Radiometric Studies of *Mycobacterium lepraemurium*¹,²

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*Mycobacterium lepraemurium* and *Mycobacterium leprae* are organisms difficult to study in the laboratory because of the impossibility of cultivating them in cell-free media. Therefore, little is known of the metabolism of these organisms and the conditions which might force or inhibit their growth *in vitro*. The studies of Tepper and Varma (14) with radioisotopes and liquid scintillation counting showed that *M. lepraemurium* was metabolically active *in vitro* (U-¹⁴C) acetate and (U-¹⁴C) glycerol were found to be assimilated and oxidized by these organisms after seven days of incubation in the Hart-Valentine elongation medium (³) or in the simple K-36 buffer of Weiss (12).

In 1969, we introduced a simple radiometric system, an ion chamber, for the detection of bacterial growth as measured by the conversion of ¹⁴C-labeled substrate to ¹⁴CO₂ (⁴). Investigations have been completed comparing the standard and radiometric technics in blood cultures (¹), in anaerobic microbiology and detection of the effect of drugs on bacterial growth (⁴). More recently, the radiometric technic was extended to the study of mycobacteria. Based on the findings of Tepper and Varma (14), it was decided to develop a more expedient method for monitoring the metabolic activity of *M. lepraemurium in vitro* (¹). The next step was to use the same method for the study of the effect of several drugs on the metabolism of these organisms in *vitro* (¹). Simultaneously, several other experiments have been done, in order to better understand the metabolic requirements and the influence of physical and biochemical factors that alter the metabolism of *M. lepraemurium* in cell-free media. The present paper reports the results of these experiments.

**MATERIALS AND METHODS**

Preparation of bacilli. *M. lepraemurium* (Hawaiian strain) was harvested from infected livers of female CBA/J or CFW mice, which had been intravenously and intraperitoneally infected 3-4 months previous with 5 x 10⁹ organisms. The livers were aseptically removed and the bacteria separated from the infected tissue according to the technic previously developed (¹). The suspensions were further diluted with sterile water to final concentrations of 8.6 x 10⁹ or 4 x 10⁸ or 2 x 10⁷ organisms/ml.

**Media.** The simple K-36 buffer of Weiss (¹²) or the complex NC-5 medium (¹⁰) were used as suspending solutions for the organisms.

**Reaction system.** The reaction systems for most of the experiments consisted of 10 ml of suspending solution in a 20 ml multidose sterile vial. In some of the experiments, when testing the effect of concentration of substrate on the ¹⁴CO₂ output, 5 ml or 1.0 ml of suspending solution in a 20 ml vial were also used. In all the experiments, 5 µCi of ¹⁴C-substrate and 0.5 ml of the bacterial suspension were used. The experimental vials were prepared at least in duplicate. Control vials were prepared in the same way, but with autoclaved bacteria added. When studying the effect of substances on the metabolism of *M. lepraemurium*, extra controls with live bacteria and without the given substance were always prepared for comparison.

**Radiometric measurement.** With the exception of the experiment designed to study the effect of incubation temperature, the vials were always incubated at 30°C. An ion chamber device (Bactec K-301, Johnston Laboratories, Cockeysville, Md.) was used to measure bacterial metabolism. Details of the operation of the measurement device have been published elsewhere (²). The

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vials were sampled at least daily (in one of the experiments also at 6, 12 and 18 hours) for 15 to 18 days. The results were expressed in "index units" where 100 units = 0.025 µCi of 14C activity. The curves represent the cumulative 14CO2 production within a certain time interval, that is, they are the integral curves of total activity.

Sterility testing. Sterility tests were performed on all positive samples and consisted of subcultures on chocolate-agar plates, on Lowenstein-Jensen medium and radiometric sterility testing with (U-14C) glucose (47). 

Assimilation of substrates. In one of the experiments, 14C-substrates incorporation into the bacteria were measured by liquid scintillation counting. After the period of incubation, all suspensions of the same substrate were pooled and filtered through sterile membrane filters (0.45 µ pore size, Millipore Corporation). The filters were washed with sterile saline until the radioactivity in the last wash was at background levels, dissolved with ethyl acetate and Permafluor II scintillation fluid (Packard Instrument Company) was added. Counting was performed in a Packard Tri-Carb scintillation spectrometer model 3003 (Packard Instrument Company).

EXPERIMENTAL

Oxidation and assimilation of 14C-substrates. Female CFW mice were used as the source of bacteria. The organisms were diluted to a final concentration of 8.6 × 109 per milliliter. Both the K-36 buffer and the NC-5 medium were used as suspending solutions. The reaction system consisted of 10 ml of suspending solution in a 20 ml multidose sterile vial, along with 5 µCi (0.5 ml) of (U-14C) acetate or (U-14C) glycerol or (U-14C) glucose or (U-14C) glycine or (14C) formate or (U-14C) pyruvate and 0.5 ml of the final suspension of bacteria (4.3 × 108 organisms/vial). All vials were incubated at 30°C and sampled at 6, 12, 18 and 24 hours after incubation and at daily intervals for 16 days thereafter. Then, the vials were prepared for assimilation studies, as described above. Results were expressed as radioactivity ratio between live and killed bacilli.

In both media, 14CO2 production from (U-14C) acetate could be easily detected after six hours, increasing progressively to reach a plateau by 13 days (Figs. 1, 2). From (U-14C) glycerol, 14CO2 production in K-36 buffer was detected by 24 hours, also increasing to a plateau by 13 days (Fig. 1); however, in NC-5 medium, only on the third day could 14CO2 be detected, increasing progressively to a lower plateau by 16 days (Fig. 2). The assimilation of (U-14C) acetate and (U-14C) glycerol paralleled the oxidation of these substrates (Figs. 3, 4). In both media, 14CO2 production from (U-14C) glucose, (U-14C) pyruvate, (U-14C) glycine or (14C) formate never exceeded background levels. All the substrates were assimilated in K-36 buffer, although assimilation was poor for (U-14C) glucose, (U-14C) pyruvate and (U-14C) glucose. In NC-5 medium, there was no assimilation of (U-14C) glucose or (U-14C) pyruvate; incorporation of (14C) formate was also very poor (Figs. 3, 4).

Influence of incubation temperature. Female CBA/J mice were used in this experi-
The bacteria were diluted to a final concentration of $2 \times 10^9$ organisms per milliliter. K-36 buffer was the suspending solution. The reaction system consisted of 10 ml of buffer in a 20 ml multidose sterile vial, along with 5 µCi (0.1 ml) of (U-14C) acetate and 0.5 ml of the final suspension of bacteria ($1 \times 10^9$ organisms/vial). Some of the vials were incubated at 30°C and some at 37°C and sampled daily for 15 days. No difference in the $^{14}$CO$_2$ production caused by incubation temperature between vials incubated at 30°C and 37°C was found over the first four days. After that, $^{14}$CO$_2$ output from vials incubated at 30°C increased progressively so that by the end of the experiment their $^{14}$CO$_2$ production was 58% higher than that obtained with vials incubated at 37°C (Fig. 5).

Influence of polysorbate 80 (Tween 80) and unlabeled oleic acid. Female CBA/J mice were the source of M. lepraemurium. The organisms were diluted to a final concentration of $4 \times 10^9$ bacteria per milliliter. Only the K-36 buffer was used as suspending solution. The reaction system consisted of 10 ml of buffer in a 20 ml multidose
sterile vial, along with 5 μCi (0.1 ml) of (U-14C) acetate, 0.5 ml of the final suspension of bacteria (2 × 10^9 organisms/vial) and 0.1 ml of the desired concentration of Tween 80 or unlabeled oleic acid. The effect of Tween 80 was evaluated at 0.005%, 0.01%, 0.025% and 0.05% per vial; for unlabeled oleic acid, only 0.005% per vial was used. The vials were incubated at 30°C and sampled daily for 18 days.

As the concentration of Tween 80 increased, the production of 14CO₂ by M. leprae murium also increased, particularly after the third day (Fig. 6). A profound inhibitory effect of unlabeled oleic acid on the 14CO₂ production from (U-14C) acetate is observed in Figure 7. Because of this effect of unlabeled oleic acid, in an additional experiment, 5 μCi (0.1 ml) of oleic acid was substituted for 5 μCi (U-14C) acetate. Figure 8 suggests that M. leprae murium seems to prefer (U-14C) oleic acid to (U-14C) acetate with respect to conversion to 14CO₂.

Influence of concentration of 14C-substrate. The preparation was exactly the same as described under the influence of polysorbate 80. The reaction system consisted of 20 ml multidose sterile vials with 1.5 or 10 ml of K-36 buffer. In all these vials 5 μCi of (U-14C) acetate (0.1 ml) and 0.5 ml of the final suspension of bacteria (2 × 10^9 organisms/vial) were used. Therefore, three different concentrations of (U-14C) acetate were used: 0.5 μCi/ml, 1.0 μCi/ml and 5 μCi/ml. The vials were incubated at 30°C and sampled daily for 18 days. There was a clear difference in the 14CO₂ production as related to the concentration of substrate. As the concentration increased, the 14CO₂ output also increased, as shown in Figure 9.

Influence of freezing. Female CBA/J mice were used in this experiment. After harvesting the infected livers, one half of the liver specimen was prepared according to the technic already described to yield a final concentration of 4 × 10^9 organisms/ml. The reaction system consisted of 10 ml of K-36 buffer in a 20 ml multidose sterile vial along with 5 μCi (0.1 ml) of (U-14C) acetate and 0.5 ml of the final bacterial suspension (2 × 10^9 organisms/vial). The vials were incubated...
The effect of concentration of (U-14C) acetate on the metabolism of M. lepraemurium in K-36 buffer.

**DISCUSSION**

The inability of M. lepraemurium to oxidize (U-14C) glucose, already discussed by Tepper and Varma (11), besides suggesting that the "hexose" portion of the glycolytic pathway is largely synthetic, further supports the concept of using this substrate for the detection of contaminants in pure suspension of M. lepraemurium. Most contaminating bacteria rapidly metabolize (U-14C) glucose to 14CO2 (47). The inability in oxidizing pyruvate found with the radiometric technic agrees with the experiments of Mori et al (9) utilizing enzyme systems. It suggests that the metabolism of (U-14C) glyceral may occur by means of a simple enzymatic action on glyceralic acid instead of more complex pathways.

The fact that M. lepraemurium does not oxidize (U-14C) glycine may be related to the inability of metabolizing pyruvate and formate. Therefore, the pathways for glycine oxidation, either through pyruvate or formic acid, probably are not available for these organisms. The reason why some substrates such as (U-14C) glycine and 14C-formate are assimilated in both media but oxidized is unknown.

The curves in Figure 5 suggest that for short-term experiments, differences in the incubation temperature may not be important. But for experiments that have to be carried out for one week or more, the incubation of the organisms at 30°C yields a much higher 14CO2 output than at 37°C.
The presence of 0.05% Tween 80 in the reaction system causes almost a tenfold increase in the $^{14}$CO$_2$ production by M. lepraemurium (Fig. 6). This may be due to the emulsifying effect of Tween 80, but could also be related to the presence of fatty acids such as oleic acid in its composition. To evaluate the possible effect of oleic acid on M. lepraemurium metabolism, unlabeled oleic acid was used initially and a profound inhibitory effect on the oxidation of (U-$^{14}$C) acetate occurred (Fig. 7). The next step was to decide whether the results in Figure 7 represented a toxic effect of oleic acid or a shifting from acetate to oleic acid metabolism by M. lepraemurium. The use of (1-$^{14}$C) oleic acid was revealing, since there is no question that these organisms metabolize avidly the fatty acid (Fig. 8). Results shown in Figures 7 and 8 are similar to the "diastatic" growth cycles (1) because (1-$^{14}$C) oleic acid is also metabolized. These findings, which actually began with an accidental use of Tween 80 in one of our early experiments in order to break up a few clumps, lead us to an in-depth investigation of the fatty acids series with encouraging preliminary results.

One explanation for the results obtained with different concentrations of (U-$^{14}$C) acetate (Fig. 9) is based on the kinetics of the assimilation process. As the concentration of a substance increases in a reaction system, its rate of transformation increases proportionally. Since the total amount of radioactive material was the same (5 μCi) in all the vials, the only difference was the volume of K-36 buffer, and therefore the concentration of (U-$^{14}$C) acetate. This may be a superficial analysis of the differences in the atmosphere of the vials and the oxygen requirements of M. lepraemurium in an in vitro system, but certainly is the simplest explanation.

Because of these findings, we have recently changed our reaction system, decreasing the volume of suspending solution to 1 ml and using 5 ml multidose serum vials, instead of 20 ml vials. By doing so, the $^{14}$CO$_2$ output from either (U-$^{14}$C) acetate or (L-$^{14}$C) fatty acids currently under investigation has been considerably increased. As a result, the detection time has decreased to less than 12 hours for a 10$^8$ inoculum or 24 hours for a 10$^9$ inoculum.

The results of Figure 10 should be interpreted qualitatively rather than quantitatively. They show that the storage of M. lepraemurium in infected tissue for 12 days at -20°C does not change its metabolic activity. However, since the curves in Figure 10 were obtained at different times, the difference in the $^{14}$CO$_2$ output between frozen and control bacilli could be due to differences in the number of organisms in the experimental vials. It is not possible to determine the infectivity of these organisms based only on the in vitro studies. Further studies are needed to assess the possible relationship between infectivity and viability as measured in vitro and to determine for how long the viability of frozen bacilli can be maintained.

The failure to demonstrate the presence of any inhibitors of the metabolism in these experiments suggests that the decline in metabolism of M. lepraemurium in K-36 buffer is due to lack of a crucial substrate or depletion of (U-$^{14}$C) acetate molecules rather than production of toxic by-products. However, the presence of such products cannot be completely excluded since the lyophilized medium had to be diluted to about 1:5 of the original concentration and it is difficult to evaluate the effect of dilution on the behavior of the possible inhibitors. Therefore, further investigation is needed to achieve a better understanding of the reasons for the decline of M. lepraemurium metabolism in K-36 buffer.

In conclusion, the radiometric method seems to be an important tool for studying the metabolic pathways, and the influence of physical and biochemical factors that enhance or inhibit the metabolism of M. lepraemurium in vitro. Several important questions on the problem of cultivation of these organisms in cell-free media can probably be rapidly answered by using the radiometric method.

**SUMMARY**

The radiometric method has been applied for studying the metabolism of M. lepraemurium and the conditions which might force or inhibit its metabolic activity in vitro. These organisms assimilate and oxidize (U-$^{14}$C) glycerol, and (U-$^{14}$C) acetate, but are unable to oxidize (U-$^{14}$C) glucose, (U-$^{14}$C) pyruvate, (U-$^{14}$C) glycine and $^{14}$C-formate.

When incubated at 30°C M. lepraemurium oxidizes (U-$^{14}$C) acetate to $^{14}$CO$_2$ faster than at 37°C. The same effect was observed...
with increasing concentrations of poly-
sorbate 80 (Tween 80), or the 14C-substrate. No change in metabolic rate was observed when the organisms were kept at -20°C for 12 days. Although tried several times, it was not possible to demonstrate any “inhibitors” of bacterial metabolism in the reaction system.

The radiometric method seems to be an important tool for studying metabolic pathways and the influence of physical and biochemical factors on the metabolism of M. lepraemurium in vitro.

**RESUMEN**

Se ha utilizado el método radiométrico para estudiar el metabolismo del M. lepraemurium y las condiciones que pueden forzar o inhibir su actividad metabólica in vitro. Estos microorganismos asimilan y oxidan (U-14C) glucero y (U-14C) acetato, pero no son capaces de oxidar (U-14C) glicero, (U-14C) piruvato, (U-14C) gliceno y (U-14C) formato. Cuando se incuba a 30°C, el M. lepraemurium oxida (U-14C) acetato más rápidamente que a 37°C. Se observa el mismo efecto con concentraciones crecientes de polisorbato 80 (Tween 80), o el substrato 14C. No se observaron cambios en la tasa metabólica cuando los microorganismos se conservan a -20°C, durante 12 días. Aunque se probó varias veces, no fue posible demostrar ningún “inhibidor” del metabolismo bacteriano en el sistema de reacción.

El método radiométrico parece ser una herramienta importante para estudiar las vías metabólicas y la influencia de factores físicos y bioquímicos en el metabolismo del M. lepraemurium in vitro.

La méthode radiométrique semble constituer un outil important pour l’étude des circuits métaboliques et pour l’analyse de l’influence exercée par les facteurs physiques et biochimiques sur les métabolismes de M. lepraemurium in vivo.

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**REFERENCES**


