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Radiometric Measurement of Differential Metabolism of Fatty Acid by Mycobacteria[1]

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A previous report from this laboratory on radiometric studies of *Mycobacterium lepraemurium* described the differential metabolism of [1-^{14}C] fatty acids by this organism ([4]).

Because some radiometric similarities had been found with *M. lepraemurium* and *M. tuberculosis* ([7,13]), the study of the [1-^{14}C] fatty acid series was extended to include the latter. The striking differences in the oxidation rates of these substrates observed between *M. lepraemurium* and *M. tuberculosis* $H_{37}Rv$ led to the hypothesis that oxidation patterns of fatty acids might provide a basis of species differentiation of the genus *Mycobacterium* ([5]). To investigate further this possibility, two additional organisms (*M. bovis* BCG and *M. tuberculosis* Erdman) were included in this study.

The present report describes the results of this investigation.

MATERIALS AND METHODS

One fully susceptible strain of *M. tuberculosis* was obtained from the Johns Hopkins Hospital Mycobacteriology Laboratory ($H_{37}Rv$). Another fully susceptible strain of *M. tuberculosis* (Erdman) and one strain of *M. bovis* (BCG) resistant to 5 μg of isoniazid were obtained from Dr. Gardner Middlebrook at the University of Maryland. These organisms were grown in 50 ml of liquid 7H9 medium with 10% ADC enrichment and 0.05% polysorbate 80. Colonies were scraped from Lowenstein-Jensen slants and transferred to the liquid growth medium where they were grown for six to eight days. The bacteria were then homogenized with a Sorvall omni-mixer at 8.5 speed scale twice for 30 sec. The number of bacteria was estimated with MacFarland barium sulfate standards ([2]). The final suspension was further diluted with sterile 7H9 to yield from 1×10^7 to 5×10^7 organisms/ml.

Experimental media. The liquid 7H9 medium with 10% ADC enrichment and no polysorbate 80 was used as the suspending solution for the organisms.

Reaction system. The reaction system for detection of $^{14}CO_2$ consisted of 0.8 ml of medium in a 5.0 ml multi-dose sterile glass vial (A. H. Thomas Co., Philadelphia, Pennsylvania, U.S.A.) with airtight aluminum seals (Wheaton Scientific, Millville, New Jersey, U.S.A.) fitted with rubber liners (Johnston Laboratories, Cockeysville, Maryland, U.S.A.), along with 0.1 ml of bacterial suspension and 0.1 ml (1.0 μCi) of ^{14}C-substrate. The following [1-^{14}C] fatty acids (Amersham Corporation, Arlington Heights, Illinois, U.S.A.) were used with all organisms: 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), and linolenic (60 mCi/mM). Lauric acid was dissolved in methanol; all other fatty acids were dissolved in ethanol. ^{14}C-Formate (2.83 mCi/mM) was included as an indicator of the metabolic activity of the organisms ([13]). All vials were prepared at least in duplicate. Control vials were prepared in the same way, but with autoclaved bacteria added.

Radiometric measurement. The vials were incubated at 37°C. An ion chamber device (Bactec R-301, Johnston Laboratories,

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Cockeysville, Maryland, U.S.A.) was used to measure the bacterial metabolism ([6]). The vials were sampled daily for at least 3 days. The results were expressed as "index units" (100 units = 0.025 μCi of ^{14}C activity). Means and standard deviations of the cumulative $^{14}CO_2$ production for each substrate over the entire period of the experiment were calculated. Results were converted to percent, using the cumulative $^{14}CO_2$ production of the best [1-^{14}C] fatty acid for each organism as 100%.

For *M. lepraemurium*, the details of the preparation of organisms, experimental media, reaction system, and radiometric measurement have been published previously ([4]).

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 ([8, 9, 10]).

Assimilation of substrates. At the end of *M. tuberculosis* $H_{37}Rv$ experiments, the incorporation of ^{14}C-substrates into the bacteria was measured by liquid scintillation counting. Details of this procedure have been published elsewhere ([4]).

RESULTS

Table 1 represents the assimilation and oxidation patterns of [1-^{14}C] fatty acids by *M. tuberculosis* $H_{37}Rv$. With some of the saturated fatty acids such as butyric, hexanoic, and lauric, there was also a direct relationship between incorporation and $^{14}CO_2$ production. The amount in autoclaved bacteria increased with the carbon chain length for saturated acids; with linoleic acid, 98% of the activity was present in heat-killed organisms and no radioactivity was found in the medium.

Table 2 summarizes the oxidation patterns of [1-^{14}C] fatty acids by mycobacteria. Results previously published on *M. lepraemurium* have been included for comparison ([4]). With this organism, $^{14}CO_2$ production progressively increased with the carbon chain length, reached a maximum with lauric and decreased to a minimum with the unsaturated molecules. In decreasing order, $^{14}CO_2$ production by this organism was greatest with lauric, decanoic, myristic, octanoic, and stearic.

TABLE 1. *Assimilation and oxidation patterns of [1-^{14}C] fatty acids by* M. tuberculosis *$H_{37}Rv$*.[a]

Substrate	Bacteria	Medium	Cumulative $^{14}CO_2$
Formate[b]	2	23	75
Control[c]	0	100	0
Butyric	36	42	22
Control	0	100	0
Hexanoic	20	42	38
Control	0	100	0
Octanoic	0	97	3
Control	1	99	0
Decanoic	0	98	2
Control	4	96	0
Lauric	6	70	24
Control	2	98	0
Myristic	0	98	2
Control	3	97	0
Palmitic	2	96	2
Control	4	96	0
Stearic	2	96	2
Control	7	93	0
Oleic	1	97	2
Control	7	93	0
Linoleic	1	98	1
Control	98	0	2
Linolenic	1	98	1
Control	2	98	0

[a] Results expressed as percent of the total reactivity initially added which was found incorporated into the bacteria, left in the medium, and converted to $^{14}CO_2$ at the end of the incubation.
[b] Containing viable bacilli.
[c] Containing the autoclaved bacilli.

With both strains of *M. tuberculosis*, hexanoic gave the greatest $^{14}CO_2$ production. However, the $H_{37}Rv$ strain also oxidized lauric, butyric, and decanoic; whereas Erdman oxidized lauric, myristic and stearic. With *M. bovis* (BCG) the best substrates were lauric, decanoic, linolenic, and linoleic, in decreasing order. With *M. tuberculosis* ($H_{37}Rv$ and Erdman) and *M. bovis* (BCG), however, none of these substrates was better than ^{14}C-formate, used as reference substrate.

By using 20% of maximal oxidation as the lower limit to consider the cumulative $^{14}CO_2$ production as positive, the results on Table 2 can be simplified (Table 3). According to Table 3, these four organisms can be differentiated by using butyric, hexanoic, and stearic acids. As expected, $^{14}CO_2$ production was not observed in the control vials and all sterility tests were negative.

TABLE 2. *Oxidation patterns of [1-^{14}C] fatty acids by mycobacteria.*[a]

Substrate	*M. lepraemurium* (Hawaiian)	*M. bovis* (BCG)	*M. tuberculosis* ($H_{37}Rv$)	*M. tuberculosis* (Erdman)
Formate	[b]	≫100	>100	>100
Butyric	0	4 ± 2	36 ± 1	0
Hexanoic	2	9 ± 2	100 ± 6	100 ± 3
Octanoic	29	8 ± 3	17 ± 3	10 ± 0
Decanoic	63	36 ± 5	23 ± 1	6 ± 0
Lauric	100	100 ± 12	76 ± 4	37 ± 15
Myristic	55	4 ± 1	18 ± 1	20 ± 3
Palmitic	17	4 ± 1	9 ± 1	9 ± 2
Stearic	26	5 ± 0	7 ± 1	13 ± 0
Oleic	13	1 ± 0	8 ± 1	9 ± 4
Linoleic	2	19 ± 3	7 ± 1	5 ± 1
Linolenic	1	21 ± 1	4 ± 1	5 ± 1

[a] Cumulative $^{14}CO_2$ production as percent of the best [1-^{14}C] fatty acid for each organism.
[b] *M. lepraemurium* does not metabolize formate ([7]).

DISCUSSION

We have previously reported and discussed the oxidation of the fatty acid series by *M. lepraemurium* ([4]). Most of our results were found to be in agreement with the studies of Kusaka who described fatty acid synthesizing enzyme activity in extracts of this organism ([14]).

There are several reports on the fatty acid synthesizing enzyme activity in extracts of *M. tuberculosis* and other mycobacteria ([12, 15, 16, 17]). Briefly, these studies have shown that several long chain fatty acids are formed, from 12 to 26 carbons, by addition of two carbons to the acceptor fatty acid's carboxyl end. Acetate, malonate, and NADH are essential for these reactions. Also, there is evidence that *M. tuberculosis* accumulates lipids during exponential growth ([1]), with a synthesis of mycolic acids that parallels the growth rate ([3]). With *M. tuberculosis* $H_{37}Rv$ there was utilization of most of the substrates as both carbon sources and energy sources (Table 1). The exceptions were formate and lauric, used mainly for energy purposes. The oxidation of formate was probably due to the presence of a formate dehydrogenase already identified in *M. phlei* ([11]). Whether the preferential oxidation of lauric over its use as carbon source may have the implications discussed with *M. lepraemurium* is unknown at present. The considerations on the possible mechanisms of elimination of toxic substances by *M. lepraemurium* cannot be extended to *M. tuberculosis* and *M. bovis,* since no radiochromatographic analysis of the media was done for the latter two. Although adsorption may have influenced assimilation of saturated long chain fatty acids (Table 1), the enzyme systems involved with oxidation of fatty acids in both

TABLE 3. *Oxidation patterns of selected [1-^{14}C] fatty acids by mycobacteria.*[a]

	M. lepraemurium (Hawaiian)	*M. bovis* (BCG)	*M. tuberculosis* ($H_{37}Rv$)	*M. tuberculosis* (Erdman)
Butyric	0	0	+	0
Hexanoic	0	0	+	+
Octanoic	+	0	0	0
Decanoic	+	+	0	0
Lauric	+	+	+	+
Myristic	+	0	0	0
Stearic	+	0	0	0

[a] + = >20% oxidation of the best [1-^{14}C] fatty acid for each organism. 0 = <20% oxidation of the best [1-^{14}C] fatty acid for each organism.

strains of *M. tuberculosis* showed preference for the six-carbon chain (hexanoic acid). On the other hand, the enzyme systems of *M. bovis* preferred the 12-carbon chain (lauric acid), as did *M. lepraemurium*.

The enzyme systems of the various organisms used in this study did not oxidate the entire [1-^{14}C] fatty acid series at the same rates. Moreover, the different oxidation rates for the various fatty acids also varied from one organism to another. Differential oxidation patterns could therefore be recognized. By using a 20% threshold of maximal oxidation rate to consider the cumulative $^{14}CO_2$ production as positive (Table 3), it becomes apparent that by selecting three convenient fatty acids it is possible to differentiate the various organisms used in this study. This simpler approach only includes butyric, hexanoic, and stearic acids. Only stearic is positive with *M. lepraemurium*; butyric and hexanoic are positive with *M. tuberculosis* $H_{37}Rv$; only hexanoic is positive with *M. tuberculosis* Erdman, and none of them are positive with *M. bovis*.

There is evidence that the uptake of isoniazid by tubercle bacilli, an enzyme-dependent phenomenon ([18]), is followed by a fall in the incorporation of [1-^{14}C] acetate into fatty acids ([12]). This suggests some relationship between the action of the drug and the metabolism of fatty acids. It is conceivable that isoniazid-resistant and isoniazid-susceptible organisms of the same strain of *M. tuberculosis* may show different patterns of fatty acid oxidation. In this case, a comparison of the oxidation patterns of selected fatty acids by these organisms would bring new information on the metabolic pathways involved with the mechanism of susceptibility and resistance to isoniazid.

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.

Two strains of *M. tuberculosis* ($H_{37}Rv$ and Erdman) and one of *M. bovis* (BCG) in 7H9 medium (ADC) with 1.0 μCi of one of the fatty acids (butyric, hexanoic, octanoic, decanoic, lauric, myristic, palmitic, stearic, oleic, linoleic, and linolenic) were studied. Results previously published on *M. lepraemurium* (Hawaiian) were also included for comparison.

Both strains of *M. tuberculosis* had maximum $^{14}CO_2$ production from hexanoic acid. Oxidation of butyric and avid oxidation of lauric acids were also found with the $H_{37}Rv$ strain but not with Erdman. In contrast, $^{14}CO_2$ production by *M. bovis* was greatest from lauric and somewhat less from decanoic acid. *M. lepraemurium* showed increasing oxidation rates from myristic, decanoic and lauric acids. Assimilation studies of *M. tuberculosis* $H_{37}Rv$ 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 strain identification of the genus *Mycobacterium*.

RESUMEN

Se desarrolló un ensayo para la cuantificación radiométrica del $^{14}CO_2$ producido por la oxidación micobacteriana de ácidos grasos marcados en el C-1 (^{14}C-1).

Se estudiaron 2 cepas de *M. tuberculosis* ($H_{37}Rv$ y Erdman) y una de *M. bovis* (BCG) en medio 7H9 (ADC) con 1.0 μCi de uno de los siguientes ácidos grasos: butírico, hexanoico, octanoico, decanoico, láurico, mirístico, palmítico, esteárico, oleico, linoleico y linolénico. Para comparación, también se incluyeron algunos resultados previamente publicados obtenidos con el *Mycobacterium lepraemurium*, cepa Hawaii.

Las dos cepas de *M. tuberculosis* tuvieron la máxima produción de $^{14}CO_2$ cuando se usó el ácido hexanoico. Con la cepa $H_{37}Rv$, pero no con la Erdman, también se observó la oxidación de los ácidos butírico y láurico. En contraste, la producción de $^{14}CO_2$ por el *M. bovis* fue máxima con el ácido láurico y algo menor con el ácido decanoico. El *M. lepraemurium* mostró un incremento en su capacidad de oxidación que fue, del ácido mirístico al ácido decanoico, hasta el ácido láurico. Los estudios de asimilación con el *M. tuberculosis* $H_{37}Rv$ confirmaron que la mayoría de los substratos oxidados fueron convertidos en biproductos en tanto que no hubo cambio en aquellos que no fueron oxidados.

Estos datos sugieren que la medición radiométrica diferencial del metabolismo de los ácidos grasos puede proporcionar una base para la identificación de cepas del género *Mycobacterium*.

RÉSUMÉ

On a mis au point une méthode expérimentale d'évaluation basée que la quantification radiométrique

automatisée du $^{14}CO_2$ produit à la suite de l'oxydation par les mycobactéries des acides-gras 1-^{14}C.

Deux souches de *M. tuberculosis* ($H_{37}Rv$ et Erdman), ainsi qu'une souche de *M. bovis* (BCG), ont été étudiées en milieu 7H9 (ADC), auquel avait été ajouté 1,0 μCi de l'un des acides-gras suivants: butyrique, hexanoïque, décanoïque, laurique, myristique, palmitique, stearique, oléïque, linoléïque, et linolénique. Des résultats publiés précédemment sur *M. lepraemurium* (Hawaii) ont également été repris à des fins de comparaison. L'une et l'autre souche de *M. tuberculosis* présentaient une production maximale de $^{14}CO_2$ avec l'acide hexanoïque. On a également observé une oxydation de l'acide butyrique (avid oxidation) des acides lauriques, avec la souche $H_{37}Rv$, mais non avec la souche Erdman. Au contraire, la production de $^{14}CO_2$ par *M. bovis* était la plus prononcée avec l'acide laurique, et un peu moins prononcée pour l'acide décanoïque. *M. lepraemurium* a témoigné d'une augmentation des taux d'oxydation avec les acides myristiques, décanoïques et lauriques. Les études d'assimilation menées sur la souche $H_{37}Rv$ de *M. tuberculosis* ont confirmé que la plupart des substrats oxydés étaient transformés en sous-produits, alors qu'aucune transformation n'était relevée avec les produits qui ne témoignaient pas d'oxydation.

Ces données suggèrent que les mesures radiométriques des différences métaboliques des acides-gras pourraient fournir une base pour l'identification des souches du genre *Mycobacterium*.

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