Radiometric Measurement of Differential Metabolism of Fatty Acids by *Mycobacterium lepraemurium*[1]

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A previous report from this laboratory on radiometric studies of *M. lepraemurium* ([2]) described the incidental finding that polysorbate 80 (Tween 80) enhanced markedly the oxidation of (U-^{14}C) acetate by this organism. It was assumed that the presence of oleic acid in the structure of polysorbate 80 probably caused this phenomenon. An investigation of the oxidation of (1-^{14}C) oleic acid revealed a $^{14}CO_2$ production comparable to that of (U-^{14}C) acetate and led to an extension of this study to the (1-^{14}C) fatty acid series.

At the same time, radiometric drug susceptibility testing of *M. tuberculosis* was under investigation with promising results ([7]). Because some radiometric similarities had been found with both organisms ([2,7]), the study of the (1-^{14}C) fatty acid series was extended to include *M. tuberculosis*. The preliminary experiments showed differences in the oxidation rates of these substrates between *M. lepraemurium* (Hawaiian) and *M. tuberculosis* $H_{37}Rv$ and led to the hypothesis that oxidation patterns of fatty acids might provide a basis of differentiation of these organisms. The present report describes the results of this investigation with *M. lepraemurium*.

MATERIALS AND METHODS

Preparation of bacilli. *M. lepraemurium* (Hawaiian) was harvested from infected livers of female CFW mice which had been both intravenously and intraperitoneally infected three to four months previously with 5×10^8 organisms. The livers were aseptically removed and the bacteria separated from the infected tissue according to a technic previously described ([11]). The suspension was further diluted with sterile water to a final concentration of 1.20×10^9 organisms/ml.

Experimental media. The simple K-36 buffer of Weiss ([12]) was used as a suspending solution for *M. lepraemurium*.

Reaction system. The reaction system for detection of $^{14}CO_2$ consisted of 1.0 ml of K-36 buffer in a 5.0 ml multidose sterile glass vial with airtight aluminum seals fitted with rubber liners, along with 0.5 ml of bacterial suspension and 0.5 ml (5.0 μCi) of ^{14}C-substrate. The following (1-^{14}C) fatty acids (Amersham Corporation) were used: butyric (25 mCi/mM), hexanoic (23.6 mCi/mM), octanoic (22.5 and 31.8 mCi/mM), decanoic (18.7 and 21 mCi/mM), lauric (28.8 mCi/mM), myristic (38 and 45 mCi/mM), palmitic (57.9 mCi/mM), stearic (58.4 and 59.7 mCi/mM), oleic (51.8 and 58 mCi/mM), linoleic (56 and 61 mCi/mM), linolenic (60 mCi/mM) and malonic (5.93 and 9.5 mCi/mM). As indicators of the metabolic activity of the organisms, (U-^{14}C) acetate (58.5 mCi/mM) and 1-^{14}C) acetate (57 mCi/mM) were used ([2]). All vials were prepared in triplicate. Control vials were prepared in the same way, but with autoclaved bacteria added.

Radiometric measurement. The vials were incubated at 30°C and sampled daily for five days ([1]). An ion chamber device (Bactec R-301, Johnston Laboratories) was used to measure bacterial metabolism ([1]). The results were expressed as "index units" (100 units = 0.025 μCi of ^{14}C activity). Mean and standard deviation of the cumulative $^{14}CO_2$ production for each substrate over the entire period of the experiment were not calculated because some of the substrates gave readings beyond the upper limit of the instrument (>3,000 "index units"). For this reason, the actual $^{14}CO_2$ output was obtained by pooling vials of the same

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substrate and calculating the difference between the radioactivity found in the vial (medium and bacteria) in the assimilation experiment (see below) and the known original radioactivity contained in the vial as determined with a carbon-14 standard. After corrections for specific activity differences, results were converted to percent, using the cumulative $^{14}CO_2$ production of the best (1-^{14}C) fatty acid (lauric) as 100 percent.

Sterility testing. Sterility tests were performed on positive samples and consisted of subcultures on chocolate-agar plates, on Lowenstein-Jensen medium and radiometric sterility testing with (U-^{14}C) glucose ([3-5]).

Assimilation of substrates. At the end of the experiment, ^{14}C-substrates incorporation into the bacteria was measured by liquid scintillation counting. The triplicate vials of the same ^{14}C-substrate were pooled and filtered through 0.22 μm pore size Millipore membrane filters. The filters were washed three times with 10.0 ml sterile saline, allowed to air dry in a liquid scintillation vial, dissolved with 5.0 ml ethyl acetate, and then 10.0 ml Permafluor II scintillation fluid (Packard Instruments Company) was added. To determine the radioactivity in the medium, 0.1 ml samples of each wash were prepared for liquid scintillation counting, also with 5.0 ml ethyl acetate and 10.0 ml Permafluor II; counts were corrected for dilution and added to obtain the total carbon-14 activity in the medium. Counting with quenching correction for all samples was performed in a Packard Tri-Carb scintillation spectrometer model 3003. Control vials were prepared and counted in the same way. Results were expressed as percent of total radioactivity in the vial. For each ^{14}C-substrate, the $^{14}CO_2$ output was double-checked by calculating the difference between the total radioactivity expected in the vials as determined with a carbon-14 standard and the recovered radioactivity (medium and bacteria).

Radiochromatographic analysis of the medium from experimental and control vials was also performed by using paper or instant thin layer chromatography according to instructions from the manufacturer of the ^{14}C-substrates. For each ^{14}C-substrate, a standard was prepared by dissolving 5 μCi (0.1 ml) of the original (1-^{14}C) fatty acid into 5 ml of K-36 buffer and then increasing the volume to 15 ml with sterile saline to match the dilution of the first wash of the medium. Paper chromatography was performed on Whatman 40 paper with acetone: t-butanol: n-butanol: NH_4OH (2:1:1:1) as solvent for acetate and n-butanol: ethanol: 3N NH_3 (4:1:5) (upper layer) as solvent for hexanoic, octanoic and decanoic acids. Instant thin layer chromatography was used with the remaining (1-^{14}C) fatty acids with petroleum ether: ether: acetic acid (80:20:1) as solvent. All strips were air dried and cut into 1.0 cm bands which were placed in liquid scintillation vials with 15 ml

TABLE 1. *Assimilation and oxidation patterns of (1-^{14}C) fatty acids by* M. lepraemurium.[a]

	Bacteria	Medium	Cumulative $^{14}CO_2$
Acetate	2	89	9
Control	0	98	2
Acetate (U-^{14}C)	30	45	25
Control	0	99	1
Malonic	0	100	0
Control	0	100	0
Butyric	0	100	0
Control	0	100	0
Hexanoic	1	97	2
Control	0	100	0
Octanoic	2	80	18
Control	0	100	0
Decanoic	7	56	37
Control	2	98	0
Lauric	3	5	92
Control	23	77	0
Myristic	14	18	68
Control	20	80	0
Palmitic	32	40	28
Control	88	12	0
Stearic	26	31	43
Control	100	0	0
Oleic	39	40	21
Control	100	0	0
Linoleic	37	61	2
Control	31	69	0
Linolenic	55	41	4
Control	69	31	0

[a] Results expressed as percent total radioactivity.

Permafluor II and counted. Radioactivity of each band was expressed as percent of total radioactivity for each strip.

RESULTS

Table 1 represents assimilation and oxidation patterns of all (1-^{14}C) fatty acids. With saturated acids, the higher the amount found in live bacteria, the higher the $^{14}CO_2$ output with the exception of lauric. The amount in heat-killed bacteria increased with the carbon chain length. Also, preferential oxidation of some substrates such as lauric, decanoic and myristic was found, as indicated by higher oxidation rates than that of acetate, used as an indicator of metabolic activity. Live cells incorporated the unsaturated fatty acids (oleic, linoleic and linolenic) in high amounts although oxidation rates varied. Malonic acid was not assimilated.

Table 2 summarizes the oxidation rates of all (1-^{14}C) fatty acids after corrections for specific activities. The $^{14}CO_2$ production progressively increased with the carbon chain length, reached a maximum at twelve carbons (lauric) and decreased to a minimum with the unsaturated molecules. In decreasing order, $^{14}CO_2$ production was greatest with lauric, decanoic, myristic, octanoic and stearic. All showed higher oxidation rates than the metabolic reference substrates, (1-^{14}C) acetate and (U-^{14}C) acetate. No oxidation was observed with malonic acid.

Figure 1 shows the oxidation pattern of selected [1-^{14}C] fatty acids. Figures 2 and 3 show the radiochromatograms obtained from the suspending buffer. Some of the substrates were not degraded; this occurred with both nonoxidized (butyric) and some oxidized substrates (hexanoic, octanoic, decanoic). Most substrates were oxidized and broken into by-products such as the acetates, lauric, myristic, palmitic, stearic, oleic, linoleic, and linolenic.

TABLE 2. *Oxidation patterns of (1-^{14}C) fatty acids by* M. lepraemurium[a] *Hawaiian.*

Acetate	5.0
Acetate (U-^{14}C)	14.0
Malonic	0.2
Butyric	0.0
Hexanoic	2.5
Octanoic	29.0
Decanoic	63.0
Lauric	100.0
Myristic	55.0
Palmitic	17.0
Stearic	26.0
Oleic	13.0
Linoleic	2.5
Linolenic	1.0

[a] Cumulative $^{14}CO_2$ production as percent of (1-^{14}C) lauric acid after corrections for specific activities.

A summary of assimilation, oxidation and radiochromatographic results, corrected for specific activities, is found in Table 3. It shows that adsorption to bacterial wall occurred when the molecular weight increased from 12 to 18 carbon chain. No assimilation was found with butyric and malonic acids. Assimilation with poor oxidation occurred with (1-^{14}C)

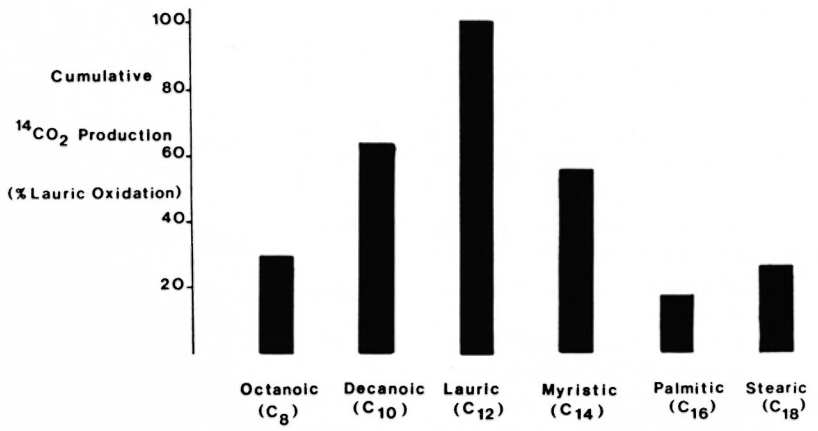

FIG. 1. Oxidation pattern of selected [1-^{14}] fatty acids by *M. lepraemurium* (Hawaiian) after corrections for specific activities.

TABLE 3. *Assimilation and radiochromatographic patterns of [1-^{14}C] fatty acids by* M. lepraemurium *in K-36 buffer.*

I. Adsorption	None:	[1-^{14}C] acetate, [U-^{14}C] acetate, butyric, hexanoic, octanoic
	Poor:	decanoic
	Marked:	lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic
II. Assimilation	None:	butyric, malonic
	Poor oxidation:	[1-^{14}C] acetate, hexanoic, linoleic, linolenic
		Poor retention, no breakdown: hexanoic, octanoic, decanoic
	Oxidation:	Poor retention, with breakdown: [1-^{14}C] acetate, lauric
		Retention and breakdown: [U-^{14}C] acetate, myristic, palmitic, stearic, oleic

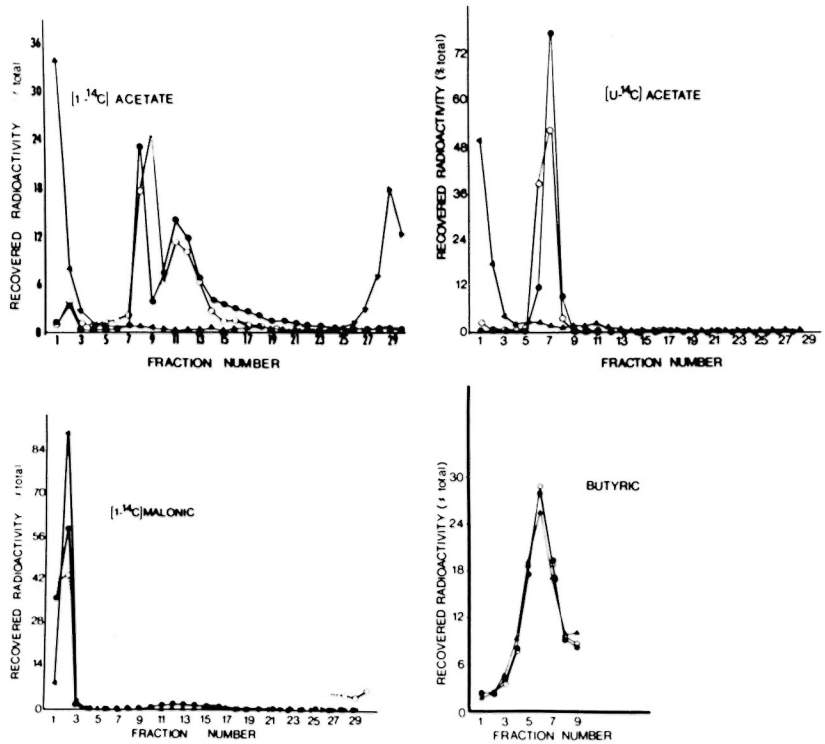

FIG. 2. Radiochromatogram of *M. lepraemurium* suspending buffer (K-36) containing [1-^{14}C] acetate, or [U-^{14}C] acetate, or [1-^{14}C] malonic, or [1-^{14}C] butyric acid, after 5 days of incubation. ▲ Experimental vials. ● Standard substrate. ○ Control vials.

acetate, hexanoic, linoleic and linolenic. Hexanoic, octanoic and decanoic were oxidized but poorly retained and not transformed into by-products; (1-^{14}C) acetate and lauric were oxidized and transformed, but retention was poor; (U-^{14}C) acetate, myristic, palmitic, stearic and oleic were oxidized, retained and transformed into by-products.

As expected, $^{14}CO_2$ production was not observed in the control vials, and all sterility tests were negative.

DISCUSSION

Studies on the oxidation of the fatty acids series by *M. lepraemurium* have not been reported. Recently, Kusaka ([8]) described fatty acid synthesizing enzyme activity in extracts of this organism. Most of the results described

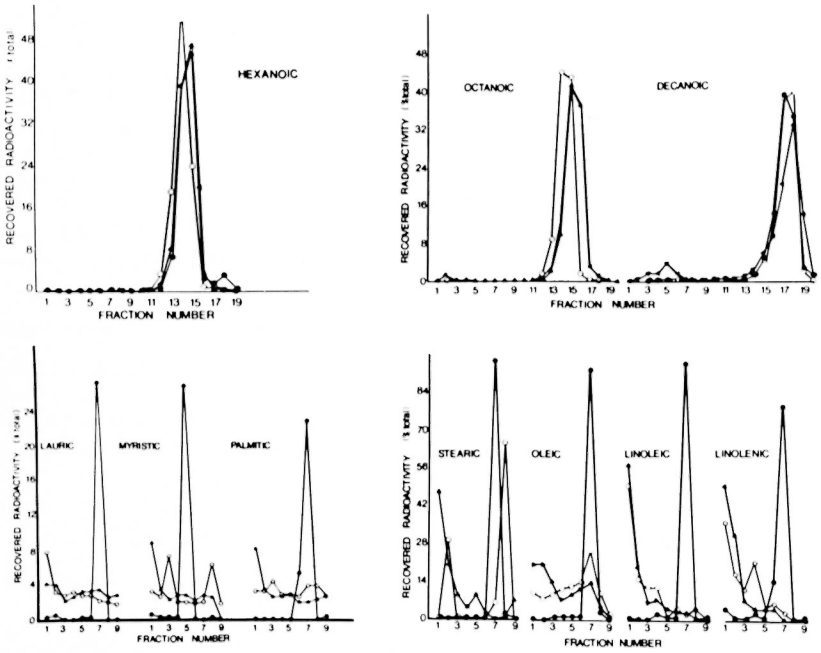

FIG. 3. Radiochromatogram of *M. lepraemurium* suspending buffer (K-36) containing [1-^{14}C] hexanoic, or octanoic, or decanoic, or lauric, or myristic, or palmitic, or stearic, or oleic, or linoleic, or linolenic acid, after 5 days of incubation. ▲ Experimental vials. ● Standard substrate. ○ Control vials.

in this experiment were in agreement with those reported by Kusaka, including the failure of *M. lepraemurium* to assimilate and oxidize malonic acid. The "malonyl CoA pathway" does not appear to be present in this organism.

The results shown in Tables 1 and 3 suggest that most of the acids tested were used as energy sources and to variable extents as carbon sources as well. The exception was lauric, primarily used for energy purposes, a pattern similar to that of formate with *M. tuberculosis* ([7]).

Adsorption of higher molecular weight fatty acids such as lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic (Tables 1, 3) probably contributed to the assimilation process. The breakdown products demonstrated by radiochromatography of most of the oxidized fatty acids were not analyzed. Although, oxidized by-products of hexanoic, octanoic and decanoic acids were not found. Some of these fatty acids were preferably incorporated into triglycerides ([10]) and retained in the cell, and some of them were transformed into by-products and excreted into the medium. The excretion of by-products into the medium may represent a mechanism of elimination of toxic substances. Toxic effects or a possible inhibitory effect on bacterial enzymes involved in lipogenesis ([6]) probably would not occur with the small amounts used in this study (e.g., the concentration of lauric acid was about 9×10^{-5}M). Whether the enzyme systems of *M. lepraemurium* are sensitive enough to recognize potentially toxic substances and either transform them into nontoxic triglycerides or excrete them as by-products is speculative.

Table 2 shows that the activity of the enzyme systems of *M. lepraemurium* involved with oxidation of fatty acids increased with the carbon chain length to a maximum at 12 carbons and then declined. Stimulatory effect on oxygen uptake by *M. tuberculosis* has been described with long chain fatty acids. A similar mechanism, if present in *M. lepraemurium*, would explain the various oxidation rates observed with octanoic, decanoic, lauric, myristic, and other acids. The preferential oxidation of lauric over its use as a carbon source could therefore be due to its toxic nature, to its stimulatory effect on oxygen uptake, to a combination of both, or to the

fact that this molecule, for some reason, is particularly suitable for the energy needs of *M. lepraemurium*.

Studies of *M. avium* have shown that palmitic acid is necessary for a maximal cell division rate and that cell division occurs when palmitic acid uptake ceases ([9,10]). When *M. avium* was pregrown to a filamentous stage in the presence of palmitic acid and then transferred to a fresh medium lacking fatty acid, the cells divided at a very slow rate and did not fragment ([9]). This means that the elongated cells were not "committed" to fragmentation in the absence of palmitic acid. These findings suggest the presence of similar "commitments" to fragmentation with a particular fatty acid in other mycobacteria. *M. avium* metabolism of the (1-^{14}C) fatty acid series with the radiometric method is currently under investigation in our laboratory and may be revealing particularly if (1-^{14}C) palmitic acid is found to be the best substrate with this organism. This finding would then support the concept that the preferred ^{14}C-substrate described in the present study (lauric acid) is eventually the fatty acid to which *M. lepraemurium* is "committed" for cell fragmentation.

Therefore, in future experiments, identification of the role of lauric acid seems to be essential because if this substrate is found to be stimulatory to *M. lepraemurium*, it could also be a potential growth-promoting factor, and perhaps more favorable multiplication conditions could be achieved in its presence.

SUMMARY

An assay system has been developed based on automated radiometric quantification of $^{14}CO_2$ produced through oxidation of (1-^{14}C) fatty acids by mycobacteria.

With this system, the Hawaiian strain of *M. lepraemurium* was studied using the K-36 buffer as a suspending solution for the organisms along with 5.0 μCi of one of the following fatty acids: acetate, butyric, hexanoic, octanoic, decanoic, lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic, and malonic. The $^{14}CO_2$ production by this organism was greatest with lauric, decanoic, myristic, octanoic, and stearic acids, in decreasing order. Assimilation studies and radiochromatograms confirmed that most of the oxidized substrates were converted into by-products with no change in those from which no oxidation was found.

These data suggest that the radiometric measurement of differential fatty acid metabolism may provide a basis of radiometric identification of *M. lepraemurium* and assessment of the growth requirements of this organism.

RESUMEN

Se presenta un sistema desarrollado para estudiar el matabolismo diferencial de diversos ácidos grasos por el *Mycobacterium lepraemurium*. El sistema se basa en la cuantificación radiométrica automatizada del $^{14}CO_2$ producido como resultado de la oxidación de ácidos grasos marcados (^{14}C-1).

En este sistema, la cepa Hawaiiana de *M. lepraemurium* se suspendió en el regulador K-36 junto con 5.0 μCi de uno de los siguientes ácidos grasos: acetato, butírico, hexanoico, octanoico, decanoico, láurico, mirístico, palmítico, esteárico, oleico, linoleico, linolénico y malónico. La producción de $^{14}CO_2$ por este organismo fue mayor con el ácido láurico y decreció en el siguiente órden: decanoico, mirístico, octanoico y esteárico. Los estudios de asimilación y los radiocromatograms, confirmaron que la mayoría de los substratos oxidados fueron convertidos en bi-productos, mientras que no hubieron cambios en aquellos en los que no hubo oxidación.

Estos datos sugieren que las mediciones radiométricas del metabolismo diferencial de los ácidos grasos pueden constituir la base para la identificación radiométrica del *M. lepraemurium* y para el estudio de las necesidades metabólicas de este organismo.

RÉSUMÉ

On a mis au point une épreuve basée sur la détermination quantitative radiométrique et automatisée du $^{14}CO_2$ produit à la suite de l'oxydation des acides gras (1-^{14}C) par des mycobactéries.

On a étudié par ce système la souche hawaienne de *M. lepraemurium*, en utilisant un tampon K-36 comme solution pour mettre en suspension les micro-organismes, avec addition de 5,0 μCi de l'un des acides gras suivants: acétate, butyrique, hexanoïque, octanoïque, décanoïque, laurique, myristique, palmitique, stéarique, oléique, linoléique, linolénique et malonique. La production de $^{14}CO_2$ par ces organismes a été la plus importante lorsque l'on utilisait l'acide laurique, et ensuite en ordre décroissant les acides décanoïque, myristique, octanoïque et stéarique. Les études d'assimilation et la radiochromatographie ont confirmé que la plupart de substrats oxydés étaient transformés en produits dérivés, alors que l'on ne notait aucune

modification dans les substrats qui n'étaient pas oxydés.

Ces données suggèrent que les mesures radiométriques du métabolisme différentiel des acides gras peuvent fournir une base pour l'identification radiométrique de *M. lepraemurium* et pour la mise en évidence des exigences requises par la croissance de cet organisme.

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