Tricarboxylic Acid Cycle in *M. lepraemurium*[1]

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Cultivation of *Mycobacterium leprae (M. leprae)* and *Mycobacterium lepraemurium (M. lepraemurium)* is extremely difficult and in the attempt to overcome this problem, the respiratory enzyme systems have been studied as a first step in the elucidation of the metabolism of *M. lepraemurium*, clarification of which may make cultivation possible. Gray *et al.* (4) have used various substances and measured the oxygen consumption in *M. le-*

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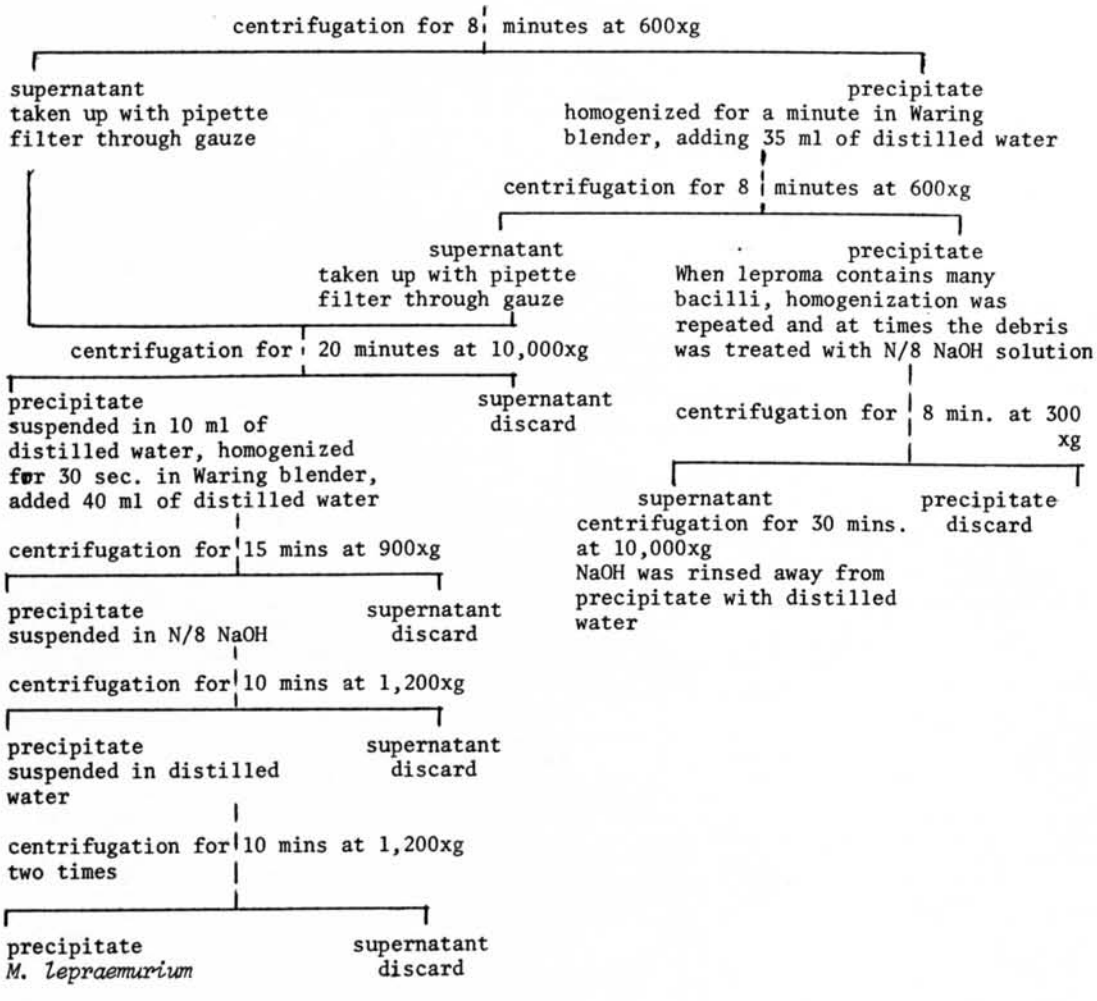

FIG. 1. Collection method for *M. lepraemurium*.

Murine lepromas, 9.7 gm (dry weight 2.25 gm), were minced with scissors, homogenized with 10 ml distilled water in a Waring blender, adding 35 ml of distilled water.

praemurium by the Warburg manometric method and reported that increase in oxygen consumption was not found with any of the substrates. Ito and Sonoda ([5]) carried out follow-up studies and also studied the factors which will intensify the oxygen consumption of *M. lepraemurium*. Mori *et al.* ([10]) found that malic dehydrogenase is present in *M. lepraemurium* by studies on the oxidation of various substrates.

The terminal electron transport system in *M. lepraemurium* will be described separately. In the present study, the enzyme systems and pathways related to the tricarboxylic acid cycle were examined by supplementing the disrupted terminal electron transport system with artificial dyes.

MATERIALS AND METHODS

Collection method for *M. lepraemurium*. Hybrid F_1 mice ([6]) C3H ♂ (not Heston strain) X ddO ♀ were inoculated subcutaneously in the chest region with 0.1 ml of a 1,000-fold diluted emulsion of murine leprosy leproma and the subcutaneous leproma which developed after four to five months was used as the study material. This leproma is poor in connective tissue so that the yield of bacteria is highly satisfactory and a simplification of Mori's method, illustrated in Figure 1, was used for collection of the bacteria.

Preparation of *M. lepraemurium* extract. The fraction of *M. lepraemurium* collected by the above method was freeze-dried, or after freezing in the centrifuge tube, the frozen bacterial cells were placed on a double layer of filter paper and stored overnight in a low temperature room to remove water. The weighed bacterial fraction was then placed in a mortar, an equal weight of quartz sand was added, and ground for two hours with chilling with ice in a cold room. After extraction with this buffer (pH 7.0) or potassium and sodium phosphate buffer (pH 7.0), the bacterial cells were removed by centrifugation at 10,000 rpm for 30 minutes at 4°C and the supernatant was used as the crude enzyme solution.

Preparation of the material for paper-chromatography. The reaction was terminated by cooling and the solution, placed in Visking's cellophane tube, was dialyzed against a small amount of distilled water in the cold room. The outer solution was changed three times at 12 hour intervals, and these outer solutions were then pooled and freeze-dried. The residue was dissolved in 0.5 ml of distilled water and 0.1 ml was used for paper chromatography.

Paper-chromatography. Organic acids were determined by the ascending method using the butanol:formic acid:water (10:2:5) solvent (A) of Lugg and Overell ([9]) or the n-amylalcohol:5 M formic acid (1:1) solvent (B) of Buch ([2]). After developing overnight, the strips were left at room temperature for four days, to adequately eliminate the formic acid, and sprayed with an alcohol solution of 0.1% bromo-cresol green. Organic acids appeared as yellow spots on a green background. Amino acids were detected after developing overnight by the ascending method using water-saturated phenol as the solvent, drying at room temperature, spraying with 0.1% water-saturated butanol ninhydrin solution and heating for ten minutes at 110°C.

Paper-strip radioactive scanning. After detection of the control cold standard compounds by the appropriate color test, the radioactivities on the paper strips were measured and compared with the spot of the standard compounds, by radioactive scanning (Nuclear Chicago Co., Actigraph).

Determination of enzyme activity. *Dehydrogenases.* Anaerobic reactions were carried out in Thünberg tubes with triphenyl tetrazolium chloride (TTC) as electron acceptor and the reaction stopped by addition of 0.1 ml of 10% trichloroacetic acid. The red color of the formazan was extracted with ethylacetate and colorimetrically determined at 480 mμ.

Fumarate hydratase (fumarase) (4.2.1.2). Fumarate was allowed to react with the enzyme solution anaerobically and the organic acid produced was isolated by paper-chromatography.

Succinate dehydrogenase (1.3.99.1). Phenazine methosulfate was added as the autooxidizable electron carrier and then incubated aerobically. The conversion of

succinate to fumarate and malate was followed by paper-chromatography for organic acids.

Aconitate hydratase (aconitase) (4.2.1.3). Citrate was produced anaerobically using cis-aconitate and DL-isocitrate as substrate and the citrate quantitatively determined by the method of Natelson ([15]).

Citrate synthase (condensing enzyme) (4.1.3.7). The production of citrate from oxaloacetate and acetyl CoA was determined quantitatively by the method of Natelson ([15]).

Isocitrate lyase (isocitritase) (4.1.3.1). Since a radio-active isocitrate could not be obtained, citrate-1,5-C^{14} was used as the substrate. Aerobic and anaerobic reaction products were separated by paper-chromatography, and radio-active reaction products were compared with the control standard samples spotted on the same paper strips. On the other hand, the aerobic metabolism of isocitrate was observed by adding phenazine methosulfate as the electron transport system and measurement of the oxygen uptake and carbon dioxide evolution by the Warburg manometric method.

Glutamate decarboxylase (4.1.1.5.). 10^{-4}mol glutamic acid containing radio-active glutamic acid-1-C^{14} 1μc were used as substrate. The glutamate decarboxylase activity was determined from the fixation of carbon dioxide in 20% potassium hydroxide solution which was placed in the small inside tube. Live bacilli and ground extract of *M. lepraemurium* were used as enzyme material and the enzymatic reaction was carried out under anaerobic conditions in a Thünberg tube. Radio-active carbon dioxide was determined by the use of a liquid scintillation counter (Beckman LS-200 B).

Oxoglutarate dehydrogenase (1.2.4.2.). α-ketoglutarate-5-C^{14} was used for oxidative decarboxylase activity of α-ketoglutarate; phenazine methosulfate for the electron transport system; and CoA, TPP, lipoic acid and NAD added as coenzymes. The production of succinate was examined by paper-chromatography for organic acid.

Oxalosuccinate decarboxylase. Oxalosuccinate was used as the substrate and carbon dioxide elimination measured with the Warburg apparatus in a nitrogen atmosphere.

δ-hydroxylaevulinate synthase (glyoxylate-oxoglutarate carboligase). For observing condensing enzyme activity between glyoxylate and α-ketoglutarate, glyoxylate-u-C^{14} and α-ketoglutarate were reacted anaerobically and the biosynthesized δ-hydroxylaevulinate determined as a spot by paper-chromatography and the relation of the peak of the isotope count examined with the actigraph.

Culture of contaminating microorganisms. Centrifuge tubes, reaction tubes, substrates and buffer solution were used without sterilization. Half a milliliter of reaction mixture and 2 ml of 0.6% soft agar were poured onto bouillon agar in a Petri dish and mixed quickly. The plates were cultured for 48 hours for possible contaminating organisms.

Materials. The following radio-active compounds were obtained from the Daiichi Chemical Company: fumaric acid-1,4-C^{14} with a specific activity 4.94 mc/mM; succinic acid-2,3-C^{14}, specific activity 4.0 mc/mM; glutamic acid-1-C^{14}, specific activity 16.0 mc/mM; sodium glyoxylate-u-C^{14}, specific activity 4.71 mc/mM; citric acid-1,5-C^{14}, specific activity 12.0 mc/mM; and α-ketoglutaric acid-5-C^{14}, specific activity 9.33 mc/mM. Oxaloacetate was a product of the Nutritional Biochemicals Co.; cisaconitate was a Mann Research Laboratories Ltd. product; DL-isocitrate, coenzyme A, pyridoxal 5′-phosphate nicotinamide adenine dinucleotide (NAD) nicotinamide adenine dinucleotide triphosphate (NADP), reduced NAD (NADH), reduced NADP (NADPH) and phenazine methosulfate were products of the Sigma Chemical Co.; lipoic acid, thiamine pyrophosphate (TTP), and oxaloacetate were products of the Tokyo Chemical Co.; α-ketoglutaric acid was a Kyowa Fermentation Co. product; fumaric acid and succinic acid were products of the Wako Pure Chemicals Co.; glyoxylic acid was a Maruwaka Kagaku product and DL-sodium malate was a product of the Ishizu Chemical Co. The δ-hydroxylaevulinic acid was kindly

supplied by Professor Moriyama. The other reagents were special grade products purchased locally.

RESULTS

Fumarate hydratase (fumarase) (4.2.1.2). In order to observe the fumarate hydratase activity by which water adds to fumaric acid to produce malic acid, fumaric acid-1,-4-C^{14} was used as the substrate and the production of radio-active malic acid was followed by paper-chromatography. As may be seen in Figure 2, prominent formation of malic acid occurred. Comparison of radio-activities shows that the reaction inclines toward the direction of malic acid formation.

Succinate dehydrogenase (1.3.99.1). Phenazine methosulfate is known to be an autoxidizable dye related to succinate dehydrogenase ([18]). As a disturbance in the terminal electron transport system is present in the cell-free extract of *M. lepraemurium*, phenazine methosulfate was added and the reaction carried out aerobically with occasional shaking. Succinate-2 3-C^{14} was used as the substrate and the radio-active reaction products were determined by paper-chromatography. As shown in Figure 3, fumaric acid and malic acid were produced. The fumaric acid produced by succinate dehydrogenase is converted to malic acid by fumarate hydratase which

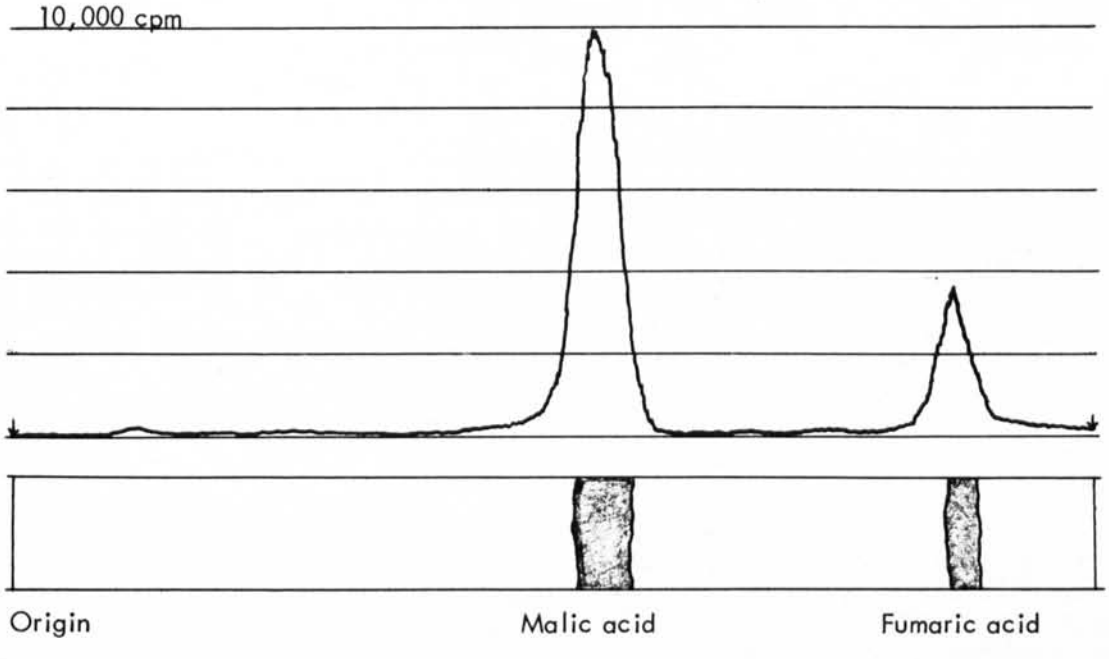

FIG. 2. Fumarate hydratase activity of *M. lepraemurium*.

0.06 mc of fumaric acid-1,4-C^{14}, specific activity 4.94 mc/mM, was used as substrate without adding carrier cold fumarate. 0.2 gm of lyophilized *M. lepraemurium* (Hawaiian strain) was ground with quartz powder and extracted with 2 ml of phosphate buffer M/100, at pH 7.0. 1.7 ml of supernant was obtained after 10,000 rpm centrifugation for 30 minutes. 0.5 ml of radioactive fumaric acid, with pH corrected to neutral, and 1.7 ml of supernatant were incubated for 300 minutes at 37°C under anaerobic conditions. Total protein of reaction mixture was 12.4 mg by phenol method. Solvent for paper-chromatography was (A). Radioactivity was scanned under collimator 1/16 inch, time count 2 seconds, scan speed ¾ inch/min., and count rate 10,000 cpm.

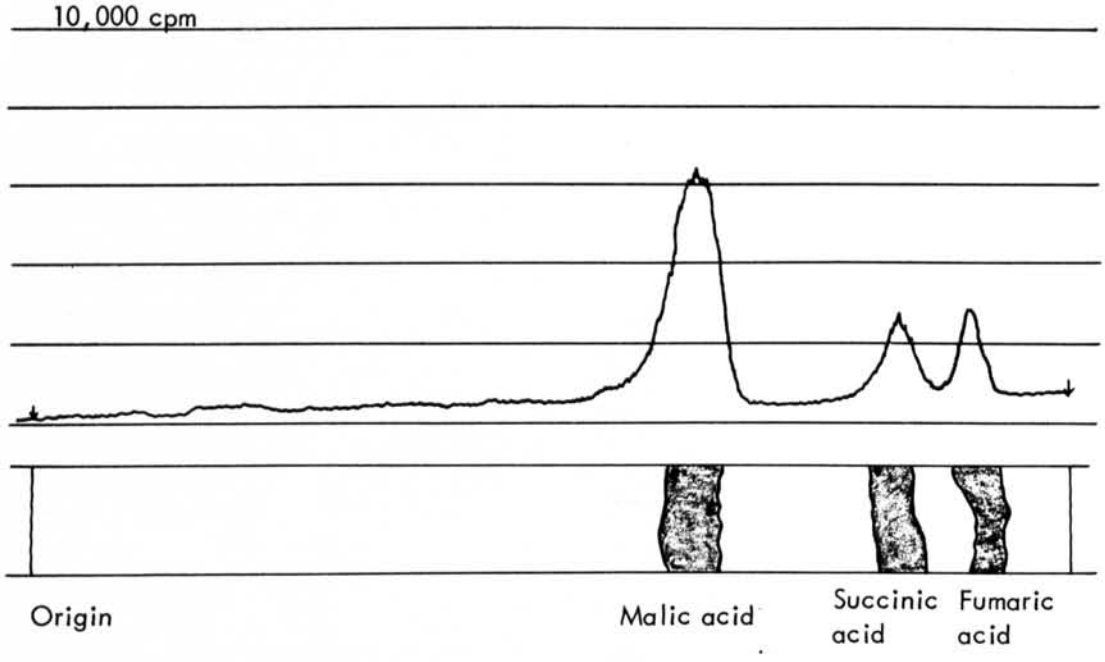

FIG. 3. Succinate dehydrogenase activity of *M. lepramurium*.

0.05 mc of succinic acid-2,3-C^{14}, specific activity 4.0 mc/mM, was used as a substrate without cold carrier succinate. *M. lepraemurium* (dry weight 1.4 gm) were suspended in 20 ml of phosphate buffer M/100, pH 7.0, and disintegrated in a French pressure cell under 500 atmospheres pressure. 2 ml of the disintegrated cell suspension was used as enzyme and 2 mg of phenazine methosulfate was added to the enzyme as an electron carrier. Incubation was carried out at 37°C for 300 minutes, occasionally shaking under aerobic condition. Solvent for paper-chromatography was (A). Radioactivity was scanned under collimator 1/16 inch, time count 2 seconds, scan speed ¾ inch/min and count rate 10,000 cpm.

was also present.

Aconitate hydratase (aconitase) (4.2.1.3). The formation of citrate from cis-aconitate and DL-isocitrate was examined. Table 1 indicates that citrate was produced from both substrates.

Citrate synthase (condensing enzyme) (4.1.3.7). As shown in Table 2, prominent formation of citrate occurred from oxaloacetate and acetyl CoA. According to Yamamura and Kusunose ([21]) in their study on citrate synthase activity using the crude extract of the Takeo strain of an avian type (saprophytic) mycobacterium, prominent citrate formation was found only with oxaloacetate but in the case of *M. lepraemurium*, citrate formation was not found.

Formazan production with substrates of the tricarboxylic acid cycle system. The tricarboxylic acid cycle system has four dehydrogenase steps, that is, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase. Pyruvate dehydrogenase leads to the formation of acetyl CoA. Table 3 shows the results of quantitative determination of formazan production by cell-free extract of *M. lepraemurium* using substrates of the TCA cycle system. It has been shown by Ochoa *et al* ([16]) that in the case of pyruvate, decarboxylation and

TABLE 1. *Aconitate hydratase activity of* M. lepraemurium.

Substrates	Citrate formed x 10^{-6}mols
—	0
Cis-aconitate 4 x 10^{-5}mols	10.0
DL-isocitrate 8 x 10^{-5}mols	4.0

0.5 gm of lyophilized *M. lepraemurium* (Hawaiian strain) was ground with glass powder, and the enzyme was extracted with 10 ml of tris buffer M/20, pH 7.6. The supernatant was used as the enzyme solution. Two ml of enzyme and 0.5 ml of substrate were incubated for 300 minutes at 37 °C under anaerobic condition. Total protein of reaction mixture was 7.3 mg by the phenol determination method.

Substrate	citrate formed x 10^{-6}mols
—	0
Cis-aconitate 4 x 10^{-5}mols	8.2
DL-isocitrte 4 x 10^{-5}mols	3.0

0.6 gm of lyophilized *M. lepraemurium* (Hawaiian strain) was ground, and extracted with 20 ml of tris buffer M/20, pH 7.2. Two ml of supernatant enzyme and 0.5 ml of substrate were incubated for 150 minutes at 37 °C under anaerobic condition. Total protein of reaction mixture was 4.3 mg by the phenol determination method.

dehydrogenation take place simultaneously to form acetyl CoA by the action of pyruvate dehydrogenase, but this activity is almost absent in *M. lepraemurium*. Formazan production is highest when malate is used as the substrate and appears as the sum of the malate dehydrogenase and malate-VK_3 (2-methyl-1,4-naphtoquinone) reductase ([1, 13]). Oxaloacetate is decarboxylated ([8]) and converted to

TABLE 2. *Citrate synthase (condensing enzyme) activity of* M. lepraemurium.

Crude extract 1.3 ml	Oxaloacetate 0.3 ml 10^{-5} mols	Acetyl CoA 0.4 ml 6 x 10^{-6} mols	Pyruvate 0.4 ml 10^{-5} mols	Citate formed x 10^{-7} mols
+	−	−	−	0
+	+	−	−	1.0
+	−	+	−	1.5
+	+	−	+	1.1
+	+	+	−	22.0

0.4 gm of lyophilized murine leprosy bacilli (Hawaiian strain) was ground with glass powder, and the enzyme was extracted with 6.0 ml of phosphate buffer M/10, pH 7.4. The supernatant was used as the enzyme solution. Total volume made up to 2 ml with distilled water, and incubation was carried out at 37 °C for 240 minutes under aerobic condition. Total protein of reaction mixture was 6.3 mg by the phenol determination method.

TABLE 3. *Dehydrogenase activities of* M. lepraemurium.

Substrates	Formazan formed x 10^{-7}mols
—	4.9
—[a]	4.8
pyruvate	5.0
malate	14.8
oxaloacetate[a]	4.8
succinate	6.5
α-ketoglutarate[a]	8.4
oxalosuccinate[a]	4.8
DL-isocitrate	7.0
cis-aconitate	10.0
citrate	11.0
pyruvate + oxaloacetate[a]	4.9
lactate	6.5
glucose	4.7
glucose-6-P	7.0
glyceraldehyde-3-P	4.8

[a] $MgCl_2$ 10^{-6} mols, lipoic acid 2 × 10^{-7} mols, TPP 2 × 10^{-7} mols and Co A 2 × 10^{-7} mols were added.

0.5 gm of lyophilized murine leprosy bacilli (Hawaiian strain) was ground with glass powder and extracted with 20 ml of phosphate buffer

M/20, pH 7.0 containing 4×10^{-6} mols/ml cysteine. The supernatant was used as the enzyme solution: 1.0 ml enzyme solution. 0.1 ml NAD 2×10^{-7} mols, 0.5 ml H_2O in main vessels, 0.2 ml substrate 2×10^{-5} mols, 0.2 ml TTC 10^{-5} mols in each side vessel. Incubation was carried out at 37 °C for 150 minutes under anaerobic condition. Total protein of reaction mixture was 1.8 mg by phenol method.

pyruvate or oxidative decarboxylation takes place by direct dehydrogenation ([20]) but there is almost no production of formazan. Some succinate dehydrogenase activity is seen but it is not prominent. This may be due to inappropriateness of triphenyl tetrazolium chloride (TTC) as an acceptor of hydrogen from succinate dehydrogenase. Despite the absence of succinate production with α-ketoglutarate as the substrate, formation of formazan is greater when α-ketoglutarate is the substrate than with succinate as the substrate. This will be discussed later in the section on isocitrate metabolism. If isocitrate, cis-aconitate and citrate are metabolized to follow the TCA cycle, dehydrogenation should take place at the isocitrate level, but from the viewpoint of formazan yield, the order is citrate, cis-aconitate and isocitrate. Thus, it is difficult to assume that formazan production depends only on isocitrate dehydrogenase. It is more logical to consider a metabolic pathway of isocitrate→cis-aconitate→citrate. It is not clear, however, what pathway from citrate results in a dehydrogenation step. If it is assumed that malate is formed by a reversal of citrate synthase and then dehydrogenation takes place, then oxaloacetate should be a good substrate for dehydrogenation. In fact, oxaloacetate can not serve as a substrate. The findings on the metabolic products of citrate, which are presented later, make it unavoidable to assume that the pathway is from citrate to isocitrate. It is believed at the present time that the weak enzymatic activity was due to an inhibition of the site of activity of the enzyme by an isomer which would not act as a substrate since the DL-form of isocitrate was used. In the presence of both pyruvate and oxaloacetate, dehydrogenation takes place after the formation of citrate but since acetyl CoA is not produced from pyruvate, there is no production of citrate with pyruvate alone, so there is almost no formation of formazan. Oxalosuccinate is converted to α-ketoglutarate by decarboxylation ([16]) and is further metabolized to the stage of dehydrogenation, but in the case of the cell-free extract of *M. lepraemurium*, formazan production is low compared to α-ketoglutarate even though oxalosuccinate decarboxylase activity is present, as may be seen in Table 4.

2-oxoglutarate dehydrogenase (1.2.4.2). Considerable formation of formazan was noted when α-ketoglutarate was the substrate, so the reaction product with α-ketoglutarate-5-C^{14} as the substrate was examined by paper-chromatography. As may be seen in Figures 4 and 5, there was no formation of succinic acid and malic acid

TABLE 4. *Decarboxylation of oxalosuccinate in cell-free extract of* M. lepraemurium.

Main chamber		Side arm A	Side arm B		
M/20 tris buffer pH 7.2	enzyme extract	4N H_2SO_4	oxalosuccinate 2×10^{-5} mols	CO_2 evolution μl	difference μl
0.5 ml	1.4 ml	0.3 ml	—	26	
0.5 ml	1.4 ml	0.3 ml	0.5 ml	65	39

3 gm of wet murine leprosy bacilli (Hawaiian strain) were ground with 3 gm of quartz powder and extracted with 27 ml of M/200, pH 7.2 tris buffer. The supernatant of 10,000 rpm centrifugation was used as the enzyme solution. The enzyme reaction was measured by the Warburg manometric method at 37.5 °C for 60 minutes. Dry weights of enzyme extract per 1.4 ml were 42 mg. Total protein of reaction mixture was 17.9 mg by the phenol method.

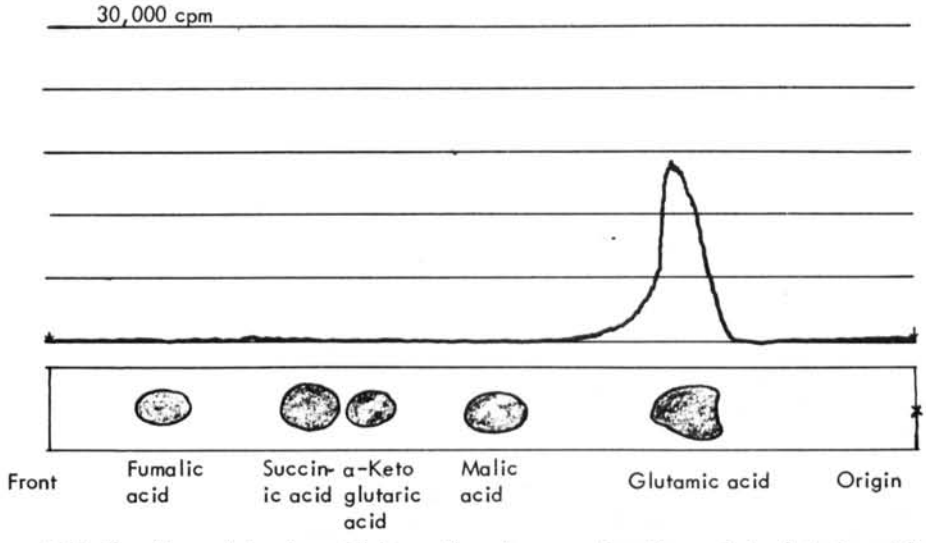

FIG. 4. Radioactive glutamic acid formation from radioactive α-ketoglutaric acid by enzyme action of *M. lepraemurium*.

0.05 mc of α-ketoglutaric acid-5-C^{14}, specific activity 9.33 mc/mM, was used as substrate without cold carrier. 1.5 gm of lyophilized *M. lepramurium* (Hawaiian strain) were ground with 2 ml of phosphate buffer M/100, pH 7.0. The ground cell suspension was used as enzyme adding 1 mg of phenazine methosulfate and 5 mg of Difco bacto yeast extract. Incubation was carried out at 37°C for 300 minutes occasionally shaking under aerobic condition. Solvent for paper-chromatography was (A). Reaction products were applied lineally on the original line and control cold standard samples were spotted on the middle of the original point. Radioactivity was scanned under collimator 1/16 inch, time count 2 seconds, scan speed ¾ inch/min and count rate 30,000 cpm.

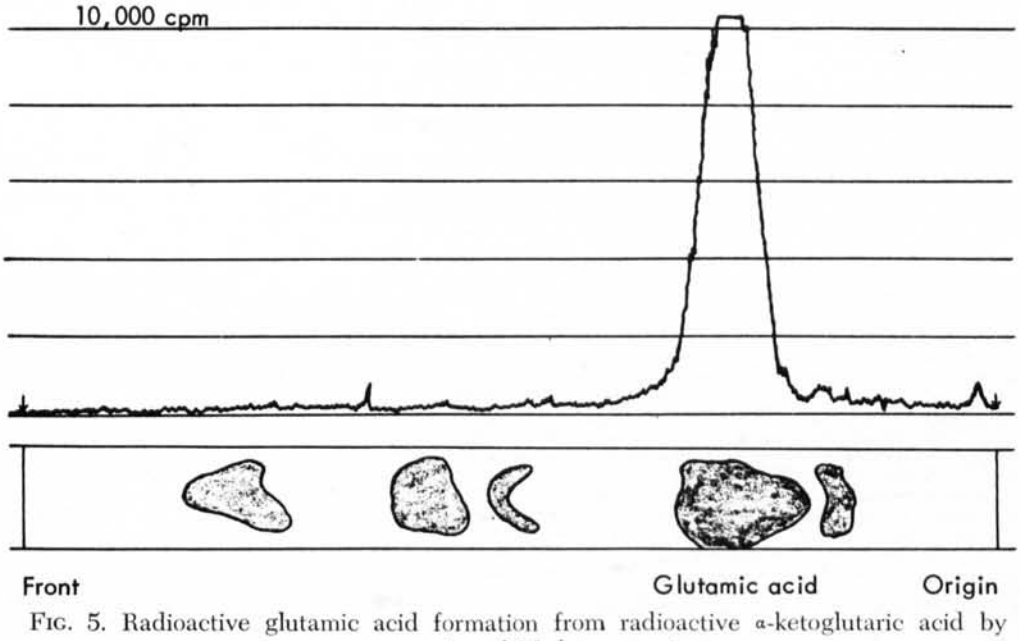

FIG. 5. Radioactive glutamic acid formation from radioactive α-ketoglutaric acid by enzyme action of *M. lepraemurium*.

Paper-chromatography was developed with water saturated phenol by the ascending method using the same material as in Figure 4.

and almost all of the α-ketoglutarate was converted to glutamate. It is believed that the amino group was introduced into α-ketoglutarate by transaminase activity since the enzyme solutions contained endogenous amino acids. When α-ketoglutarate dehydrogenase activity was determined with TTC, the dehydrogenase activity did not depend on α-ketoglutarate dehydrogenase but rather on glutamate dehydrogenase since α-ketoglutarate was converted to glutamate.

Glutamate-1-carboxy-lyase (glutamate decarboxylase) (4.1.1.15). The pathway of glutamate to γ-aminobutyric acid by the action of glutamate decarboxylase and that of γ-aminobutyric acid to succinic semialdehyde by transamination between γ-aminobutyrate and α-ketoglutarate and that of succinic semialdehyde to succinate by oxidation have been proven with the avian type Takeo strain (saprophytic acid-fast bacillus) by Shoji *et al.* [19]. The α-ketoglutarate metabolism in *M. lepraemurium* was studied by examination of glutamate decarboxylase activity but, as shown in Table 5, there was no production of γ-aminobutyrate and there was no release of radio-active carbon dioxide from glutamic acid-1-C^{14} with either cell-free extract or living cells as noted in Table 6.

Isocitrate dehydrogenase (1.1.1.42). In the TCA cycle of *M. lepraemurium*, there is a disturbance in α-ketoglutarate metabolism which accumulates almost wholly as glutamate, hence the glyoxylate cycle was investigated by determining isocitrate-lyase activity to observe the formation of the carbon source metabolic cycle. It has been reported that organisms which have proliferated under anaerobic conditions have a strong enzyme activity by which isocitrate is broken down to succinate and glyoxylate [17]. Moriyama *et al* [14] report that with the avian type Takeo strain (saprophytic acid-fast bacillus), isocitrate is decomposed to succinate and glyoxylate and the glyoxylate binds with α-ketoglutarate to form δ-hydroxylaevulinate.

A sample of radio-active isocitrate could not be obtained so the aerobic and anaerobic metabolic products of citrate-1,5-C^{14} were analyzed by paper-chromatography. Reaction under aerobic conditions gave a peak radioisotope count at the Rf position of glutamate, α-ketoglutarate, succinate, δ-hydroxylaevulinate, malate, fumatate and isocitrate, as may be seen in Figure 6. Figure 7 shows the result of anaerobic reaction and the reaction products are glutamate, succinate, δ-hydroylaevulinate and unreacted citrate. Succinate has been produced from isocitrate anaerobically, that is, succinate and glyoxylate are produced anaerobically by the action of isocitrate-lyase and the gloxylate reacts with α-ketoglutarate to form δ-hydroxylaevulinate, as shown in Figure 8. A strange phenomenon at this point is the production of a large

TABLE 5. *Glutamate decarboxylase activity of* M. lepraemurium.

Enzyme 1 ml	Acetate buffer M/20, pH 4.8	Substrate	γ-aminobutyric acid
Crude extract	1 ml	—	—
Crude extract	1 ml	glutamate 10^{-5} mols	—
Crude extract	1 ml	radio-active glutamate[a]	—
Crude extract + cell debris	1 ml	glutamate 10^{-5} mols	—

[a] Reaction product of Figure 4.

0.4 gm of lyophilized *M. lepraemurium* (Hawaiian strain) was ground with glass powder, and the enzyme was extracted with 5 ml of distilled water. The supernatant and cell debris were used as enzyme. Pyridoxal-5′-P 10^{-7} mols were added to each tube; 3 ml of reaction mixture was incubated for 300 minutes at 37 °C under anaerobic condition. The formation of γ-aminobutyric acid was observed as a ninhydrin positive spot or radio-active peak on paper chromatography, developed with water saturated phenol. Total protein of the reaction mixture was 17.5 mg by the phenol method.

TABLE 6. *Decarboxylation of glutamic acid-1-C-14 in* M. lepraemurium.

Buffer solution 0.5 ml	Enzyme solution or bacterial suspension	L-glutamate 10^{-4} mol DL-glutamate-1-C^{14} 162,200 cpm	Radio-active CO_2 cpm
pH 4.7[a]	H_2O 0.4 ml	0.1 ml	156
pH 4.7[a]	bacteria[c] 0.4 ml	0.1 ml	120
pH 7.2[b]	H_2O 0.4 ml	0.1 ml	146
pH 7.2[b]	bacteria[c] 0.4 ml	0.1 ml	156
pH 4.7[a]	H_2O 1.4 ml	0.1 ml	124
pH 4.7[a]	enzyme[d] 1.4 ml	0.1 ml	100
pH 7.2[b]	H_2O 1.4 ml	0.1 ml	118
pH 7.2[b]	enzyme[d] 1.4 ml	0.1 ml	108

Specific activity of DL-glutamic acid-1-C^{14} was 16.0 mc/mM.
[a] M/10 acetate buffer
[b] M/20 tris buffer
[c] Dry weights of bacilli 24.7 mg
[d] Dry weights of enzyme 26.3 mg, protein 5.6 mg

1.5 gm of wet *M. lepraemurium* (Hawaiian strain) was ground with quartz powder and extracted with 15 ml of distilled water. The supernatant of 10,000 rpm centrifugation was used as the enzyme solution. Thünberg tubes were used for enzyme reaction and radio-active carbon dioxide was fixed on 0.2 ml of 20% KOH put into small test tube inside the Thünberg tube. The reaction mixture was incubated for 60 minutes (bacterial suspension), 120 minutes (supernatant enzyme) at 37 °C under vacuum.

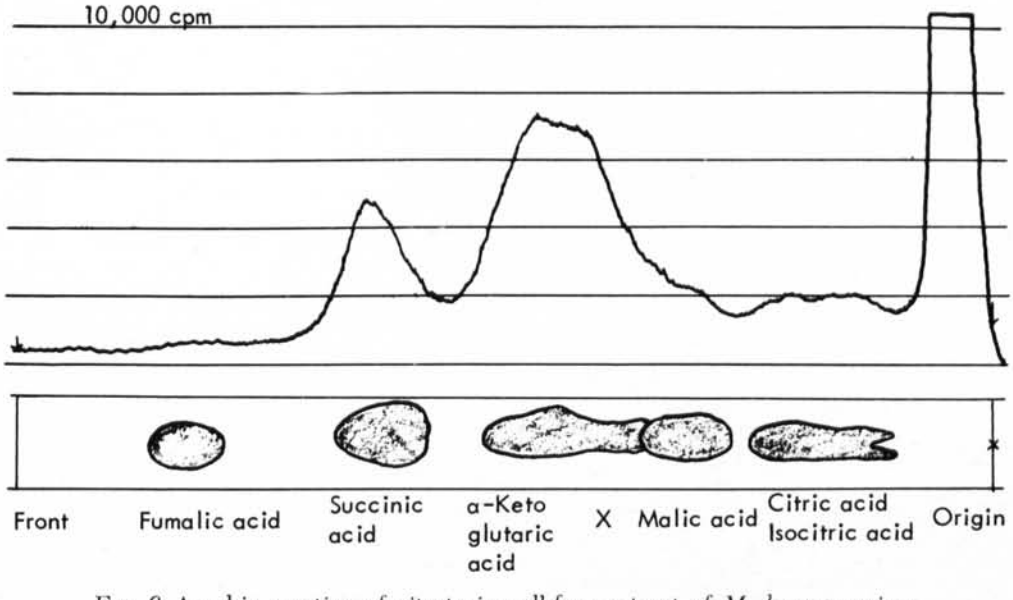

FIG. 6. Aerobic reaction of citrate in cell-free extract of *M. lepraemurium*.

0.05 mc of citric acid-1,5-C^{14}, specific activity 12.0 mc/mM, was used as substrate without cold carrier citrate. 3 gm, wet weight, *M. lepraemurium* (Hawaiian strain) were ground with 27 ml of tris buffer M/100, pH 7.2. The supernatant 1.5 ml (dry weight 31.5 mg) was used as enzyme. Total protein of the reaction mixture was 13.5 mg by the phenol method. Incubation was carried out at 37°C for 225 minutes under aerobic condition. Solvent for paper chromatography was (A). Control cold standard samples were spotted on the original point. Radioactivity was scanned under collimator 1/16 inch, time count 10 seconds, scan speed ¾ inch/min and count rate 10,000 cpm.

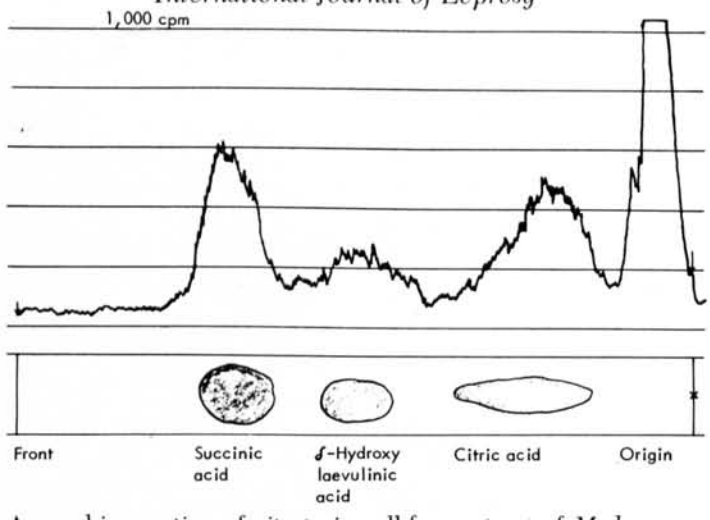

FIG. 7. Anaerobic reaction of citrate in cell-free extract of *M. lepraemurium*.

Cold citrate, 4 x 10^{-5} mols containing 0.02 mc of citric acid-1,5-C^{14}, specific activity 12 mc/mM, was used as substrate. 2 gm, wet weight, *M lepraemurium* (Hawaiian strain) were ground with 20 ml of tris buffer M/100, pH 7.2. The supernatant of 10,000 rpm centrifugation, 2.0 ml (dry weight 40.0 mg) was used as enzyme. Total protein of reaction mixture was 17.1 mg by the phenol method. Incubation was carried out at 37°C for 190 minutes under anaerobic condition. Solvent for paper-chromatography was (B). Control cold standard samples were spotted on the original point. Radioactivity was scanned under collimator 1/16 inch, time count 10 seconds, scan speed ¾ inch/min and count rate 1,000 cpm.

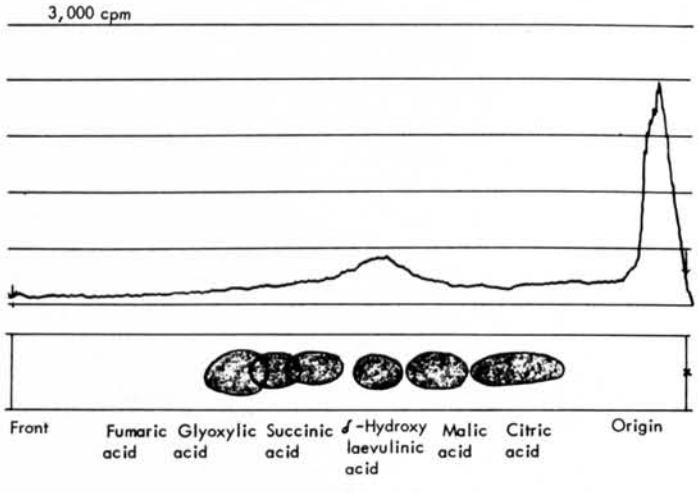

FIG. 8. Anaerobic reaction of glyoxylate and α-ketoglutarate in cell-free extract of *M. lepraemurium*.

3.25 x 10^{-5}mols of glyoxylate and the same mol quantity of α-ketoglutarate containing 0.01 mc of glyoxylate-u-C^{14}, specific activity 4.71 mc/mM, were used as substrate. 2.5 gm, wet weight, *M. lepraemurium* (Hawaiian strain) were ground with 25 ml of tris buffer M/100, pH 7.4. The supernatant 5 ml (dry weight 92.5 mg), total protein 39.5 mg were used as enzyme with addition of 5 x 10^{-6}mols of $MgSO_4 7H_2O$, 5 x 10^{-6}mols of cystein and 10^{-6}mols of thiamin pyrophosphate. Incubation was carried out at 37°C for 210 minutes under anaerobic condition. Solvent for paper-chromatography was (B). Control cold standard samples were spotted on the original point. Radioactivity was scanned under collimator 1/16 inch, time count 10 seconds, scan speed ¾ inch/min and count rate 3,000 cpm.

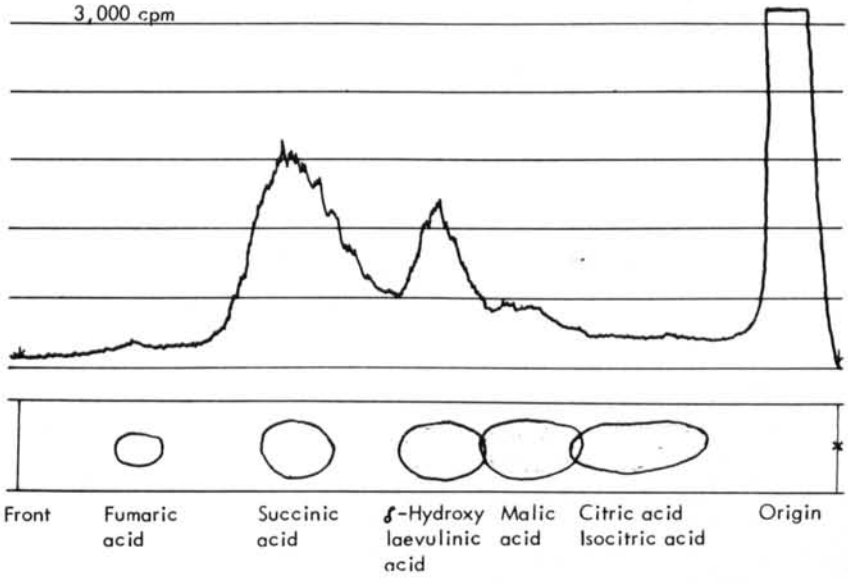

FIG. 9. Anaerobic reaction of citric acid and acetyl CoA in a cell-free extract of *M. lepraemurium*.

5 x 10^{-6}mols of acetyl CoA and 0.05 mc of citric acid-1,5-C^{14}, specific activity 12.0 mc/mM, were used as substrates. 0.5 gm, wet weight, *M. lepraemurium* (Hawaiian strain) was ground with 4 ml of phosphate buffer M/100, pH 7.0. The supernatant of 10,000 rpm centrifugation 1.8 ml (dry weight 36.0 mg) was adjusted to pH 7.8 and 5 x 10^{-6}mols of $MgSO_4 7H_2O$ and 5 x 10^{-6}mols of cystein were added. Total protein of reaction mixture was 15.4 mg by the phenol method. Incubation was carried out at 37°C for 180 minutes under anaerobic condition. Solvent for paper-chromatography was (B). Control cold standard samples were spotted on the original point. Radioactivity was scanned under collimator 1/16 inch, time count 10 seconds, scan speed ¾ inch/min and count rate 3,000 cpm.

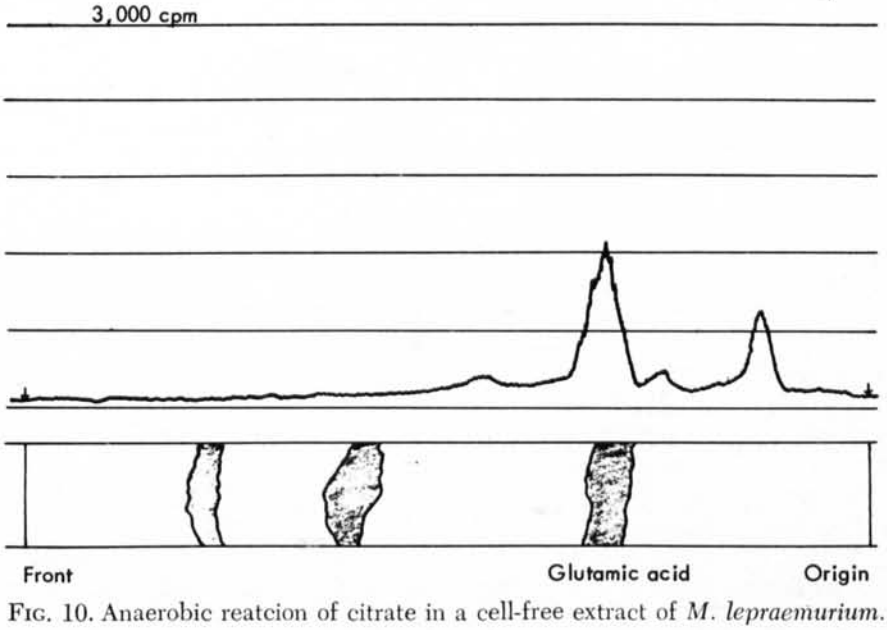

FIG. 10. Anaerobic reatcion of citrate in a cell-free extract of *M. lepraemurium*.

Paper-chromatography was developed with water saturated phenol by the ascending method using the same materials as in Figure 9.

amount of glutamate anaerobically from citrate-1,5-C^{14} as shown in Figure 9 and Figure 10. The mechanism for this is not known. The formation of large amounts of α-ketoglutarate and δ-hydroxylaevulinate by the aerobic metabolism of citrate indicates the simultaneous production of α-ketoglutarate by the action of isocitrate dehydrogenase.

Observation of isocitrate metabolism by measurement of oxygen consumption and carbon dioxide evolution with the Warburg manometric method on addition of phenazine methosulfate as the electron transport system reveals a ratio of O_2:CO_2=2:3, as seen in Table 7. When isocitrate is metabolized by the TCA cycle, the process of α-ketoglutarate dehydrogenase does not proceed with the cell-free extract of *M. lepraemurium*, the ratio of O_2:CO_2 becomes 1:2. When metabolism occurs by the action of isocitrate-lyase and δ-hydroxylaevulinate synthase, succinate is oxidized to oxaloacetate and δ-hydroxylaevulinate to α-ketoglutarate. The summarized O_2:CO_2 is calculated to be 1:1. If both reactions occur simultaneously, then O_2:CO_2 will become 2.5:3 and close to the experimental value. Since oxidation requires more steps compared to decarboxylation and a considerable time is needed for reaction to proceed to completion, it is considered that the ratio O_2:CO_2 is about 2:3.

TABLE 7. *Isocitrate metabolism of cell-free extract of* M. lepraemurium.

M/20 tris buffer[a] pH 7.2	Enzyme extract	KOH 20%	4N H_2SO_4	Isocitrate 5×10^{-5} mols	Gas μl	Difference μl
0.5 ml	1.4 ml	0.1 ml	—	—	O_2 up take 22.96	
0.5 ml	1.4 ml	—	0.2 ml	—	CO_2 evolution 63.20	
0.5 ml	1.4 ml	0.1 ml	—	0.5 ml	O_2 up take 46.76	23.8
0.5 ml	1.4 ml	—	0.2 ml	0.5 ml	CO_2 evolution 99.10	36.0

All conditions are the same as in Table 4.
[a] One mg phenazine methosulfate was contained in 0.5 ml.

Malate synthase (malic enzyme) (4.1.3.2). The presence of malate synthase activity, by which malate is formed by a condensation reaction between glyoxylate and acetyl CoA in the glyoxylate pathway, was examined with cell-free extract of *M. lepraemurium*. Glyoxylate u-C^{14} and acetyl CoA were used as substrates anaerobically and the reaction products determined by paper-chromatography. Figures 11 and 12 show the results. There was no production of malate, and only glutamate and unknown substances were found.

Cultivation test for contaminating microorganism in reaction mixture. Since the enzyme activities of *M. lepraemurium* were generally low, detectable enzyme activities could not be determined without long incubation periods. As we were afraid that these enzyme activities might be the result of contaminating microorganism activity, a cultivation test for contaminating microorganisms was carried out with each reaction mixture. Only a few contaminating bacteria were found in reaction mixtures and their number did not increase with the lapse of incubation times. These small contaminants can be disregarded as having any significant effect on the enzyme reactions (Table 8).

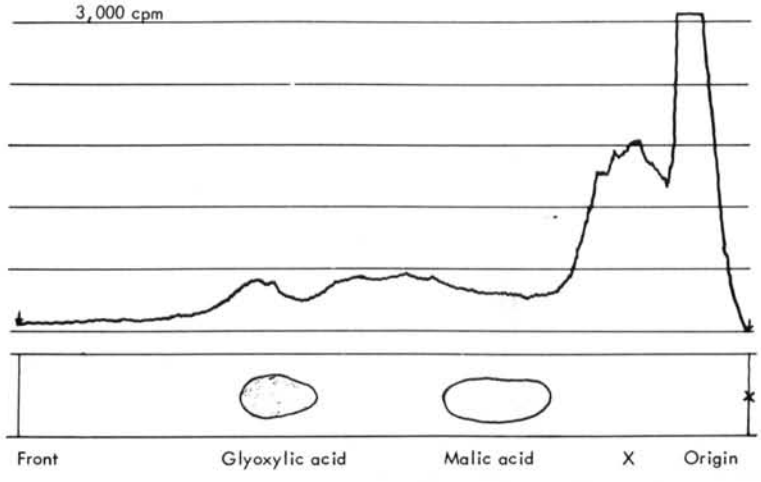

FIG. 11. Anaerobic reaction of glyoxylate and acetyl CoA in a cell-free extract of *M. lepraemurium*.

0.025 mc of glyoxylate-u-C^{14}, specific activity 4.71 mc/mM, and 5 x 10^{-6}mols of acetyl CoA were used as substrates. 0.5 gm, wet weight, *M. lepraemurium* (Hawaiian strain) was ground with 4 ml of phosphate buffer M/100, pH 7.0. The supernatant of 10,000 rpm centrifugation, 1.8 ml (dry weight 36.0 mg) was adjusted to pH 7.8 and 5 x 10^{-6}mols of $MgSO_4 7H_2O$ and 5 x 10^{-6}mols of cystein were added. Total protein of reaction mixture was 15.4 mg by the phenol method. Incubation was carried out at 37°C for 180 minutes under anaerobic condition. Solvent for paper-chromatography was (B). Cold standard samples were spotted on the original point. Radioactivity was scanned under collimator 1/16 inch, time count 10 seconds, scan speed ¾ inch/min and count rate 3,000 cpm.

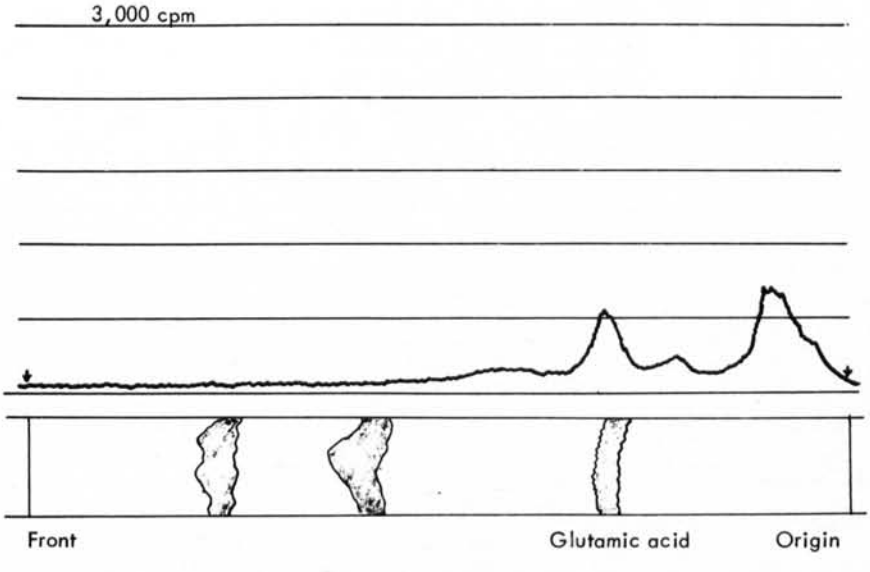

FIG. 12. Anaerobic reaction of glyoxylate and acetyl CoA in cell-free extract of *M. lepraemurium*.

Paper-chromatography was developed with water saturated phenol by the ascending method using the same materials as in Figure 11.

TABLE 8. *Contaminating microorganisms in reaction mixtures.*

Incubation time hours	Number of micro-organism/ml at aerobic reaction	Number of micro-organism/ml at anaerobic reaction
0	22	22
1	18	26
2	10	32
3	22	20
4	10	18
5	16	16
after dialysis for 36 hours	occasional microorganism	18

Wet weight 3 gm of *M. lepraemurium* were ground with quartz powder and the enzyme was extracted with 22 ml of pH 7.0, M/20 tris buffer. The supernatant 7 ml of 10,000 rpm centrifugation were incubated with 5.7 mg of sodium citrate at 37 °C under aerobic and anaerobic condition. Each milliliter of reaction mixture was withdrawn from reaction tubes every hour. 0.5 ml was cultivated on agar plate and 0.5 ml of same reaction mixture was diluted tenfold with 4.5 ml bouillon and 0.5 ml was cultivated. Colony count was carried out after 48 hours cultivation. Dry weights of 1 ml dialyzed supernatant were 11.3 mg and its protein was 4.8 mg by the phenol method.

DISCUSSION

Since oxidative decarboxylation of pyruvate was not found in cell-free extracts of *M. lepraemurium*, the source of acetyl CoA for the TCA cycle may be derived from the β-oxidation of fatty acids. A-ketoglutarate, similarly does not undergo oxidative decarboxylation, but if α-ketoglutarate is formed from isocitrate, it may be stored as glutamate in the bacterial cell. The TCA cycle in *M. lepraemurium* was blocked at the α-ketoglutarate step, hence substrate metabolism may take place by the isocitrate-lyase pathway, a side pathway of the TCA cycle. The succinate produced by the isocitrate-lyase system was metabolized by the TCA cycle. Glyoxylate, which was formed at the same time, condensed with α-ketoglutarate to form δ-hydroxylaevulinate, but succeeding metabolic steps are unknown. If the δ-hydroxylaevulinate is oxidized to α-ketoglutarate, fatty acids should be utilized in catalytic amounts for energy production. Since citrate and succinate are utilized by cell-free extracts of *M. lepraemurium*, aerobic metabolism should take place and oxygen consumption rise when citrate or succinate are added to the whole cell. However, oxygen consumption corresponding to the supplemented substrates was not found. As the bacilli were washed repeatedly with water, the surface structures may have been disrupted. If transport proteins are essential for the incorporation of these substrates, they would not be utilized when the transport systems are non-functional. However, *in vivo* grown *M. lepraemurium* may possess transport systems for citrate or succinate. On the other hand, *M. lepraemurium* might utilize other substrates for energy production *in vivo* rather than citrate or succinate.

SUMMARY

1. *Mycobacterium lepraemurium* possesses fumarate hydratase activity and malate is produced from fumarate.

2. It possesses succinate dehydrogenase activity and fumarate is produced from succinate.

3. There is aconitate hydratase activity, and citrate is produced from cis-aconitate and isocitrate.

4. Citrate-synthase activity is present and citrate is produced from acetyl CoA and oxaloacetate. Citrate formation was not found when pyruvate was substituted for the acetyl CoA.

5. A-ketoglutarate is produced by isocitrate dehydrogenase in *Mycobacterium lepraemurium*.

6. The 2-oxoglutarate dehydrogenase activity of *Mycobacterium lepraemurium* was examined, but production of succinate from α-ketoglutarate was not found.

7. *Mycobacterium lepraemurium* produces glutamate from α-ketoglutarate.

8. Glutamate decarboxylase activity was investigated but formation of γ-aminobutylic acid was not observed and release of radio-active carbon dioxide from glutamate-1-C^{14} was not found either with the live cell or the cell-free extract.

9. *Mycobacterium lepraemurium* decomposes isocitrate to succinate and glyoxylate by the action of isocitrate lyase. The glyoxylate does not bind with malate but binds with α-ketoglutarate to form δ-hydroxylaevulinate.

RESUMEN

1. El *Mycobacterium lepraemurium* posee actividad fumarato hidratasa y del fumarato se produce malato.

2. Posee actividad succinato deshidrogenasa y se produce fumarato a partir del succinato.

3. Hay actividad aconitato hidratasa y se produce citrato del cis-aconitato e isocitrato.

4. La actividad citrato-sintetasa está presente y se produce citrato a partir del acetil CoA y oxaloacetato. No se encontró formación de citrato cuando se substituyó piruvato en vez de acetil CoA.

5. El α-cetoglutarato es producido por la isocitrato deshidrogenasa en el *Mycobacterium lepraemurium*.

6. Se examinó la actividad de 2-oxoglutarato deshidrogenasa del *Mycobacterium lepramurium*, pero no se encontró producción de succinato a partir de α-cetoglutarato.

7. El *Mycobacterium lepraemurium* produce glutamato a partir de α-cetoglutarato.

8. Se invetigó la actividad glutamato decarboxilasa pero no se observó formación de ácido γ-aminobutílico y no se encontró liberación de dióxido de carbono radioactivo a partir del glutamato-1-C^{14} ya sea con las células vivas o con el extracto libre de células.

9. El *Mycobacterium lepraemurium* descompone el isocitrato a succinato y glioxilato por la acción de isocitrato liasa. El glioxilato no se une con malato pero si se une con un α-cetoglutarato para formar δ-hidroxilevulinato.

RÉSUMÉ

1. *Mycobacterium leprae* possède une activité au point de vue de l'hydratase de fumarate; de malate est produit à partir du fumarate.

2. Cet organisme possède une activité au point de vue de la succinate déhydrogenase; du fumate est produit à partir du succinate.

3. On constate une activité au point de vue de l'aconitate hydratase; du citrate est produit à partir du cis-aconitate et de l'isocitrate.

4. Une activité au point de vue de la citrate-synthase est présente; du citrate est produit à partir de l'acétyl CoA et de l'oxaloacétate. La formation de citrate n'est pas observée lorsqu'on substitue du pyruvate à la place de l'acétyl CoA.

5. De l'α-cétaglutarate est produit par l'isocitrate déhydrogenase chez *Mycobacterium lepraemurium*.

6. On a étudié *Mycobacterium lepraemurium* en ce qui concerne l'activité au point de vue de la 2-oxoglutarate déhydrogenase; aucune production de succinate à partir dé α-cétoglutarate n'a été observée.

7. *Mycobacterium lepraemurium* produit du glutamate à partir de l'α-cétoglutarate.

8. On a exploré l'activité au point de vue de la glutamate décarboxylase; on n'a pas observé de formation d'acide γ-aminobutylique, ni de libération de dioxyde de carbone radio-actif, à partir du glutamate-1-C^{14}, ni dans les cellules vivantes, ni dans les extraits sans cellules.

9. *Mycobacterium lepraemurium* décompose l'isocitrate en succinate et en glyoxylate par action de l'isocitrate lyase. Le glyoxylate ne se combine pas avec le malate, mais se combine avec l'α-cétoglutarate pour former du d-hydroxylaevulinate.

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