Radiometric Measurement of Metabolic Activity of *Mycobacterium lepraemurium*

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A sensitive and nondestructive radiometric method has been applied to the detection of metabolism of *Mycobacterium lepraemurium*, as a model for the study of the metabolism and substrate requirements of *M. leprae*. The method is based on the measurement of the $^{14}$CO$_2$ produced through the bacterial conversion of [U-$^{14}$C]acetate or [U-$^{14}$C]glycerol by $7 \times 10^8$ bacteria suspended in 10 ml of either a simple buffer system (K-36) or a complex medium (NC-5). Metabolism of the bacilli was easily detected within 3 days after inoculation and was measured daily. NC-5 medium supported metabolism of *M. lepraemurium* for several weeks longer than the simple K-36 buffer. The radiometric technique shows promise as a rapid and efficient system for evaluating the metabolism of mycobacteria without introducing any changes in the physiologic state of the organisms, studying their metabolic pathways, determining conditions potentially favorable for multiplication of these organisms in vitro, and studying their susceptibility to inhibition by drugs.

*Mycobacterium lepraemurium*, the agent of murine leprosy, has been used extensively as a model for human lepromatous leprosy in studies of pathology, immunology, and chemotherapy (9). Laboratory study of *M. leprae* and *M. lepraemurium* has been greatly hindered by the fact that neither organism can be maintained in culture in cell-free media. Thus, little is known of the metabolism of these organisms and the conditions which might force their growth in vitro. The use of radioactive substrates has shown promise in studies of the substrate requirements of *M. lepraemurium*. In 1968, E. Camargo (unpublished data) found [U-$^{14}$C]aspartic acid to be more easily assimilated than [U-$^{14}$C]thymidine. More recently the assimilation of several radioactive substrates by *M. lepraemurium* has been studied by Tepper (8) and by Tepper and Varma (9). For these investigations, the bacilli were suspended in the Hart-Valentine elongation medium (5) or in a simple buffer system (10) and assayed by liquid scintillation detection for both the $^{14}$CO$_2$ production and $^{14}$C-substrate incorporation. Although 7 days of incubation of the bacilli (8) were required before detectable levels of $^{14}$CO$_2$ were produced, these investigations demonstrated that acetate and glycerol were oxidized and assimilated by *M. lepraemurium*, and these findings suggested a starting point for the present study.

In 1969, we introduced a simple radiometric system, an ionization chamber, for the detection of bacterial growth as measured by the conversion of carbon-14-labeled substrate to $^{14}$CO$_2$ (4). Since that time, preliminary investigation supporting this concept has been completed on a comparison of the standard and radiometric microbiologic techniques in blood cultures (2, 3), radiometric applications to anaerobic microbiology, and radiometric detection of antibiotic effect on bacterial growth (1). These studies demonstrated that the radiometric detection of bacterial growth using $^{14}$C-labeled substrates is rapid and sensitive for many species of bacteria and technically much easier than conventional liquid scintillation counting.

The purpose of this report is to extend the application of the ionization chamber system to the study of the metabolism of *M. lepraemurium* and to test its application for determining conditions potentially favorable for multiplication of this organism in vitro.

**MATERIALS AND METHODS**

**Preparation of bacilli.** The Hawaiian strain of *M. lepraemurium* was harvested from female CBA/5 mice (Jackson Laboratories) which had been infected intraperitoneally and intravenously 3 to 4 months previously with $5 \times 10^8$ bacteria. Infected liver and lesions in the pelvic and omental fat were removed.
aseptically and the bacteria were freed from the tissue components according to the technique described by Tepper and Varma (9). Approximately 10^11 bacteria were obtained from 10 mice and the bacilli were suspended in sterile water to a final concentration of 1.4 × 10^11 bacteria per ml.

**Media.** The simple K-36 buffer of Weiss (10) (0.1 M KCl, 0.01 M NaCl, 0.05 M KH₂PO₄, pH 7.0) provided an environment with a high K⁺/Na⁺ ratio. A complex nutrient medium (NC-5) was prepared according to the technique of Nakamura (6); in some of the experiments, glycerol was omitted from the preparation.

**Reaction system.** A 10-ml amount of suspending medium was placed in a 20-ml multidose vial, along with 5 μCi of [U-¹⁴C]acetate or [U-¹⁴C]glycerol (New England Nuclear Corp.). A 0.5-ml amount of the final suspension of bacteria was inoculated (7 × 10^9 bacteria per flask) into each vial. All flasks were prepared in triplicate. Controls were obtained that consisted of the identical substrates and medium, but with autoclaved bacteria added.

**Radiometric measurement.** The vials were incubated at 30°C and the ¹⁴CO₂ produced by bacterial metabolism was measured radiometrically. The measurement device, the Bactec-301 (Johnston Laboratories, Inc., Cockeysville, Md.), consisted of an ionization chamber, a vacuum pump, and a set of sampling needles. The needles penetrated the rubber stopper in the top of the multidose experimental vial, and the ¹⁴CO₂ was aspirated into the ionization chamber under vacuum. The atmosphere in the vial was replaced with a culture gas that consisted of 8% CO₂ and 92% O₂. Radioactivity is measured by the ionization chamber, and the results are expressed as "index units." Each index unit was 0.025 μCi.

**Assimilation of substrates by bacteria.** In some of these experiments, ¹⁴C-substrate incorporation into the bacteria was measured by liquid scintillation counting. For these experiments, 10⁶ bacteria and 2 μCi of substrate were added to either the K-36 buffer or NC-5 medium with or without glycerol. After 19 days of incubation, the suspensions were filtered through sterile membrane filters (0.45-μm pore size; Millipore Corp.). The filters were washed with sterile saline until the radioactivity in the last wash was at background levels. Filters were then dissolved with ethyl acetate and Permaflour II scintillation fluid (Packard Instrument Co.) was added. Counting was performed in a Packard Tri-Carb scintillation spectrometer model 3003 (Packard Instrument Co.).

**Sterility testing.** Sterility tests were performed on all samples and consisted of subculture in chocolate agar, radiometric sterility testing with [U-¹⁴C]glucose (1-4), modified Ziehl-Nielsen staining (7), and subculture in Lowenstein-Jensen medium (BBL).

**RESULTS**

**Conversion of [U-¹⁴C]acetate and [U-¹⁴C]glycerol to ¹⁴CO₂.** After inoculation, metabolism of *M. lepraeumurium* resulted in readily measureable ¹⁴CO₂ production in both the K-36 buffer and NC-5 medium. The curves shown in Fig. 1 and 3 represent the differential ¹⁴CO₂ production, i.e., the amount of ¹⁴CO₂ produced within a definite time interval and plotted as an index reading on the ionization chamber scale. The curve in Fig. 2 represents the cumulative ¹⁴CO₂ production within the same time interval shown in Fig. 1 and is plotted as the integral curve of total activity. In the K-36 medium containing radioactive acetate, the full scale was reached by 7 days with a subsequent decline thereafter (Fig. 1). The same medium containing radioactive glycerol showed a peak at 80% of full scale by 7 days and subsequently declined (Fig. 1). In the NC-5 medium containing radioactive acetate, the full scale deflection (100) of the ionization chamber was reached by 3 days and maintained at this level for 16 days, declining progressively thereafter (Fig. 3). In the same medium containing radioactive glycerol, it took 11 days to reach full
scale but this activity was then maintained for 44 days (Fig. 3).

\[ ^{14}\text{CO}_2 \] production was not observed in the control flasks containing autoclaved bacteria, and all sterility tests were negative. After 60 days of incubation in NC-5 medium bacterial counts showed no evidence of replication; no elongation of the bacteria was observed.

**M. lepraemurium assimilation of [U-^{14}C]acetate and [U-^{14}C]glycerol.** Since the \[ ^{14}\text{CO}_2 \] measurements provided information on the oxidation of substrate by *M. lepraemurium*, the assimilation of substrates into bacterial macromolecules was also obtained by evaluating incorporation of radioactivity into the cells. The activity of cells exposed to [U-^{14}C]acetate and [U-^{14}C]glycerol in the K-36 buffer and NC-5 medium with and without glycerol was compared (Fig. 4). Nineteen days after incubation, the washed bacilli contained significant amounts of radioactivity when incubated with [U-^{14}C]acetate; incorporation of [U-^{14}C]glycerol was much higher, particularly when no unlabeled glycerol was added to NC-5 medium.

**DISCUSSION**

The results of this study have provided further support for the use of radiometric techniques for the study of host-dependent mycobacteria. In addition to confirming the observations of Tepper and Varma (9) that *M. lepraemurium* can utilize exogenous substrates in vitro, this technique has provided a simple and more expedient method for the monitoring of the metabolic activity of these organisms. The increase in the specific activity of the \[ ^{14}\text{C} \]-substrates to 5 \( \mu \text{Ci} \) and the sampling of the \[ ^{14}\text{CO}_2 \] oxidation product directly permits the repeated sampling for metabolic activity of individual cell suspensions during the entire course of the long incubations required with *M. lepraemurium*. This technique has proven sensitive enough to measure the metabolism of *M. lepraemurium* within a few hours and therefore has the potential for screening larger numbers of substrates and environmental conditions with these bacteria which must be laboriously and carefully separated from the host tissues.

Furthermore, the results presented in this study have shown radiobioremetry to be a biochemical tool for demonstrating growth favoring nutritional or physical environments for *M. lepraemurium*. Two environmental conditions were tested, a simple buffer system (K-36) and a more complex growth medium (NC-5). Nakamura (6) has reported limited multiplication of *M. lepraemurium* in the NC-5 medium; however, serial subculture of *M. lepraemurium* in the NC-5 medium has not been accomplished. Our data readily demonstrated the NC-5 medium to be a more favorable environment than the K-36 buffer for the metabolism of acetate and glycerol by *M. lepraemurium*. When compared to the results in K-36 system, *M. lepraemurium* showed higher oxidative and assimilative capacity when the two substrates were added to the NC-5 medium. This occurred in the absence of demonstrable multiplication or elongation of the bacteria under the conditions of our experiments. Subsequent improvements to the medium should show similar improvements in the metabolism of *M. lepraemurium*.

The radiometric technique described provides a method for the study of the metabolism of *M. lepraemurium* and a rational approach to the design of a medium which will support the continued multiplication of this organism. For *M. lepraemurium*, other potential applications of this method include radiometric testing of metabolic pathways, the effect of various media on the metabolism, and the susceptibility to inhibition by drugs.

The results also suggest that the same proce-
dure can be applied to the study of *M. leprae* and other host-dependent microorganisms.

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