# Oxidation of Substrates by Host Grown *Mycobacterium leprae* and *Mycobacterium lepraemurium* and by *In Vitro* Grown Mycobacteria Cultured from Human, Armadillo and Murine Lepromas[1]

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Human and murine leprosy bacilli are commonly classified as noncultivable mycobacteria. They are regarded as being host dependent, metabolically deficient, intracellular parasites ([10-13, 31, 32, 42]). We have presented experimental evidence that both *M. leprae* and *M. lepraemurium* have a complete and functional tricarboxylic acid cycle and electron transport chain ([15, 18, 20, 25, 26]) and the metabolic machinery to oxidize exogenous substrates ([15, 22, 23]). Indirect but strong evidence has been presented that substrate oxidation by host grown *M. lepraemurium* is coupled with energy generation ([1, 15 20, 25]). Culture media were prepared in which oxidizable substrates, respectively, served as prospective energy sources and glycerol served as source of carbon ([21, 24, 39]). All media were supplemented with sheep serum. In the presence of several of the oxidizable substrates, unidentified strains of mycobacteria were cultured regularly from human, armadillo and rat lepromata in our laboratories. A simple medium containing yeast extract, glycerol and sheep serum permitted the most reproducible results. From 18 rat lepromas ([22]), three human leprotic nodules ([23]) and leprous tissue of an armadillo ([23]), slow growing mycobacteria were cultured. Subcultures were easily and *ad infinitum* obtained on the homologue media. A culture of mycobacteria was obtained from both a human and armadillo subcutaneous leprous nodule on a hyaluronic acid-based medium ([21, 24]). The primary cultures did not grow on conventional culture media used to grow mycobacteria. After several subcultures, however, most of the cultures became adapted to and grew on Lowenstein, Dubos and Sauton media ([22]). They were identifiable with simple bacteriologic technics as slow-growing scotochromogens belonging to the scrofulacea species ([19]). Mycobacteria cultured from rat lepromas produced, but limited, leprotic lesions in the rat. Killed suspensions of mycobacteria cultured from human leprotic lesions provoked, often ulcerating, but Arthus type reactions in tuberculoid as well as lepromatous leprosy patients when injected intradermally. It therefore became questionable as to whether the obtained cultures are identical with the etiologic agents of human and rat leprosy ([19, 35]).

The question also arises as to whether the obtained cultures are secondary laboratory contaminants. Careful examination of working procedures, glassware, live stock and great variety of controls did not reveal the presence of scrofulacea contaminants in our laboratories. The same scotochromogenic cultures were obtained from human lepromata supplied from different continents. Cultures of the same basic characteristics were obtained from 17 rat lepromas removed from 17 batches of *M. lepraemurium* infected rats and also from biopsy material sent from outside laboratories. These observations raised the possibility that the obtained cultures might be related or probably identical with the etiologic agents of human or rat leprosy ([19]). Previous results have shown that host grown *M. leprae* and *M. lepraemurium* are metabolically extremely competent with the capacity to oxidize several substrates ([15, 22, 23]). The obtained results indicated that these so far noncultivable mycobacteria should

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grow on culture media once the appropriate substrates are offered which the bacilli are able to metabolize. The potent respiratory metabolism and oxidizing capacity of these microorganisms might permit the adaptation to new substrates once the primary culture is achieved. With new substrates, utilized in extracellular environment, the bacilli might acquire characteristics, different from those they presented when isolated from the host. Oxidation of intermediates of the tricarboxylic acid cycle, glycolysis and oxidation of exogenous substrates by host grown *M. leprae* and *M. lepraemurium* were compared to the same parameters of mycobacteria cultured from human, armadillo and rat leprous tissues as well as an authentic strain of *M. scrofulaceum* and *M. bovis,* BCG. The results obtained are presented in this communication.

## MATERIALS AND METHODS

**Microorganisms.** Seven cultures of mycobacteria were used in this study.

1. *In vivo* grown *M. leprae* isolated from leprous tissue of an armadillo:[3] *M. leprae* A6 ([18, 23]).

2. An unidentified strain of mycobacteria cultured from leprous tissue of the same armadillo: M. A6 ([23]). Subcultured on KI-1 media (S5-S10).

3. An unidentified strain of mycobacteria, designated as the M. Dakar strain isolated from an untreated lepromatous leprosy patient ([21]). Subcultures on KI-1 for more than six months.

4. *In vivo* grown *M. lepraemurium* isolated from four-month-old subcutaneous rat leproma, Hawaiian strain maintained for 25 years in Sprague-Dawley rats.

5. A strain of mycobacteria cultured from a four-month-old rat leproma: M. 1m 56 ([22]).

6. An authentic strain of *M. scrofulaceum* from the collection of the Royal Edward Chest Hospital, Montreal, Canada.

7. *M. bovis,* BCG.

The *in vitro* grown strains: M. A6, M. Dakar and M. 1m 56 were subcultured in a liquid medium ([21, 23]) containing: 8.2 gm $KH_2PO_4$; 0.5 gm $Na_2HPO_4$; 30.0 gm glycerol and 2.0 gm yeast extract "Difco" in one liter distilled water. The medium was distributed into 50 ml screw cap tubes or Roux bottles. Test tubes contained 10 ml and the bottles 120 ml of the medium. These were autoclaved for 30 minutes. Prior to inoculation, 10% (v/v) filter sterilized sheep serum and 200 units per ml of sodium penicillin G were added to the media. The inoculated media were incubated at 34°C.

**Preparation of cell suspensions from in vitro grown cultures.** At different times of incubation the cells were collected by centrifugation at –4°C. The cells were washed twice with 0.1 M potassium phosphate buffer, pH 6.0, and were resuspended in the same buffer. To prepare cell suspensions of BCG, cells were grown on Sauton medium for seven days and were harvested by centrifugation. The cells were washed twice with 0.1 M potassium phosphate buffer, pH 7.0, and were resuspended in the same buffer. BCG cell suspensions were homogenized thoroughly in a ground glass homogenizer immediately before being used for respiration determinations.

All cell suspensions were standardized photometrically. The homogenized bacilli were diluted with 0.1 M potassium phosphate buffer, pH 6.8, so that a 1:10 dilution of the cell suspension gave 300 Klett units at 540 mm which corresponded to 10 mg dry weight of cells in 1 ml of final cell suspension.

Dry weights of cell suspensions were determined by drying 1 ml samples of the cell suspensions to constant dry weight in an air oven at 105°C. Each preparation was stained by the Ziehl-Neelsen technics following oxidation with periodic acid. Acid-fastness and absence of secondary contaminations were determined microscopically.

Preparation of *in vivo* grown *M. lepraemurium* and *M. leprae* cell suspensions has been described elsewhere ([15, 18]).

**Measurement of oxidation of substrates.** To measure the rate of oxidation of the substrates, conventional Warburg manometric technics were employed ([44]). Experiments were conducted at 34°C with air as the gas phase. Each Warburg flask contained 50 mM of 0.1 M potassium phosphate buffer, pH 5.6, and 1.0 ml of cell suspension in the main

[3] Inoculated approximately 31 months previously, intradermally and intraperitoneally with pooled suspensions of mycobacteria isolated from several Oriental lepromatous patients. The armadillo originated from Texas, Fort Hood area. It was infected and maintained at the ALM Leprosy Atelier, University of Hawaii, Honolulu, Hawaii.

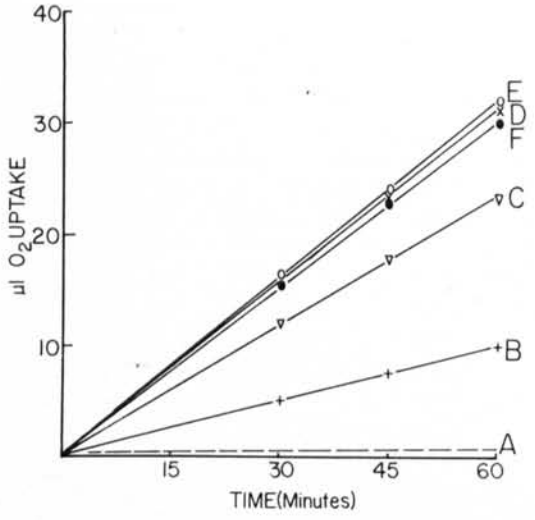


FIG. 1. Effect of age of M. Dakar culture on the oxidation of yeast extract. Curves A, B, C, D, E and F represent, respectively, 2, 5, 14, 21, 30 and 40 day old cultures.

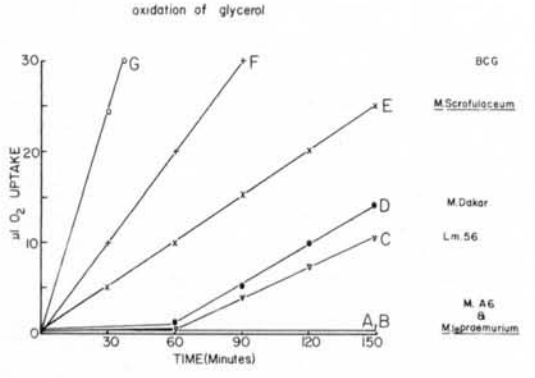


FIG. 2. Oxidation of glycerol by *in vivo* grown *M. leprae* and *M. lepraemurium* as well as by *in vitro* grown mycobacteria. Curves A and B show, respectively, oxygen uptake by whole cell suspensions of host grown *M. lepraemurium* and *M. leprae*. Curves C, D and F indicate, respectively, oxygen uptake by whole cell suspensions of *in vitro* grown M. 1m 56, M. Dakar and M. A6. Curves E and G represent oxygen uptake by *M. scrofulaceum* and *M. bovis*, BCG, respectively.

compartment and 10 mM of substrate in the side arm. When yeast extract was used as substrate, 0.2 ml of a 10% solution was used. $CO_2$ was absorbed by 0.2 ml of 20% KOH in the center well. The total volume of liquid in the Warburg flasks was 2.0 ml. Additional control in all experiments consisted of simultaneous determination of endogenous respiration. The flasks were equilibrated for 15 minutes prior to the addition of the substrate from the side arm.

## RESULTS

**Substrate oxidation as influenced by the age of the cultures.** Cells were harvested at various time intervals and the rate of substrate oxidation was investigated. All tables and figures are corrected for endogenous respiration. Figure 1 shows that yeast extract was not oxidized by two day old *in vitro* grown cultures of the Dakar strain (curve A) and was poorly oxidized by five day old cells (curve B). The oxygen uptake increased with the age of the culture until about three weeks. Yeast extract was actively oxidized by cell suspensions prepared after 14 and 21 days of incubation (curves C and D). The rate of oxidation of yeast extract remained more or less the same whether the cells were harvested after 30 days (curve E) or 40 days (curve F). The cells harvested the fifth day after inoculation showed poor endogenous respiration, but this increased with time and was optimal when the cells were about three weeks old. The endogenous respiration, as well as substrate oxidation, declined slowly if the cultures were grown for more than 40 days. Maximal respiration occurred at pH 5.5-6.0. A gradual decrease in growth was observed when the pH was increased up to 9.0. The optimal conditions for respiratory studies were found to be pH 5.5-6.0 and a temperature of 34°C for *M. scrofulaceum*, M. 1m 56 and M. Dakar, as well as *in vivo* grown M. A6 and *M. lepraemurium*.

**Oxidation of substrates of the culture media.** Oxidation of glycerol by the cultures is shown in Figure 2. Host-grown *M. leprae* A6 (curve A) and *M. lepraemurium* (curve B) did not oxidize glycerol during 150 minutes of incubation. During the first 60 minutes of incubation, the initial rate of glycerol oxidation by whole cell suspensions of the *in vitro* grown M. Dakar and M. 1m 56 strains was extremely low, close to null. After about 60 minutes the rate of oxidation increased slightly and remained linear throughout the period of the experiment (curves C and D). Glycerol was oxidized without any latency period and at a relatively high rate by *M. scrofulaceum* cell suspensions (curve E). M. A6 oxidized glycerol at a fast rate (curve F) and the same substrate was oxidized ex-

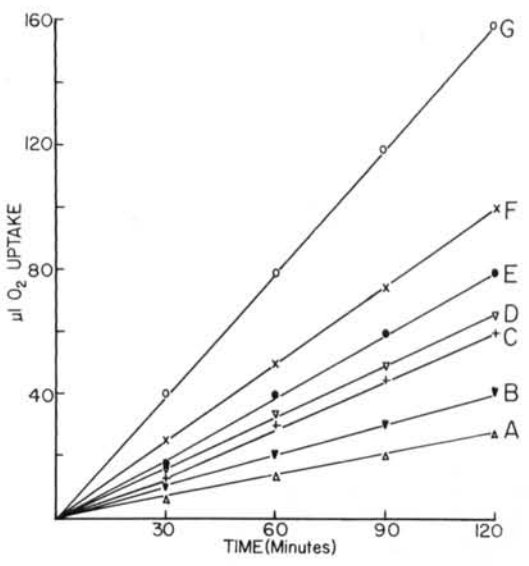


FIG. 3. Oxidation of yeast extract by *in vivo* grown *M. leprae* and *M. lepraemurium* as well as by *in vitro* grown mycobacteria. Curves A and B show, respectively, oxygen uptake by *in vivo* grown cell suspension of *M. lepraemurium* and *M. leprae*. Curves C, D, E, F and G indicate, respectively, oxygen uptake by *in vitro* grown whole cell suspensions of M. 1m 56, *M. bovis*, BCG, M. Dakar, M. A6 and *M. scrofulaceum*.

tremely fast by BCG cell suspensions (curve G). In experiments repeated several times, the *in vivo* grown cells never oxidized glycerol. Although glycerol was only slightly oxidized by M. Dakar and M. 1m 56, oxygen uptake values higher than endogenous respiratory activity were constantly obtained. *M. bovis*, BCG always showed the highest oxidation of glycerol and the *in vitro* grown M. A6 constantly showed a high rate of glycerol oxidation. Oxidation of yeast extract as a substrate by the different mycobacteria is shown in Figure 3. Host-grown *M. lepraemurium* (curve A) and *M. leprae* A6 (curve B) oxidized yeast extract at a slow rate. The same substrate was somewhat better oxidized by *in vitro* grown M. 1m 56 (curve C), BCG (curve D) and M. Dakar (curve E). M. A6 (curve F) and *M. scrofulaceum* (curve G) oxidized yeast extract at extremely fast rates.

The oxygen uptake increased almost immediately upon the addition of sheep serum to the cell suspensions. However, this was found not to be a biological oxidation of sheep serum, but the change in pressure occurred due to an immediate change in pH. Upon an addition of 0.3 ml of sheep serum, the initial pH of the reaction mixture increased from 5.5 to pH 6.0. The rapid change of pH ceased in about eight minutes and the subsequent oxygen uptake occurred at a rate similar to that of the endogenous respiration.

**Oxidation of the intermediates of the glycolytic and tricarboxylic acid cycles.** None of the intermediates of the glycolytic cycle were oxidized by purified cell suspensions of *M. lepraemurium* ([20]). Results in Table 1 show that although glucose was not oxidized, pyruvate and phosphoenolpyruvate were oxidized at a very low rate by the *in vitro* grown M. 1m 56. Due to the limited amount of cells available, from early host-grown *M. leprae* A6, oxidation of these substrates has not yet been studied. However, in addition to glycerol some other members of the glycolysis cycle such as pyruvate and phosphoenolpyruvate were oxidized by whole cell suspensions of *in vitro* grown M. Dakar and M. A6 as well as *M. scrofulaceum* and *M. bovis*, BCG. Of the several substrates of the tricarboxylic acid cycle, only succinate has been shown to be oxidized by frozen and thawed cell suspensions of *M. lepraemurium* ([20]). Table 1 shows that succinate was poorly oxidized by normal (not frozen) cell suspensions of *in vivo* grown *M. lepraemurium*, but strongly oxidized by host grown *M. leprae* A6. Cell suspensions prepared from the cultivated M. 1m 56 strain oxidized succinate, acetate and oxalacetate very slowly. *In vitro* grown M. A6 oxidized citrate, succinate, α-ketoglutarate and oxalacetate slowly. Acetate was oxidized by the same culture at an extremely fast rate. The *in vitro* grown M. Dakar oxidized the same substrates except citrate, at a very slow rate. Substrates of the tricarboxylic acid cycle were oxidized by *M. scrofulaceum* and BCG oxidized the same substrates except for succinate and α-ketoglutarate.

**Oxidation of sulfur containing compounds.** Table 2 shows that SH containing compounds such as L-cysteine, dithioerythritol and DL-penicillamine were actively oxidized by whole cell suspensions of *M. lepraemurium* prepared from four month old rat lepromas as well as *M. leprae* isolated from armadillo lepromas. Likewise, these substrates were oxidized readily by *M. scrofulaceum* cell suspensions. The same substrates were oxidized at a comparatively slow rate by *in vitro* grown M. Dakar strain as well as

TABLE 1. *Oxidation of intermediates of glycolytic and tricarboxylic acid cycles by whole cell suspensions of mycobacteria.*

| | Mycobacteria | Growth | Exogenous $O_2$ uptake $\mu$l/two hours: Substrates | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Glucose | Pyruvate | Phosphoenolpyruvate | Oxalacetate | Acetate | α-Ketoglutarate | Succinate | Citrate |
| Rat leprosy | *M. lepraemurium* | *In vivo* | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 |
| | M. 1m 56 | *In vitro* | 0 | 8 | 8 | 11 | 10 | 0 | 14 | 0 |
| Human leprosy | *M. leprae* A6 | *In vivo* | 0 | ----------Not Done*---------- | | | | | 89 | |
| | M. A6 | *In vitro* | 0 | 48 | 7 | 56 | 150 | 16 | 22 | 5 |
| | M. Dakar | *In vitro* | 0 | 38 | 15 | 20 | 25 | 10 | 13 | 0 |
| Others | *M. scrofulaceum* | *In vitro* | 0 | 61 | 12 | 50 | 61 | 32 | 48 | 30 |
| | BCG | *In vitro* | 0 | 32 | 17 | 20 | 44 | 0 | 5 | 24 |

*Not enough material available.

TABLE 2. *Oxidation of sulfur compounds by intact cell suspensions of mycobacteria.*

| | Mycobacteria | Growth | Exogenous $O_2$ uptake $\mu$l/two hours: Substrates | | |
|---|---|---|---|---|---|
| | | | L-cysteine | dithioerythritol | penicillamine |
| Rat leprosy | *M. lepraemurium* | *In vivo* | 80 | 45 | 35 |
| | M. 1m 56 | *In vitro* | 22 | 22 | 14 |
| Human leprosy | *M. leprae* A6 | *In vivo* | 112 | 110 | 94 |
| | M. A6 | *In vitro* | 51 | 46 | 14 |
| | M. Dakar | *In vitro* | 23 | 20 | 13 |
| Others | *M. scrofulaceum* | *In vitro* | 65 | 49 | 33 |
| | BCG | *In vitro* | 94 | 26 | 18 |

by M. 1m 56; the rate of oxidation was the same by both cultures of mycobacteria. Although L-cysteine was oxidized at a rapid rate, dithioerythritol and penicillamine were oxidized at a slow rate by BCG cells.

**Oxidation of polysaccharides.** Whole cell suspensions of *M. lepraemurium* failed to oxidize hyaluronic acid and related compounds ([20]). Although the M. Dakar strain has been cultivated *in vitro* in hyaluronic acid-based medium, whole cell suspensions prepared from the cultures did not oxidize hyaluronic acid and other polysaccharides such as heparin dextran and galactomannane. Similarly, the same polysaccharides were not oxidized by whole cell suspensions of *M. scrofulaceum,* M. 1m 56 and *M. bovis,* BCG.

TABLE 3. *Oxidation of oleic acid and Tween by mycobacteria.*

| | Mycobacteria | Growth | Exogenous $O_2$ uptake $\mu$l/two hours | | |
|---|---|---|---|---|---|
| | | | Substrates | | |
| | | | Oleic acid | Tween 80 | Tween 20 |
| Rat leprosy | *M. lepraemurium* | *In vivo* | 0 | 43 | 48 |
| | M. 1m 56 | *In vitro* | 158 | 245 | 277 |
| Human leprosy | *M. leprae* A6 | *In vivo* | 0 | 0 | Not done |
| | M. A6 | *In vitro* | 256 | 248 | 256 |
| | M. Dakar | *In vitro* | 170 | 113 | 134 |
| Others | *M. scrofulaceum* | *In vitro* | 230 | 182 | 277 |
| | BCG | *In vitro* | 0 | 60 | 92 |

**Oxidation of fatty acids.** Results in Table 3 show that oleic acid was not oxidized by *in vivo* grown *M. lepraemurium* and *M. leprae* A6. While *M. lepraemurium* oxidized Tween 80 and Tween 20 at a slow rate, Tween 80 was not oxidized by *in vivo* grown *M. leprae* A6. All the *in vitro* grown cultures M. 1m 56, M. A6 and M. Dakar isolated from rat, armadillo and human leprotic lesions respectively, oxidized oleic acid, Tween 80 and Tween 20 at an extremely rapid rate similar to *M. scrofulaceum*. BCG did not oxidize oleic acid but burned Tween 80 and Tween 20 at a slow rate. Results are summarized in Table 3.

## DISCUSSION

The cultures isolated by the Skinsnes group ([40]) and our team ([16, 24]) were identified as belonging to the *M. scrofulaceum* complex, similar to *M. marianum*. We proposed ([19]) that the *in vivo* grown *M. leprae* as well as *M. lepraemurium* once adapted to extracellular *in vitro* substrates might behave culturally like the scotochromogenic strains. The primary purpose of our investigations is to investigate this working hypothesis. For metabolic studies, adequate amounts of purified cells, free from inhibitors, are required and in the past it has not been possible to isolate enough cells from human lepromas to conduct such studies. We have been able to isolate sufficient amounts of *M. leprae* from an infected armadillo and high amounts of *M. lepraemurium* from rat lepromas.

We grew strains isolated from a lepromatous leprosy case (M. Dakar), a strain from a *M. leprae* infected armadillo as well as a culture isolated from rat lepromas, in a medium containing glycerol, yeast extract and sheep serum. These strains have yielded regularly about 1 gm (wet weight) of cells from 2.5 liters medium, incubated at 34°C for about three weeks. Availability of sufficient *in vitro* grown mycobacterial cells have made the present respiratory studies possible. Such studies are necessary to understand the fundamental metabolic properties of the cultures. Our manometric studies have shown that yeast extract and glycerol are not oxidized by young cells of all of the three strains of *in vitro* grown mycobacteria, whereas the same substrates were oxidized by three week old bacilli ([19]). We have previously reported ([19]) that most of the *in vitro* grown M. Dakar cells remained non-acid-fast up to the fourteenth day while at the end of the third week nearly all the bacilli showed definite acid-fastness. In the present studies, changes in the ability to oxidize the substrates might be due to the absence of necessary enzymes in young cells, or adaptation to the substrates offered in the media.

In the course of investigations on the effect of various substrates on growth, it was found that both the human strain (Dakar) as well as the strain cultured from murine leprosy grew only when yeast extract, glycerol and sheep serum were present in the medium; in the absence of any of these substances no growth occurred in the primary cultures. The role of sheep serum in the media is not clear. It might contribute an essential nutrient for the organisms or protect the cells from inhibitors or toxic metabolites present

in the medium during the growth. Similar suggestions have been made concerning the role of serum in other media [3].

Yeast extract is slowly oxidized by host grown *M. lepraemurium* suspensions (Fig. 2) while the rate of oxygen uptake is significantly increased by M. lm 56 grown *in vitro* during many subcultures. Yeast extract is most commonly used for the growth of several microorganisms and is the richest source of growth factors. It also probably provides essential amino acids and other sources of nitrogen. Smith *et al* [41] reported that yeast extract was required for growth of *Thermoplasma acidophilum* and the growth promoting activity was proteinous in character. Based on their results, they suggested that yeast extract acts as ion scavenger for some trace metal requirements, it protects the organism from the high H+ concentration and is involved in ion transport. The present study shows that yeast extract is oxidized quite readily by the *in vivo* grown *M. leprae* and *M. lepraemurium* and also by the *in vitro* grown M. Dakar and M. lm 56, as well as by an authentic strain of *M. scrofulaceum* and BCG. Yeast extract therefore might serve as a source of energy for the above mycobacteria. Since both *in vivo* grown mycobacteria oxidized yeast extract, it is safe to propose that *M. leprae* and *M. lepraemurium* will burn yeast extract in a culture medium for growth and multiplication.

Glycerol has been extensively employed as the primary source of carbon in the cultivation of mycobacteria [6, 17, 21, 27, 29, 38, 47]. The major pathway employed by mycobacteria for the degradation of glycerol is the glycolytic pathway and the tricarboxylic acid cycle [2, 7, 17, 28, 30, 37, 43, 45, 46]. Succinate oxidation by *in vivo* grown *M. lepraemurium* has been shown to be mediated by the tricarboxylic acid cycle involving the electron transport chain [20]. In the present experiments, it became evident that the host-grown *M. leprae* A6 has a most potent enzymatic capacity to oxidize succinate. In contrast to *in vivo* grown *M. lepraemurium*, as shown in Table 1, the general ability of the *in vitro* grown M. Dakar, M. A6, M. lm 56, *M. scrofulaceum* and BCG to oxidize some intermediates of the tricarboxylic acid cycle suggests the involvement of this cycle during glycerol oxidation by these mycobacteria. Glycerol is probably degraded by whole cell suspensions of these cultures via the triosephosphate portion of the glycolytic pathway through pyruvate, acetyl CoA and the tricarboxylic cycle. A similar pathway has been proposed for the degradation of glycerol by other mycobacteria [7, 17, 43]. Several investigators have failed to show oxidation of the members of the tricarboxylic acid cycle by intact cells on *in vitro* grown mycobacteria [8, 9, 14]. However, cell-free preparations from these mycobacteria were able to oxidize a number of substrates of this cycle [8, 30, 38, 45, 46]. Without the use of cell-free extracts, it is difficult to determine whether the low oxygen uptake values shown in Table 1 are due to slow penetration of these substrates.

*In vitro* grown mycobacteria are known to require sulfur for their growth [5, 33, 34]. The oxidation of sulfhydryl containing compounds such as L-cysteine, DL-penicillamine and dithioerythritol by the *in vivo* grown *M. leprae* and *M. lepraemurium* as well as by *in vitro* grown M. lm 56, M. A6, M. Dakar, *M. scrofulaceum* and BCG suggests that sulfur or SH-groups are provided to sulfhydryl enzymes involved in the transport system of these mycobacteria. A marked growth promoting effect of some sulfur compounds for the growth of *M. leprae* has recently been reported by Olitzki [33]. Considerable increase in the amount of ATP has been shown when *M. lepraemurium* was incubated in the presence of some sulfur compounds [4].

Hyaluronic acid was initially thought to provide nutrient or energy or both for the growth of *M. leprae* [40]. However, our studies have shown conclusively that the Honolulu strain (HI-75), the Dakar strain as well as M. lm 56 isolated from rat lepromas, grew well in the absence of hyaluronic acid [22], at least after growth adaptation to standard media. Our respiratory studies have also shown that resting cells of these strains, grown either in the presence or absence of hyaluronic acid, did not oxidize this compound. Likewise, constituents of mucopolysaccharides such as D-glucuronic acid, N-acetyl-D-glucosamine, chondroitin sulfate as well as other mucopolysaccharides such as heparin and galactomannane were all inactive.

*In vivo* grown *M. lepraemurium* and the *in vitro* cultures obtained from rat lepromas exhibited some differences in substrate oxidation. As shown in Table 1, none of the inter-

mediates of glycolysis and the tricarboxylic cycle were oxidized by the *in vivo* grown murine bacillary suspensions. However, a culture isolated from rat lepromas and cultivated on glycerol, yeast extract and sheep serum oxidized, though at a limited rate, several members of both the glycolysis and the TCA cycle. Yeast extract was poorly oxidized by the *in vivo* grown *M. leprae* and *M. lepraemurium* while it was actively oxidized by the *in vitro* cells cultivated from armadillo and rat leprous tissue.

Similarities were found between the *in vitro* grown strains isolated from human, armadillo and rat leprous tissues in that they are scotochromogenic, oxidized yeast extract, glycerol and several other members of the glycolytic and Krebs' cycle as well as sulfur containing compounds. The results might indicate that both the human and the rat strains once adapted to life outside the host on artificial media, behave like authentic *M. scrofulaceum.* There are striking similarities in the *in vitro* grown scotochromogenic mycobacteria, namely M. A6, M. Dakar, M. 1m 56 and *M. scrofulaceum,* except that *M. scrofulaceum* exhibited higher oxidative activities. Our experiments did not bring us closer to the identification of the mycobacteria isolated from human, armadillo or rat leprous tissue. The cultured cells constantly exhibited a higher metabolic activity than the *in vivo* grown cells. The *in vitro* grown cells oxidized, for instance, glycerol at a considerable rate, while the *in vivo* grown cells did not utilize the same substrate. The question still remains unanswered as to whether the host-grown *M. lepraemurium* is identical to M. 1m 56. Though we cannot claim, on these bases, the successful *in vitro* cultivation of *M. lepraemurium,* the obtained results do not exclude this possibility. Host-grown *M. lepraemurium* oxidized yeast extract and M. 1m 56 was grown on the same substrate. After adaptation in several subcultures the cells oxidized yeast extract at a higher rate. Glycerol was not oxidized by the host-grown cells and glycerol oxidation by M. 1m 56 is of such a low grade that the energy generated from this substrate may have no practical significance. On the other hand, authentic *M. scrofulaceum* readily oxidizes glycerol. Oxidation of intermediates of the glycolytic and tricarboxylic acid cycles by *in vivo* grown *M. lepraemurium* is similar if not identical to the same parameters of M. 1m 56. These properties of *M. lepraemurium* and M. 1m 56 are entirely different from the same characteristics of *M. scrofulaceum,* the substrate oxidizing properties of M. 1m 56 and *M. lepraemurium* are similar to each other but not at all identical with the oxidative properties of *M. scrofulaceum.*

*M. lepraemurium* did not oxidize oleic acid but burned Tween 80 and Tween 20. Mycobacteria cultured from rat leprous tissue burned both oleic acid and Tween at a high rate. These results do not necessarily show that the two strains are different. It is known that host-grown tubercle bacilli are easily stained by Sudan black B, while the same cells cultured *in vitro* remained unstained by the same colorant ([39]). These differences in staining properties indicate that fatty acid metabolism of *in vivo* and *in vitro* grown mycobacteria may be entirely different. *In vivo* grown *M. lepraemurium* cells accessible for the surface active Tweens and oxidation occurred. The *in vitro* grown cells were probably penetrated by both types of molecules, oleic acid and the Tweens and permitted the high rate of oxidation of both substrates. There is a definite transition between oleic acid oxidation of *in vivo* grown *M. lepraemurium* and *in vitro* grown M. 1m 56 and *M. scrofulaceum.* In this respect *M. lepraemurium* and *M. scrofulaceum* represent the two antipodes with M. 1m 56 differing from both in the rate of oleic acid oxidation. Concerning the oxidation of sulfur compounds, the host-grown *M. lepraemurium* oxidized these substrates at the same high rate as *M. scrofulaceum.* The *in vitro* grown M. 1m 56 oxidized the same substrates at a relatively low rate and thus show a definite difference from *M. scrofulaceum.* Even if *in vitro* grown *M. lepraemurium* belong to the *M. scrofulaceum* species, the striking differences between M. 1m 56 and authentic *M. scrofulaceum* in this respect cannot be denied.

The successful cultivation of *M. leprae* from human and armadillo leprous tissue, cannot be claimed on the basis of these metabolic studies. Without doubt, oxidation of yeast extract, glycerol, oleic acid and Tween, the sulfur compounds and oxidation of intermediates of the glycolytic and tricarboxylic acid cycles of the authentic *M. scrofulaceum* and the *in vitro* grown M. Dakar and M. A6 are absolutely identical. *In vivo* grown *M.*

*leprae* A6 failed to oxidize oleic acid and Tween and did not oxidize glycerol. The *in vitro* grown M. A6 oxidized glycerol and all the other substrates tested at the highest rate among the mycobacteria tested, comparable to the oxidative potency of *M. bovis*, BCG. The results support the possibility that the *in vitro* grown human leprosy bacillus is a scotochromogenic strain belonging to the *M. scrofulaceum* complex.

Important differences were registered with respect to the metabolic activity of *in vivo* grown *M. leprae* and *M. lepraemurium*. Succinate was not oxidized by rat leprosy bacillus but was strongly oxidized by *M. leprae* isolated from the armadillo. The sulfur compounds were constantly better oxidized by *M. leprae* A6 than by *M. lepraemurium*. Tween was not oxidized by *M. leprae* A6 but was actively oxidized by *M. lepraemurium*. There were also striking differences between *in vitro* grown cells cultured from rat leprosy and the *in vitro* grown mycobacteria cultured from human and armadillo leprous tissue. The substrates were not at all or weakly oxidized by M. Im 56 but were strongly oxidized by M. A6 and M. Dakar. Mycobacteria cultured from leprous tissue of the armadillo constantly exhibited a high metabolic activity when compared to the respiration of any of the other strains of mycobacteria.

Results clearly show that *M. lepraemurium* and *M. leprae* possess entirely different metabolic characteristics, except for the oxidation of Tween 80. *M. leprae* is constantly a metabolically more active and competent microorganism than *M. lepraemurium*. Similarly, mycobacteria cultured from an armadillo infected with *M. leprae* possess a considerably more potent oxidative ability than mycobacteria cultured from rat leprotic tissues. Mycobacteria cultured from the leprous tissues of the armadillo are also metabolically more competent and potent than mycobacteria cultured from human leprous tissue.

We may, therefore, conclude:

1. *In vitro* adapted mycobacteria derived from an *M. leprae* infected armadillo and an *M. lepraemurium* infected rat are metabolically highly competent, have metabolic characteristics similar to the commonly recognized strains of scrofulacea, which suggest that they are members of the scrofulaceum complex.

2. Both *in vivo* grown *M. leprae* and the corresponding culture of mycobacteria derived from an *M. leprae* infected armadillo possess considerably more competent and potent oxidative ability than *in vivo* grown *M. lepraemurium* and the culture derived from *M. lepraemurium* infected rats.

3. Mycobacteria cultured from an *M. leprae* infected armadillo were metabolically more competent than mycobacteria cultured from lepromatous patients.

4. The substrate oxidizing properties of host-grown *M. leprae* and *M. lepraemurium* and of mycobacteria cultured from *M. lepraemurium* infected rats and from leprous tissues of humans and armadillos are similar but not at all identical with those of a standard strain of *M. scrofulaceum*.

Based on present and previous results, we propose that cultivation trials of *M. leprae* should be centered on leprous tissue from *M. leprae* infected armadillos. We maintain the hypothesis that *M. leprae* and *M. lepraemurium* once adapted to extracellular life may behave like a scotochromogenic, slow-growing strain of scrofulaceum or that some strains of *M. scrofulaceum* may produce in the susceptible host a disease identified by the morbid pathologists as human or rat leprosy. The same strains of *M. scrofulaceum* adapted to intracellular life might become noncultivable on conventional culture media used for mycobacteria. However, even during intracellular life, the same strains of scrofulacea retain their ability to oxidize certain specific substrates such as yeast extract, sulfur compounds or succinate and, once the same substrates are offered to them in the culture media, they may be adapted again to extracellular life and be identifiable as a slow-growing scotochromogenic culture. However, in many respects they show striking differences depending on the host from which they are isolated or cultured as has become evident in our present experiments.

## SUMMARY

Oxidative activities of armadillo-grown *M. leprae* and rat-grown *M. lepraemurium* as well as of the *in vitro* grown mycobacteria cultured from human, armadillo and murine lepromas were investigated and compared with authentic strains of *M. scrofulaceum* and *M. bovis*, BCG. Yeast extract was oxidized at a slow rate by *in vivo* grown *M. lep-*

*rae* and *M. lepraemurium,* but was actively utilized by all the cultures cultivated on artificial medium. Although no oxidation of glycerol by host-grown mycobacteria occurred, after a period of 60 minutes it was utilized at a slow rate by *in vitro* grown *Mlm* (M. 1m 56) and *M. leprae* (M. Dakar). However, glycerol was actively oxidized by *M. leprae* (M. A6, armadillo derived), *M. scrofulaceum* and BCG. None of the intermediates of the glycolytic cycle as well as of the tricarboxylic acid cycle were oxidized by purified cell suspensions of *M. lepraemurium* but succinate was readily oxidized by *M. leprae* cell suspensions. Resting cell suspensions of all the *in vitro* grown cultures have been shown to increase their oxygen consumption in the presence of several members of the glycolytic and Krebs' cycles. Sulfur compounds, e.g., cysteine, dithioerythritol and penicillamine were readily oxidized by all the *in vivo* and *in vitro* grown mycobacteria used in this study. While oleic acid was inactive to human and murine bacillary suspensions as well as to BCG, it was readily oxidized by M. 1m 56, M. A6, M. Dakar, and *M. scrofulaceum.* All the *in vitro* grown cultures caused considerable increase in oxygen consumption over the endogenous value in the presence of Tween 80 and Tween 20 but the same substrates were slowly oxidized by murine leprosy bacilli. Comparative rates of oxidation of several substrates by host-grown and *in vitro* grown mycobacteria are discussed.

## RESUMEN

Se estudiaron las actividades oxidativas del *M. leprae* crecido en el armadillo, del *M. lepraemurium* crecido en ratas y de las micobacterias crecidas *in vitro* a partir de lepromas humanos, murinos y de armadillos. Estas actividades oxidativas se compararon con las de cepas auténticas del *M. scrofulaceum* y del *M. bovis* (BCG). El *M. leprae* y el *M. lepraemurium* crecidos *in vivo* oxidaron lentamente al extracto de levaduras pero éste fue oxidado en forma muy activa por todos los cultivos crecidos *in vitro.* Aunque las micobacterias crecidas en su huésped natural no oxidaron al glicerol, éste fue utilizado en forma muy lenta después de 60 min por el *M. lepraemurium* (M.1m 56) y por el *M. leprae* (M. Dakar) crecidos *in vitro.* Sin embargo, el *M. leprae* (M.A6, derivado del armadillo), el *M. scrofulaceum* y el BCG, sí oxidaron activamente al glicerol. Las suspensiones purificadas del *M. lepraemurium* no oxidaron a ningún intermediario del ciclo glicolítico o del ciclo de los ácidos tricarboxílicos, pero las suspensiones del *M. leprae* sí oxidaron al succinato. Las suspensiones de todos los cultivos crecidos *in vitro* incrementaron su consumo de oxígeno en presencia de varios componentes de los ciclos glicolítico y de Krebs. Todas las micobacterias usadas en este estudio, crecidas *in vitro* o *in vivo,* oxidaron eficientemente a los compuestos azufrados cisteína, ditioeritritol y penicilamina. El ácido oléico no fue utilizado por el BCG ni por las suspensiones de las micobacterias humana o murina, mientras que este ácido sí fue oxidado por M.1m 56, M.A6, M. Dakar y *M. scrofulaceum.* Todos los cultivos crecidos *in vitro* presentaron un incremento considerable en su consumo de oxígeno en presencia de Tween 80 y Tween 20, pero estos substratos sólo fueron oxidados lentamente por el bacilo de la lepra murina. Se discuten y comparan las capacidades de oxidación de varios substratos por micobacterias crecidas *in vitro* e *in vivo.*

## RÉSUMÉ

Les capacités oxydatives de *M. leprae* et *M. lepraemurium* cultivées dans leur hôte, de même que celles de mycobactéries cultivées *in vitro* à partir de lépromes humains, murins et de tatou furent étudiées et comparées avec des souches authentiques de *M. scrofulaceum* et *M. bovis,* BCG. L'extrait de levure était oxydé à un faible taux par les cultures *in vivo* de *M. leprae* et *M. lepraemurium,* mais fut utilisé rapidement par toutes les souches cultivées sur milieu synthétique. Bien qu'il n'y avait aucune oxydation de glycérol par les mycobactéries cultivées dans l'hôte, après une période de latence de 60 minutes, ce composé est oxydé à un faible taux par M. 1m 56 et M. Dakar, des souches cultivées *in vitro.* Par contre, le glycérol est rapidement oxydé par M. A6, *M. scrofulaceum* et BCG. Aucun des intermédiaires du sentier de la glycolyse, ni du cycle des acides tricarboxyliques n'était oxydé par les suspensions cellulaires purifiées de *M. lepraemurium,* mais le succinate était facilement oxydé par les suspensions de *M. leprae.* Toutes les suspensions cellulaires de bacilles en phase stationnaire cultivés *in vitro* ont manifesté un accroissement dans la consommation d'oxygène en présence de composés du sentier de la glycolyse et du cycle de Krebs. Les substrats sulfurés, tels que la cystéine, le dithioérythritol et la pénicillamine sont rapidement oxydés par toutes les mycobactéries cultivées *in vivo* ou *in vitro* utilisées dans cette étude. Alors que l'acide oléique était inactif avec les suspensions bacillaires de *M. leprae, M. lepraemurium* et aussi de BCG, il était rapidement oxydé par M. 1m 56, M. A6, M. Dakar et *M. scrofulaceum.* Toutes les souches cultivées *in vitro* manifestaient une augmentation dans la consommation d'oxygène par rapport à la respiration endogène en présence de Tween 80 et Tween 20,

mais ces substrats n'étaient que faiblement oxydés par les bacilles de lèpre murine. Les taux d'oxydation de plusieurs substrats utilisés par les mycobactéries cultivées dans l'hôte et *in vitro* sont comparés et discutés.

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