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Problems in the Cultivation of *Mycobacterium Leprae* Related Cultivation and Biochemical Studies with *Mycobacterium Lepraemurium*

Byron S. Tepper[1]

The inability to grow *Mycobacterium leprae in vitro* is a major impediment which seriously restricts both fundamental and applied research on human leprosy. Until these organisms are grown in culture, even the simplest, basic bacteriologic techniques for studying infectious disease cannot be used in leprosy. In addition to the most difficult problem of finding suitable conditions and nutritional combinations for the growth of *M. leprae in vitro* in cell-free media or in tissue culture systems, cultivation studies are complicated further by the problem of obtaining adequate numbers of bacilli from suitable patients. Laboratory infections in the hamster, the mouse footpad and thymectomized-irradiated mice with *M. leprae*, have greatly expanded the potential for clinically oriented research, but do not yet provide sufficient bacilli for bacteriologic and biochemical research.

Mycobacterium lepraemurium, the agent of a leprotic disease in murine rodents, has been studied extensively in the search for factors required by the noncultivated mycobacteria. Like *M. leprae*, it has not been cultured in cell-free media. However, *in vitro*, cultivation of *M. lepraemurium* has been achieved in macrophages and fibroblasts. This superior potential in cell cultures suggests a somewhat greater possibility for success in cell-free media and for definition of nutrients and conditions for growth which might subsequently be useful to *M. leprae*. Since the murine bacillus can be maintained as a laboratory infection in rats or mice, it is possible to obtain an adequate, dependable supply of bacilli for bacteriologic and biochemical studies. The basic premise in utilizing this model system is analogous to the use of avian malaria as a model for human malaria, or animal cancer as a model for human cancer.

For several years in our laboratory, we have made an intensive effort to devise a cell-free medium which would support the multiplication of *M. lepraemurium in vitro*. In these attempts we have applied the best of existing knowledge concerning the metabolism and growth of mycobacteria, other fastidious microorganisms, and tissue cells in the selection of nutrients, cofactor and growth factor supplements, and environmental conditions. No increases in *M. lepraemurium* were observed in any of over 250 media tested. Similar experiences have been accumulated by Hanks, Reich and Morrison with *M. leprae*. This type of approach suffers from the disadvantage of not providing information on either the beneficial or detrimental effects of the medium components or conditions which could be applied to subsequent experiments. It would seem that a more profitable approach is afforded by cultivation trials designed to monitor some morphologic or physiologic response of the organism to the different environmental conditions.

The elongation of *M. lepraemurium*, the phenomenon of filamentous "growth" without multiplication, first observed by Hart and Valentine ([4]), occurred in many cultivation experiments. Similar elongated or filamentous forms of many other bacteria, including cultivatable mycobacteria, occur when these organisms are in an unfavorable environment. These organisms may recover and subsequently divide, provided the filament-inducing condition is alleviated before the second mean generation time of the organism. If it is assumed that elongation in *M. lepraemurium* also results from unbalanced syntheses in an unfavorable environment, then suppression of elongation should indicate a more favorable medium. In subsequent experiments with

[1] B. S. Tepper, Ph.D., Microbiologist, Leonard Wood Memorial, and Associate Professor of Pathobiology, Johns Hopkins University-Leonard Wood Memorial Leprosy Research Laboratory, School of Hygiene and Public Health, Baltimore, Maryland.

M. lepraemurium, the effect of different media on the length of the bacilli was measured and compared to controls. Table 1 shows the effect of selected modifications of the Hart-Valentine medium on the elongation of the Hawaiian strain of *M. lepraemurium*. The high osmotic pressure provided by the 7.4 per cent of sucrose in the Hart-Valentine medium was found to be unnecessary for the elongation of this strain of *M. lepraemurium*. The other modifications listed caused only transient repressions of the rate of elongation. On the seventh day, the length of the cells in the enriched media, in all cases, was less than that in the control medium. However, by the fifteenth day, which represents twice the mean generation time of *M. lepraemurium in vivo*, experimentals and controls had identical lengths. These experiments show that enrichments of the Hart-Valentine medium can effect elongation, but they also suggest that multiple deficiencies in the bacilli and in the medium must be satisfied to prevent the unbalanced syntheses and for multiplication to occur.

A more fruitful approach to the cultivation problem may lie in our current studies of the metabolism of *M. lepraemurium*. Although Draper and Hart (1) have shown that the synthesis of cellular constituents accompanied the elongation process, they did not determine the medium components utilized in the synthesis of these macromolecules. The experiments described below will demonstrate the advantage of applying tracer techniques to identify the substrates used for energy production and biosynthesis by *M. lepraemurium in vitro*.

The bacilli used in these analytical experiments were harvested from pelvic and omental fatty pads of female CFW mice (Carworth, Inc.) infected intraperitoneally three to four months previously with the Hawaiian strain of *M. lepraemurium*. The bacilli were carefully purified from the host tissue by the differential centrifugation technique of Nishimura *et al.* (8). A final digestion with the proteolytic enzyme Pronase insured the inactivation and removal of residual host protein. The washed bacilli were then inoculated into Hart-Valentine medium, without sucrose, containing a substrate labeled with radioactive carbon. The reaction system in the 25 × 125mm. tubes, shown in Figure 1, contains the medium, a C^{14}-labeled substrate, and 6.1×10^9 bacilli in a total volume of 30 ml. The Van Slyke-Cullen aeration tubes were pinched shut to prevent the escape of met-

TABLE 1. *The effect of selected additions to the Hart-Valentine medium on the elongation of* M. lepraemurium in vitro.

Medium	Average length of cells (μ)		
	Inoculum	7 day	15 day
Control = Basal medium (B)[a]	2.55	3.47	4.29
B + sucrose (7.4%)	2.55	3.49	4.35
Control	1.87	3.18	4.09
B + adenine/purine	1.87	2.62	3.97
B + yeast extract	1.87	2.71	4.11
Control	1.92	3.56	4.58
B + ammonium chloride	1.92	2.70	4.26
B + ammonium acetate	1.92	2.55	3.85
Control	1.80	3.69	4.23
B + trace metals	1.80	2.89	4.22

[a] Basal medium in % w/v = Casamino acids (Difco), 1.6; asparagine, 0.75; Na_2HPO_4, 0.14; KH_2PO_4, 0.06; sodium citrate, 0.08; $MgSO_4 \cdot 7H_2O$, 0.03; glycerol, 2.9; and albumin, 0.18 with a final pH of 6.2.

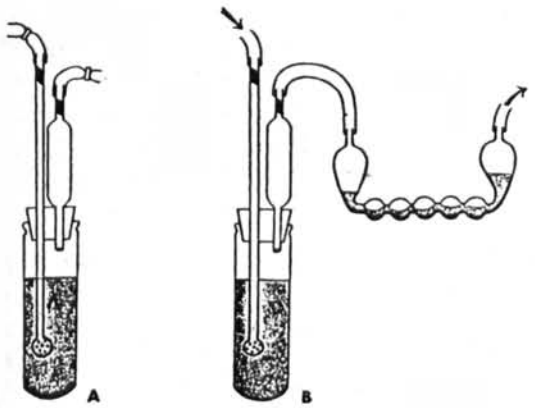

FIG. 1. Apparatus for exposing *M. lepraemurium* to radioactive substrates and for collection of metabolic carbon dioxide. A. Test tube containing medium, C^{14}-labeled substrate and bacilli with aeration tubes sealed during incubation. B. After incubation, a CO_2-collector containing NaOH is attached and the system flushed with air to trap C^{14}-labeled carbon dioxide.

abolic carbon dioxide and then the tubes were incubated at 37°. After seven days of incubation, the respiratory carbon dioxide was flushed from the reaction tubes, trapped in NaOH in a CO_2 collector and the isotope content determined. The bacilli were then washed free of the radioactive substrate and assayed for incorporated substrate-C^{14}. Uninoculated media containing the C^{14}-labeled substrates served as controls. All assays for radioactivity were made in a scintillation counter.

During the seven day incubation of *M. lepraemurium* in the Hart-Valentine medium, the bacilli elongated, on average, to 1.6 times their original length. The fact that the utilization of glycerol, acetate and asparagine accompanied this elongation is shown in Table 2. The results of this experiment have shown that glycerol and acetate were oxidized and also incorporated into cellular constituents by this organism. Although Ito and Sonoda (5) had reported asparagine to stimulate oxygen consumption in *M. lepraemurium,* the isotope data indicate that asparagine, itself, was not oxidized, but served solely as a source of carbon for cell synthesis. The stimulatory effect of asparagine on the respiration of this organism requires further study.

The results of this experiment have shown that isotope tracer techniques can provide an insight into the potentialities of *M. lepraemurium in vitro.* More importantly, the evidence of Gray (2), Hanks (3), Ito and Sonoda (5), Tamemasa and Tsutsumi (9) and Kusaka (6) had led to the general conclusion that *M. lepraemurium* derives no benefit from substrates *in vitro.* The new results clearly demonstrate that *M. lepraemurium* is capable of oxidizing and assimilating exogenous substrates.

The rates for the utilization of the exogenous substrates, under the conditions of the experiment shown in Table 2, were extremely low. They were, in fact, lower than rates observed with noncultivated

TABLE 2. *Oxidation and incorporation of C^{14}-labeled substrates by* M. lepraemurium *during 7 days in the elongation medium of Hart and Valentine.*

Substrate[a]	CO_2		Cells[b]		Cells/CO_2 ratio
	dpm	μM substrate	dpm	μM substrate	
Glycerol	12,380	12.96	7,278	7.72	0.60
Control	5,840				
Acetate	682,410	0.18	15,490	0.04	0.22
Control	17,780				
Asparagine	1,310	—	1,040	0.30	
Control	1,410				

[a] Specific activities of substrate in the incubation medium; glycerol-U-C^{14} = 955 dpm/μM; asparagine-U-C^{14} = 3500 dpm/μM; acetate-U-C^{14} = 3.87 × 10^6 dpm/μM.
[b] 0.8 mgm. dry weight (6.1 × 10^9 cells).

rickettsiae and chlamydiae, which have been investigated by similar radioisotopic techniques. The low rates observed may have resulted because of the multiplicity of substrates available in the elongation medium and because the conditions for the metabolic reactions were not optimal.

In collaboration with Dr. K. G. Varma a more optimal but less complex reaction system was devised. These more favorable conditions for the determination of the metabolism of *M. lepraemurium* were used to repeat the original observations and to determine the utilization of four additional substrates. The results in Table 3 demonstrate that all of the substrates tested were utilized to some extent by *M. lepraemurium*. The advantage of the isotope technique, besides allowing the detection of extremely low metabolic rates, is that it permits the distinction between oxidation and assimilation of the exogenous substrates. Even though glucose, asparagine, and methionine were not detectably oxidized, their incorporation into cellular material was established. The failure of *M. lepraemurium* to oxidize glucose was a surprising result of this experiment which is presently unexplainable but is under study.

The data in Table 3 provide ample evidence that the detectable metabolic reactions of *M. lepraemurium* are sufficiently diverse and active for the systematic determination of the biochemical capabilities of this organism. They strongly suggest that *M. lepraemurium* is capable of protein and lipid synthesis *in vitro*. The demonstrated oxidation of glycerol, acetate, fumarate, and α-ketoglutarate is of particular significance. During investigations of cell-free extracts of *M. lepraemurium*, Mori, Kohsaka and Tanaka ([7]) have recently shown the presence of all the enzymes of the tricarboxylic acid cycle, except pyruvate and α-ketoglutarate oxidases. The presence of isocitrate lyase suggested the activity of a modified tricarboxylic acid cycle, the glyoxalate cycle. They suggested that acetyl-CoA is generated in this organism by the degradation of fatty acids and that the

TABLE 3. *Oxidation and incorporation of isotopically labeled substrates by* M. lepraemurium *during 7 days exposure in K-36 buffer at pH 7.0*[a].

Substrate[b]	CO_2		Cells		Cells/CO_2 ratio
	dpm	nM substrate	dpm	nM substrate	
Acetate-1-C^{14}	356,703	80.88	26,723	6.10	0.08
Control	1,007				
Acetate-2-C^{14}	403,146	91.04	60,397	13.70	0.15
Control	2,481				
Glycerol-U-C^{14}	203,375	10.18	82,147	4.44	0.44
Control	17,397				
Glucose-U-C^{14}	11,226	—	3,210	0.28	—
Control	15,295				
α-ketoglutarate-5-C^{14}	38,160	0.95	2,013	0.06	0.06
Control	21,789				
Fumarate-1,4-C^{14}	118,650	3.45	5,515	0.21	0.06
Control	26,722				
Asparagine-U-C^{14}	4,493	—	365,574	0.92	—
Control	7,267				
Methionine-S^{35}	—	—	37,961	0.26	—

[a] Each flask contained 5 μc of labeled substrate, 0.84 mgm. bacterial cell N, and K-36 buffer of Weiss (10) at pH 7.0, total volume 10 ml.

[b] Specific activity of substrates-acetate-1-C-4^{14}, 2.0 μc/μM; acetate-2-C^{14}, 2.0 μc/μM; glycerol-U-C^{14}, 8.34 μc/μM; glucose-U-C^{14}, 5.2 μc/μM; α-ketoglutarate-5-C^{14}, 17.1 μc/μM; fumarate-1,4-C^{14}, 12.0 μc/μM; asparagine-U-C^{14}, 179 μc/μM; and methionine-S^{35}, 150 μc/μM.

α-ketoglutarate produced is used solely for the synthesis of glutamate.

A consideration of the oxidation of acetate via the tricarboxylic acid cycle shows that the radioactive carbon dioxide could arise from both acetate-1-C^{14} and acetate-2-C^{14} only if α-ketoglutarate is oxidized. Thus the conclusion from our data that a complete tricarboxylic acid cycle is operating in *M. lepraemurium* seems inescapable.

From the results obtained in these studies, it now seems likely that the isotope techniques will provide at least three guidelines for cultivation studies: (a) determination of the biochemical capabilities of *M. lepraemurium*, (b) identification of deficient systems which must be supplemented in an *in vitro* environment, and (c) a method for monitoring a search for more optimal physiologic environments. We now feel confident of a means for identifying more favorable environments specifically designed to meet the growth requirements of *M. lepraemurium*.

SUMMARY

Efforts to cultivate *M. lepraemurium* by providing nutrients, growth-factors and environmental conditions essential for the growth of mycobacteria, fastidious microorganisms and tissue cells, have not resulted in detectable multiplication of this organism *in vitro*. This approach was discontinued because it suffers from the disadvantage of not providing information on the beneficial or detrimental effects of the media or conditions which could prove useful in subsequent experiments.

Current experiments have centered on the biochemistry of *M. lepraemurium* in order to base future cultivation trials on a knowledge of the metabolic capabilities or deficiencies of this organism. Initial studies, using isotope tracer techniques, have tested the capacity of *M. lepraemurium* to oxidize and assimilate glycerol, asparagine and acetate. Glycerol and acetate are oxidized by *M. lepraemurium*, and carbon from these compounds is incorporated into cell constituents. Asparagine is not oxidized but is incorporated into the cell. The methods used for these determinations and the significance of the results in terms of cultivation trials will be discussed.

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