpads of mice.

On the other hand, both DH and Mahadevan media seem to be supporting the *in vitro* growth of *M. leprae*. At the end of 16 weeks of incubation, there was a significant increase in cell mass (400–600% of the original) as measured by all three criteria. The cells harvested failed to show any growth on any of the sterility test media, thus ruling out any possibility of these cells being other readily cultivable mycobacteria. These cells lost their acid-fastness when exposed to pyridine prior to staining, showed normal activity of oxidizing DOPA and gave standard growth curves in the footpads of mice 8 months after inoculation.

Table 6. *In vitro* growth of *M. leprae* in various media (pooled data)*.

Media	AD	MY	Mahadevan	Skinsness	7H9	Dubos
Counts	315–572	91–104	244–510	84–98	87–99	90–98
ATP	378–610	0	350–576	0	0	0
DNA	332–548	8–19	317–565	0	0	0
[^{3}H] Thymidine uptake	390–610	0	359–593	0	0	0
Pyridine extraction	+	±	+	±	±	±
DOPA	+	−ve	+	−ve	−ve	−ve
MFP†	1·36–5·83	−ve	0·73–3·42	−ve	−ve	−ve

* All values expressed as percent of original at 0 hour. All values for samples analysed 16 weeks after inoculations. Range of values from five different experiments.

† Mouse footpad harvests 8 months post infection ($\times 10^6$ *M. leprae*/footpad).

Data on Table 7 are representative of one of six experiments in the above series with DH and Mahadevan media wherein the results of three assays at periodic intervals are presented. There is a steady drop in the levels of bacterial ATP and DNA, and also a drop in ^{3}H-thymidine uptake for up to 4 weeks after which there followed a slow but steady increase in bacterial biomass, that reached optimum levels between 14 and 16 weeks. However, after 16 weeks there was no further increase in biomass, and the cells started deteriorating as shown from a rapid decline in the levels of all three growth parameters.

The cells harvested from DH and Mahadevan media from each of the six experiments above at the end of 16 weeks, were used to inoculate fresh DH and

Table 7. *In vitro* growth of *M. leprae* in DH and Mahadevan media. (Weekly findings of one representative experiment.)*

Weeks		0	2	4	6	8	10	12	14	16	18	20
DH medium:	ATP	100	80	72	95	138	242	368	475	494	398	117
	DNA	100	96	91	104	159	265	393	441	464	429	286
	MFP†									2·4		
Mahadevan medium:	ATP	100	83	77	102	149	265	378	454	468	331	74
	DNA	100	98	95	112	172	301	426	478	486	440	308
	MFP									1·1		

* All values expressed as percent of original at 0 hour.

† Footpad harvest 8 months post-infection ($\times 10^6$ *M. leprae*/footpad).

Table 8. *In vitro* cultivation of *M. leprae* in DH and Mahadevan media-transfer studies. (Weekly findings of a representative experiment.)*

Weeks		0	2	4	6	8	10	12
DH medium:	ATP	100	76	61	42	24	17	7·5
	DNA	100	95	90	81	69	54	43
Mahadevan medium:	ATP	100	71	55	38	19	8	4·8
	DNA	100	96	91	79	53	39	29

† All values expressed as percent of original at 0 hour.

Mahadevan media respectively. The inoculum sizes were the same as in primary cultures and all cultures were incubated at 34°C. The results pooled from five different experiments are presented in Table 8. During the 12-week period, ATP levels of *M. leprae* declined progressively to 3–10% of the original levels, while the capacity of *M. leprae* cells for ^{3}H-thymidine uptake was lost completely between the sixth and tenth week, thus suggesting that those cells were becoming more and more metabolically inactive throughout the incubation period. The cells harvested at the end of 12 weeks failed to oxidize DOPA as well as failing to multiply in the footpads of mice (Table 9).

Table 9. *In vitro* cultivation of *M. leprae* in DH and Mahadevan media-transfer studies.*

Media	DH	Mahadevan
Counts	92–107	93–106
ATP	3·2–9·4	3·8–6·2
DNA	31–58	22–41
[^{3}H] uptake Thymidine	0	0
†	−ve	−ve

Pooled data from five experiments. Inoculums were the cells removed at the end of 16 weeks from cultures of each of the five experiments of primary isolation (Table 6).

* All values expressed as percent of original at 0 hour and represent samples analysed at the end of 12 weeks.

† Footpads harvested 8 months post-infection.

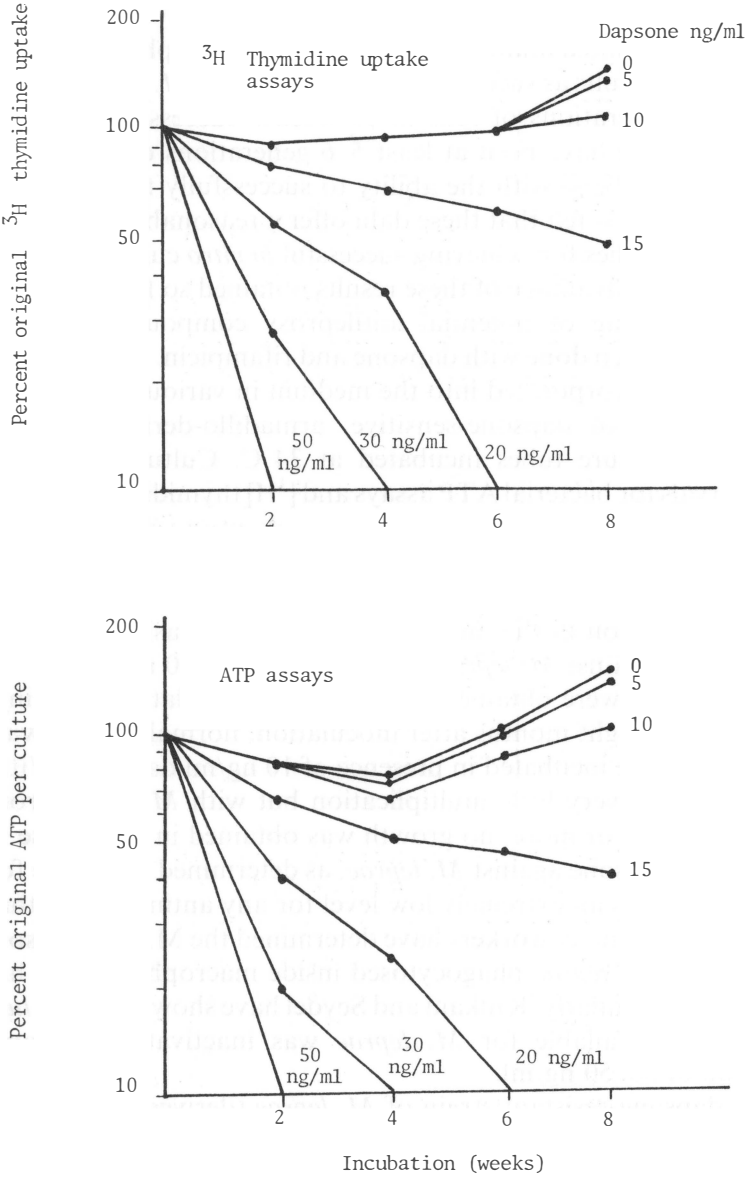

Figure 3. *In vitro* effect of dapsone on *M. leprae*.

Thus, it can be stated that there was a limited but definite multiplication of *M. leprae* in DH and Mahadevan media, but not in any of the other four media tested. Because of the techniques adopted, the declumping phenomenon can be ruled out. The question is whether the cells utilized nutrients and other substrates from the culture media or were able to multiply to a limited extent using their endogenous energy. However, the important point is that only DH and

Mahadevan media provided the necessary environments to the bacilli to survive for 4 months with limited multiplication. It should be emphasized that we are not considering these results as successful *in vitro* growth of *M. leprae* since there were less than three generations of cells in 16 weeks. Successful growth cannot be claimed unless there have been at least 5–6 generations of growth (30–60-fold increase in cell numbers) with the ability to successfully transfer these primary isolates. However, it is felt that these data offer a reasonable base upon which to conduct further studies for achieving successful *in vitro* cultivation of *M. leprae*.

We have taken advantage of these results obtained so far to develop a model for *in vitro* screening of potential antileprosy compounds and preliminary experiments have been done with dapsone and rifampicin. DH medium was used and dapsone was incorporated into the medium in various concentrations.

Known strains of dapsone-sensitive, armadillo-derived *M. leprae* were inoculated and culture tubes incubated at 34°C. Cultures were removed at periodic intervals for bacterial ATP assays and [^{3}H] thymidine uptake. The results are presented in Figure 3. No inhibitory effects were seen when the dapsone concentration was 10 ng/ml or less. At the end of 6 weeks, *M. leprae* became non-viable in presence of 20 ng/ml dapsone and this period decreased with increase in dapsone concentration in the medium. Thus, on the basis of these results, the MIC of dapsone against *M. leprae* is between 20 and 30 ng/ml. *M. leprae* from each set of cultures were obtained 8 weeks after inoculations and injected into footpads of mice. Eight months after inoculation, normal growth was obtained when *M. leprae* were incubated in presence of 10 ng/ml dapsone. With 15 ng/ml dapsone, there was very little multiplication but with *M. leprae* from cultures containing 20 ng/ml or more, no growth was obtained in the mouse footpads.

The MIC of dapsone against *M. leprae*, as determined by mouse footpad has been 3 ng/ml. This is an extremely low level for any antimicrobial that is in use today. Mahadevan and coworkers have determined the MIC of dapsone by an *in vitro* assay using *M. leprae* phagocytosed inside macrophages and have shown MIC of 28 ng/ml. Similarly, Kulkani and Seydel have shown that *M. lufu*, the best model organism available for *M. leprae* was inactivated by dapsone at a concentration of 30–50 ng/ml.

When a dapsone-resistant strain of *M. leprae* (derived from a nude mouse) was used in the above studies, dapsone at a concentration of 50 ng/ml did not have any inhibitory effects even after 12 weeks.

Using both dapsone-sensitive and dapsone-resistant strains of *M. leprae* in studies similar to the above, the MIC of rifampicin against *M. leprae* was found to be between 200 and 250 ng/ml (Figure 4).

So, that is where we are now and we plan to go forward from here, improving our medium to obtain continuous *in vitro* growth of *M. leprae*.

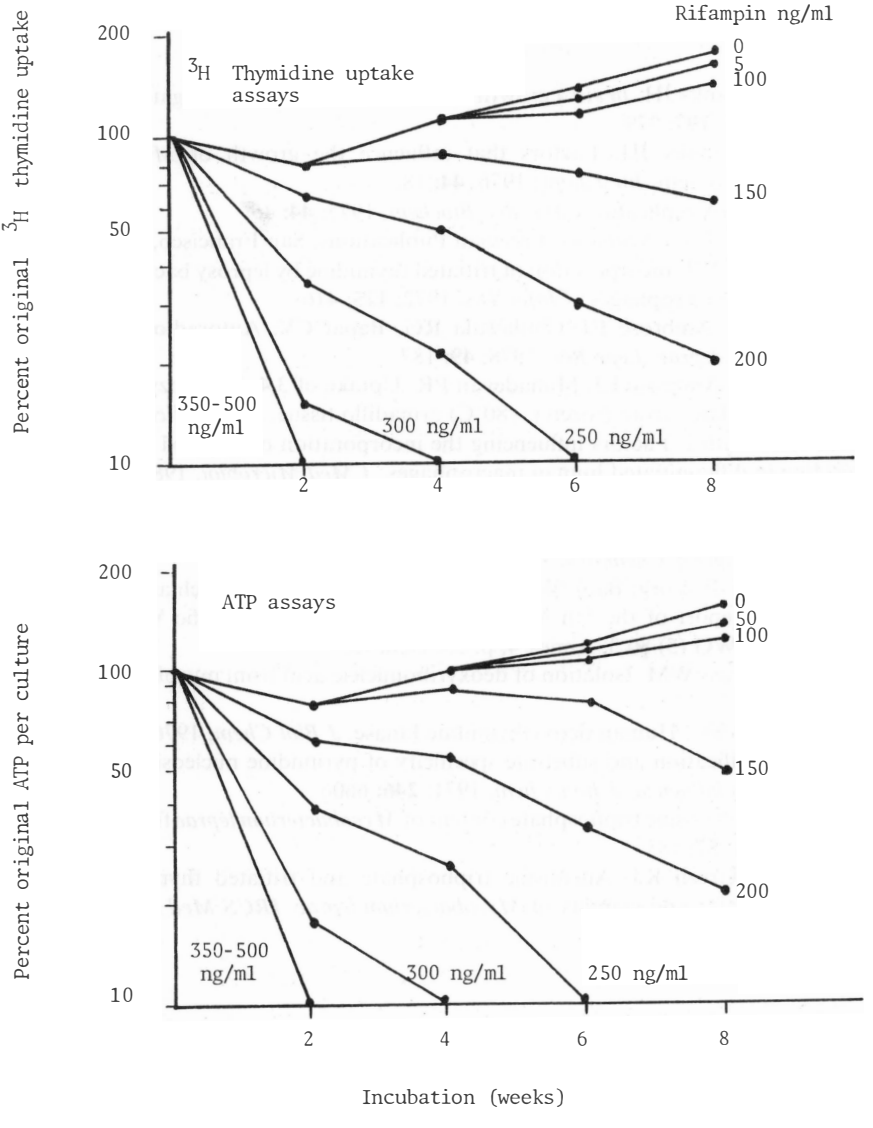

Figure 4. *In vitro* effect of rifampicin on *M. leprae*.

Acknowledgment

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