 Volume 44, Number 3
Printed in the U.S.A.

Radiometric Studies of *Mycobacterium lepraemurium*[1, 2]

E. E. Camargo, S. M. Larson, B. S. Tepper and H. N. Wagner, Jr. [3]

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 ([11]) with radioisotopes and liquid scintillation counting showed that *M. lepraemurium* was metabolically active *in vitro*. (U-^{14}C) acetate and (U-^{14}C) glycerol were found to be assimilated and oxidized by these organisms after seven days of incubation in the Hart-Valentine elongation medium ([8]) 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 ^{14}C-labeled substrate to $^{14}CO_2$ ([6, 7]). Investigations have been completed comparing the standard and radiometric technics in blood cultures ([5]), 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 ([11]), it was decided to develop a more expedient method for monitoring the metabolic activity of *M. lepraemurium in vitro* ([2]). 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.

[1] Received for publication 26 June 1975.

[2] Supported by U.S.P.H.S. Grant MG-10548 and AI-09763. Fundacão de Amparo A Pesquisa do Estado de São Paulo and Centro de Medicina Nuclear da Universidade de São Paulo, Brazil.

[3] E. E. Camargo, M.D., Fellow; S. M. Larson, M.D., Assistant Professor; B. S. Tepper, Ph.D., Associate Professor; and H. N. Wagner, Jr., M.D., Professor: Division of Nuclear Medicine and the Leonard Wood Memorial Leprosy Research Laboratory, The Johns Hopkins Medical Institutions, Baltimore, Maryland.

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 5×10^8 organisms. The livers were aseptically removed and the bacteria separated from the infected tissue according to the technic previously developed ([11]). The suspensions were further diluted with sterile water to final concentrations of 8.6×10^9 or 4×10^9 or 2×10^9 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 system 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 $^{14}CO_2$ 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 ^{14}C-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 R-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 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 ^{14}C activity. The curves represent the cumulative $^{14}CO_2$ 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-^{14}C) glucose ([4,7]).

Assimilation of substrates. In one of the experiments, ^{14}C-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 ^{14}C-substrates. Female CFW mice were used as the source of bacteria. The organisms were diluted to a final concentration of 8.6×10^9 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-^{14}C) acetate or (U-^{14}C) glycerol or (U-^{14}C) glucose or (U-^{14}C) glycine or (^{14}C) formate or (U-^{14}C) pyruvate and 0.5 ml of the final suspension of bacteria (4.3×10^9 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, $^{14}CO_2$ production from (U-^{14}C) acetate could be easily detected after six hours, increasing progressively to reach a plateau by 13 days (Figs. 1, 2). From (U-^{14}C) glycerol, $^{14}CO_2$ production in K-36 buffer was detected by 24 hours, also increasing to a plateau by 13 days (Fig. 1);

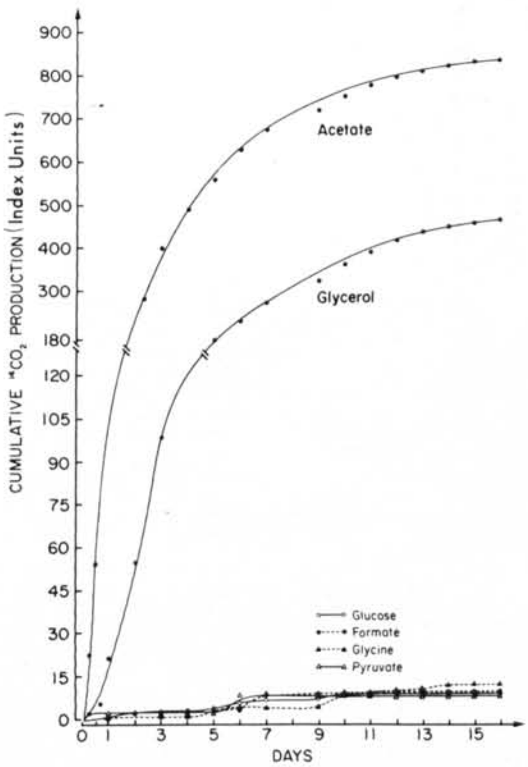

FIG. 1. Oxidation of ^{14}C-substrates by *M. lepraemurium* in K-36 buffer.

however, in NC-5 medium, only on the third day could $^{14}CO_2$ be detected, increasing progressively to a lower plateau by 16 days (Fig. 2). The assimilation of (U-^{14}C) acetate and (U-^{14}C) glycerol paralleled the oxidation of these substrates (Figs. 3, 4).

In both media, $^{14}CO_2$ production from (U-^{14}C) glucose, (U-^{14}C) pyruvate, (U-^{14}C) glycine or (^{14}C) formate never exceeded background levels. All the substrates were assimilated in K-36 buffer, although assimilation was poor for (U-^{14}C) glucose, (U-^{14}C) glycine and (U-^{14}C) pyruvate. In NC-5 medium, there was no assimilation of (U-^{14}C) glucose or (U-^{14}C) pyruvate; incorporation of (^{14}C) formate was also very poor (Figs. 3, 4).

Influence of incubation temperature. Female CBA/J mice were used in this experi-

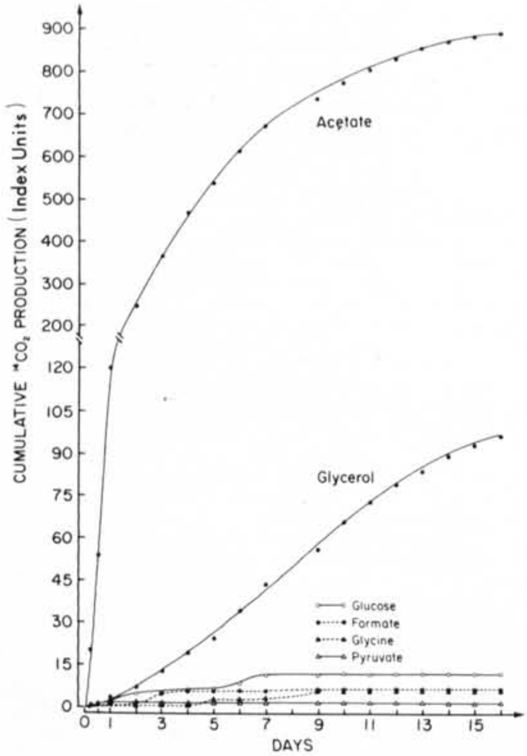

FIG. 2. Oxidation of ^{14}C-substrates by *M. lepraemurium* in NC-5 medium.

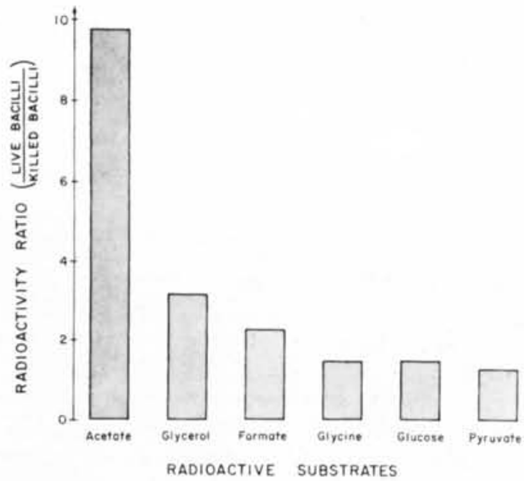

FIG. 3. Assimilation of ^{14}C-substrates by *M. lepraemurium* in K-36 buffer.

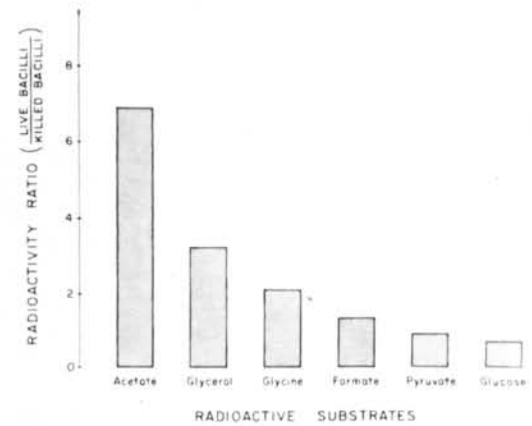

FIG. 4. Assimilation of ^{14}C-substrates by *M. lepraemurium* in NC-5 medium.

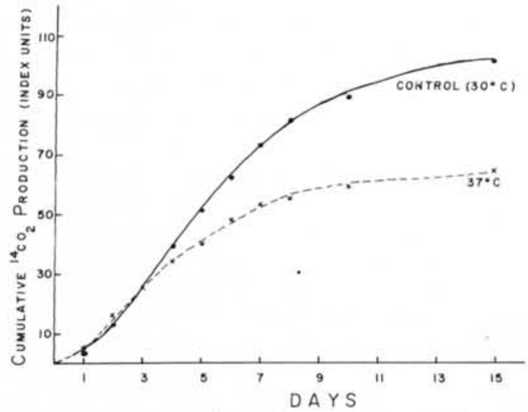

FIG. 5. Metabolism of (U-^{14}C) acetate by *M. lepraemurium* in K-36 buffer. Differences in $^{14}CO_2$ production caused by incubation temperature.

ment. The bacteria were diluted to a final concentration of 2×10^9 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-^{14}C) acetate and 0.5 ml of the final suspension of bacteria (1×10^9 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 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 4×10^9 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-^{14}C) 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 $^{14}CO_2$ by *M. lepraemurium* also increased, particularly after the third day (Fig. 6). A profound inhibitory effect of unlabeled oleic acid on the $^{14}CO_2$ production from (U-^{14}C) acetate is observed in Figure 7. Because of this effect of unlabeled oleic acid, in an additional experi-

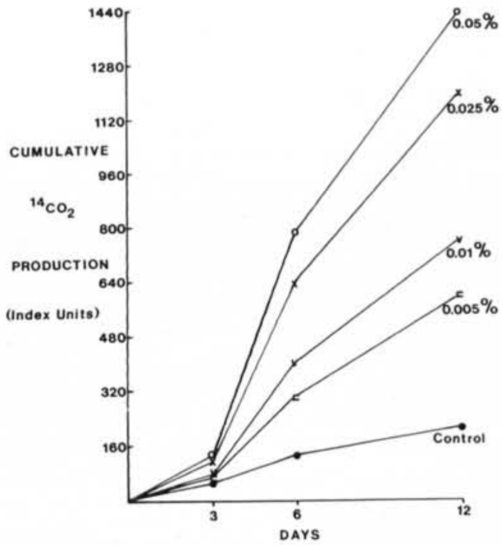

FIG. 6. Effect of Tween 80 on the metabolism of (U-^{14}C) acetate by *M. lepraemurium* in K-36 buffer.

ment, 5 μCi (1-^{14}C) of oleic acid was substituted for 5 μCi (U-^{14}C) acetate. Figure 8 suggests that *M. lepraemurium* seems to prefer (1-^{14}C) oleic acid to (U-^{14}C) acetate with respect to conversion to $^{14}CO_2$.

Influence of concentration of ^{14}C-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-^{14}C) acetate (0.1 ml) and 0.5 ml of the final suspension of bacteria (2 × 10^8 organisms/vial) were used. Therefore, three different concentrations of (U-^{14}C)

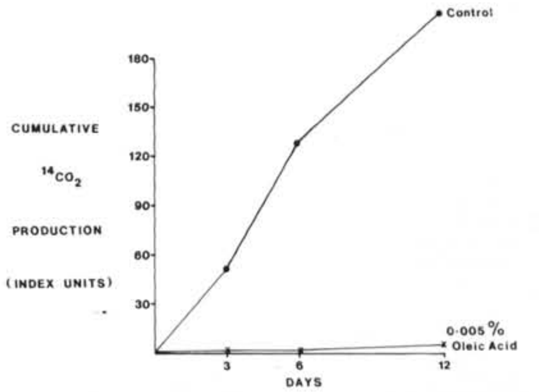

FIG. 7. Effect of unlabeled oleic acid on the metabolism of (U-^{14}C) acetate by *M. lepraemurium* in K-36 buffer.

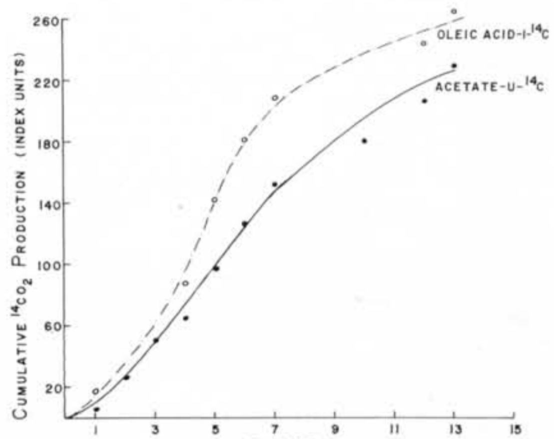

FIG. 8. Comparison between the metabolism of (U-^{14}C) acetate and (1-^{14}C) oleic acid by *M. lepraemurium* in K-36 buffer.

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 $^{14}CO_2$ production as related to the concentration of substrate. As the concentration increased, the $^{14}CO_2$ 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-^{14}C) acetate and 0.5 ml of the final bacterial suspension (2 × 10^9 organisms/vial). The vials were incubated

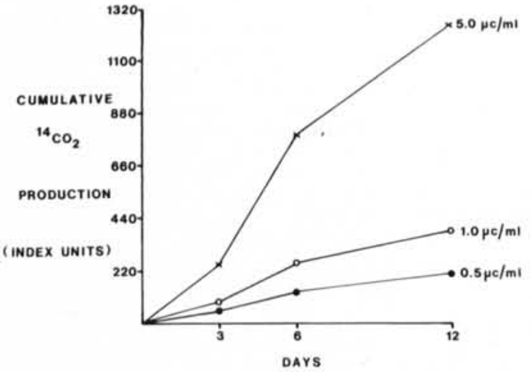

FIG. 9. Effect of concentration of (U-^{14}C) acetate on the metabolism of *M. lepraemurium* in K-36 buffer.

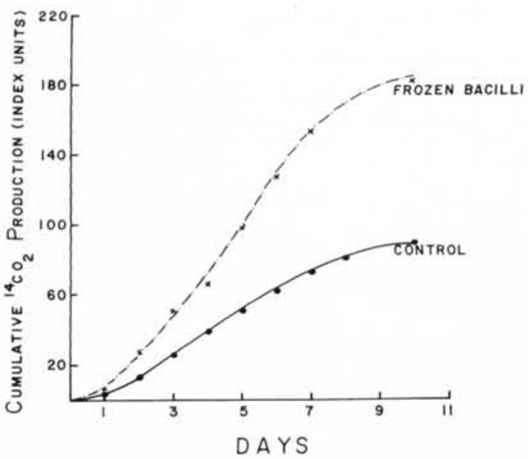

FIG. 10. Comparison between frozen and unfrozen suspensions of *M. lepraemurium*.

at 30°C and sampled daily for ten days. The $^{14}CO_2$ output obtained from these vials was used as control for the rest of the experiment. The other half of the liver specimen was kept in a sterile plastic bottle at -20°C for 12 days. After that, this material was prepared, incubated and sampled in the same way as the control material. After three days, there already was a higher $^{14}CO_2$ output from vials containing frozen bacteria and this increased progressively (Fig. 10), but may have been due to differences in the number of organisms in the experimental vials. The important finding is that the freezing process up to 12 days did not inhibit the metabolism of *M. lepraemurium*.

The effect of inhibitors. In one study, the metabolism of *M. lepraemurium* fell off rapidly in K-36 buffer ([2]). Because of that, the possible presence of "inhibitors" produced by bacterial metabolism was evaluated as follows. After 20 days of incubation at 30°C, the detectable metabolism of 10^9 organisms had ceased. The solution containing the bacteria was freed of microorganisms by filtering through a 0.22 μ pore size membrane filter (Millipore Corporation). The filtrate was lyophilized and redissolved with sterile water to a final concentration of approximately 1 μCi of (U-^{14}C) acetate/ml. One milliliter of this solution was added to some extra control vials prepared for this purpose in the experiment on the influence of freezing. The addition of lyophilized medium to the reaction system failed to demonstrate the presence of any inhibitors of metabolism. Actually, this same experiment was repeated three times, with the same results.

DISCUSSION

The inability of *M. lepraemurium* to oxidize (U-^{14}C) 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-^{14}C) glucose to $^{14}CO_2$ ([4,7]).

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-^{14}C) glycerol may occur by means of a simple enzymatic action on glyceric acid instead of more complex pathways.

The fact that *M. lepraemurium* does not oxidize (U-^{14}C) 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-^{14}C) glycine and ^{14}C-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 $^{14}CO_2$ 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 "diauxic" growth cycles ([3]) 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 (1-^{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 10^9 inoculum or 24 hours for a 10^8 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 polysorbate 80 (Tween 80), or the ^{14}C-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-^{14}C) glicerol y (U-^{14}C) acetato, pero no son capaces de oxidar (U-^{14}C) glucosa, (U-^{14}C) piruvato, (U-^{14}C) glicina y ^{14}C-formato.

Cuando se incuba a 30°C, el *M. lepraemurium* oxida (U-^{14}C) acetato más rápidamente que a 37°C. Se observa el mismo efecto con concentraciones crecientes de polisorbato 80 (Tween 80), o el substrato ^{14}C. No se observan cambios en la tasa metabólica cuando los microorganismos se conservan a -20°C, durante 12 días. Aunque se probó varias veces, no fué 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.*

RÉSUMÉ

La méthode radiométrique a été appliquée à l'étude du métabolisme de *M. lepraemurium* et des conditions qui peuvent permettre ou inhiber son activité métabolique *in vitro.* Les organismes étudiés assimilent et oxydent le glycérol U-^{14}C, l'acétate U-^{14}C, mais sont par contre incapables d'oxyder le glucose U-^{14}C, le pyruvate U^{14}C, la glycine U^{14}C et le formate U^{14}C.

Lorsqu'on incube *M. lepraemurium* à 30°C ce micro-organisme oxyde l'acétate U^{14}C en $CO_2{}^{14}$ plus rapidement qu'il ne le fait à 37°C. Le même effet est observé lorsqu'on utilise des concentrations progressives de polysorbate 80 (Tween 80), ou un substract au carbone^{14}. Aucune modification du taux métabolique n'a été observé lorsque les organismes sont conservés à moins 20°C pour 12 jours. Malgré des essais répétés plusieurs fois, il n'a pas été possible de démontrer aucune inhibition du métabolisme bactérien dans les systèmes de réaction.

La méthode radiométrique semble constituer un outillage 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.*

Acknowledgments. The authors are indebted to Michele Melville and Suzanne B. Banghart for their technical assistance.

REFERENCES

1. Camargo, E. E., Larson, S. M., Tepper, B. S. and Wagner, H. N., Jr. A radiometric method for predicting the effectiveness of chemotherapeutic agents in murine leprosy. Int. J. Lepr. **43** (1975) 234-238.
2. Camargo, E. E., Larson, S. M., Tepper, B. S. and Wagner, H. N., Jr. Radiometric measurement of metabolic activity of *Mycobacterium lepraemurium.* Appl. Microbiol. **28** (1974) 452-455.
3. Dawes, E. A. Bacterial growth. *In:* Quantitative Problems in Biochemistry, E. A. Dawes, ed., 3rd edit., Baltimore: Williams and Wilkins, 1965, pp 202-221.
4. De Blanc, H. J., Jr., Charache, P. and Wagner, H. N., Jr. Automated radiometric measurement of antibiotic effect on bacterial growth. Antimicrob. Agents Chemother. **2** (1972) 360-366.
5. DeBlanc, H. J., Jr., DeLand, F. H. and Wagner, H. N., Jr. Automated radiometric detection of bacteria in 2,967 blood cultures. Appl. Microbiol. **22** (1971) 846-849.
6. DeLand, F. H. and Wagner, H. N., Jr. Automated radiometric detection of bacterial growth in blood cultures. J. Lab. Clin. Med. **75** (1970) 529-534.
7. DeLand, F. H. and Wagner, H. N., Jr. Early detection of bacterial growth with carbon-14 labeled glucose. Radiology **92** (1969) 154-155.
8. Hart, P. D. 'A. and Valentine, R. C. Growth (without multiplication) of *Mycobacterium lepraemurium* in cell-free medium. J. Gen. Microbiol. **32** (1963) 43-53.
9. Mori, T., Kohsaka, K. and Tonaka, Y. Tricarboxylic acid cycle in *M. lepraemurium.* Int. J. Lepr. **39** (1971) 796-812.
10. Nakamura, M. Multiplication of *Mycobacterium lepraemurium* in cell-free liquid medium containing α-ketoglutaric acid and cytochrome C. J. Gen. Microbiol. **73** (1972) 193-195.
11. Tepper, B. S. and Varma, K. G. Metabolic activity of purified suspensions of *Mycobacterium lepraemurium.* J. Gen. Microbiol. **73** (1972) 143-152.
12. Weiss, G. Adenosine triphosphate and other requirements for the utilization of glucose by agents of the psittacosis-tracoma group. J. Bacteriol. **90** (1965) 243-253.