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Terminal Electron Transport System of *M. lepraemurium*[1]

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Gray ([7]) measured the oxygen consumption with various substrates by the Warburg manometric method in an attempt to clarify the carbon source utilized by *M. lepraemurium* but reported that except for blood serum, negative results were obtained with all the other substrates. It is known, however, that considerable endogenous respiration takes place in *M. lepraemurium* and substrate oxidation is believed to occur intracellularly. Permeability of the substrate through the cell membrane was considered as a possible limitation but extracts gave no oxygen consumption with added substrates. Under the assumption that this phenomenon was due to an absence of cytochromes, the absorption bands of cytochromes were examined spectroscopically and no cytochrome could be found ([14]). Kusaka and Sato ([12, 13]) have also conducted detailed investigation on cytochromes in *M. lepraemurium* and reported that cytochrome absorption bands could not be found.

It is believed that the presence or absence of cytochromes will greatly influence attempts to cultivate *M. lepraemurium*, hence the respiratory mechanisms, by which endogenous respiration takes place, are problems relevant to the cultivation of murine leprosy bacilli. Non-cytochrome oxidation pathways include oxidation by flavoproteins and by oxygenases. The presence of the malate-vitamin K_3 system was verified in relation to the former, but this system was inactive in the oxidation of other substrates. There are no reports on the relationship of oxygenases to the respiratory systems but oxygenase might be coupled to the terminal electron transport system in aerobic organisms not possessing cytochrome. The authors ([16]) have devised a collection method for *M. lepraemurium* by which almost all tissue contaminants are removed, thus permitting a study of the cytochromes in *M. lepraemurium*, which results in B_1 and A_2-types being found.

The present paper updates previous findings ([12, 13]) and reports on the cytochromes in *M. lepraemurium*.

MATERIALS AND METHODS

Collection method for *M. lepraemurium*. *M. lepraemurium* were collected from murine leprosy mouse leproma by Mori's method ([15]).

Preparation of ground extract, particles and supernatant. The frozen bacterial fraction was placed on two sheets of filter paper, dehydrated overnight in a low temperature room, weighted, placed in a mortar, an equal amount of quartz sand added, and ground well. For separation of particles, 0.25 M sucrose containing M/15 postassium and sodium phosphate buffer (pH 7.0) solution was added. The ground extract was centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant cell-free extract was ultracentrifuged at 40,000 rpm for 60 minutes. The resulting precipitate was used as the particulate fraction and the clear middle layer in the tube as the supernatant fraction. The supernatant fraction was collected by making a hole in the bottom of the tube and drawing off the clear portion. The fatty fraction floating on top was discarded.

Weighing of the extract. The bacterial extract was placed in a Visking cellophane tube, dialyzed well against distilled water, dried and accurately weighed.

Determination of oxygen uptake and carbon dioxide evolution. The conventional Warburg manometric method was used.

Determination of NADH-cytochrome C reductase activity. The supernatant of the ultra-centrifuged cell-free extract of *M. lepraemurium* was used as the enzyme solu-

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tion. NADH (reduced NAD) was supplied with the NAD (nicotinamide adenine dinucleotide), alcohol and alcohol dehydrogenase system. Horse heart cytochrome C was used for the electron acceptor and the reduction of cytochrome C measured at 550 mμ.

Determination of RQ (respiratory quotient) and QO_2(oxygen quotient). Conventional manometric methods were used.

Determination of the difference spectra of cytochromes and CO-binding cytochromes. The Cary Model 14 recording spectrophotometer was used. Particulate fractions were suspended in M/10, phosphate buffer (pH 7.0) containing 1% sodium desoxycholate. Sodium hydrosulfite was added to a suspension of the particles of *M. lepraemurium* for reduction and the difference spectra determined. Sodium hydrosulfite was added to both cuvettes to produce the reduced form. Carbon monoxide gas was passed through one cuvette and the difference spectra of CO-binding was measured.

Reduction and reoxidation of B_1 and A_2-type cytochromes of *M. lepraemurium*. The Shimadzu multipurpose autorecording spectrophotometer was used to determine the reduced form of B_1 and A_2-type cytochromes. Quartz cuvettes, 4 mm wide with a light path of 10 mm, were used under anaerobic conditions. Reduction of cytochrome by reduced vitamin K_3 (2-methy-1, 4-hydronaphthoquinone), 2-amino-4-hydroxy-6, 7-dimethyl-5,6,7,8-tetrahydropteridine, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine, NADH and NADPH (reduced NAD-phosphate) was carried out anaerobically. Reoxidation of reduced B_1 and A_2-type cytochromes was done by passage of air.

Irradiation with ultraviolet light. The particulate fraction was washed with M/10 sodium and potassium buffer pH 7.0 containing 0.25 M sucrose by ultracentrifugation. The washed particle, on a Petri dish in an ice bath, was irradiated with ultraviolet light (ACME Material Identifier, Shimadzu Seisakusho) for 30 minutes.

Reagents. Nicotinamide adenine dinucleotide (NAD) reduced nicotinamide adenine dinucleotide (NADH) reduced nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome C from horse heart and alcohol dehydrogenase from yeast were Sigma Chemical Co. products. Sodium malate and sodium desoxycholate were Ishizu Chemical Co. products. Vitamin K_3 (2-methyl-1, 4-naphthoquinone) and potassium cyanide were Katayama Chemical Co. products. Sodium azide and sodium hydrosulfite were products of the Nakarai Chemical Co. 2-amino-4-hydroxy-6, 7-dimethyl-5,6,7,8-tetrahydropteridine and 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine were kindly supplied by Professor Akino.

RESULTS

Oxidation of NADH. Segal and Bloch ([18]) reported that tubercle bacilli grown *in vivo* differ from *in vitro* grown organisms in that the respiration is analogous to *M. lepraemurium*. Hart and Valentine ([8]) reported that *M. lepraemurium* grown on a specially devised medium for 2-3 weeks become elongated without multiplication. We have investigated the NADH oxidizing activity of particles from elongated *M. lepraemurium* incubated on the artificial medium for three weeks. The results are shown in Figure 1 and Table 1 shows the

TABLE 1. *Elongation of* M. lepraemurium *(Osaka No. 1) in Hart-Valentine medium.*

Length of bacillus in μ	Initial population % with indicated length	After three weeks % with indicated length
0.5	2	2
1.0	37	27
1.2	13	3
1.5	27	20
2.0	12	22
2.2	3	1
2.5	4	4
3.0	1	5
3.5	1	2
4.0	0	5
5.0	0	4
5.5	0	1
6.0	0	3
7.0	0	1
Total enumerated	100	100

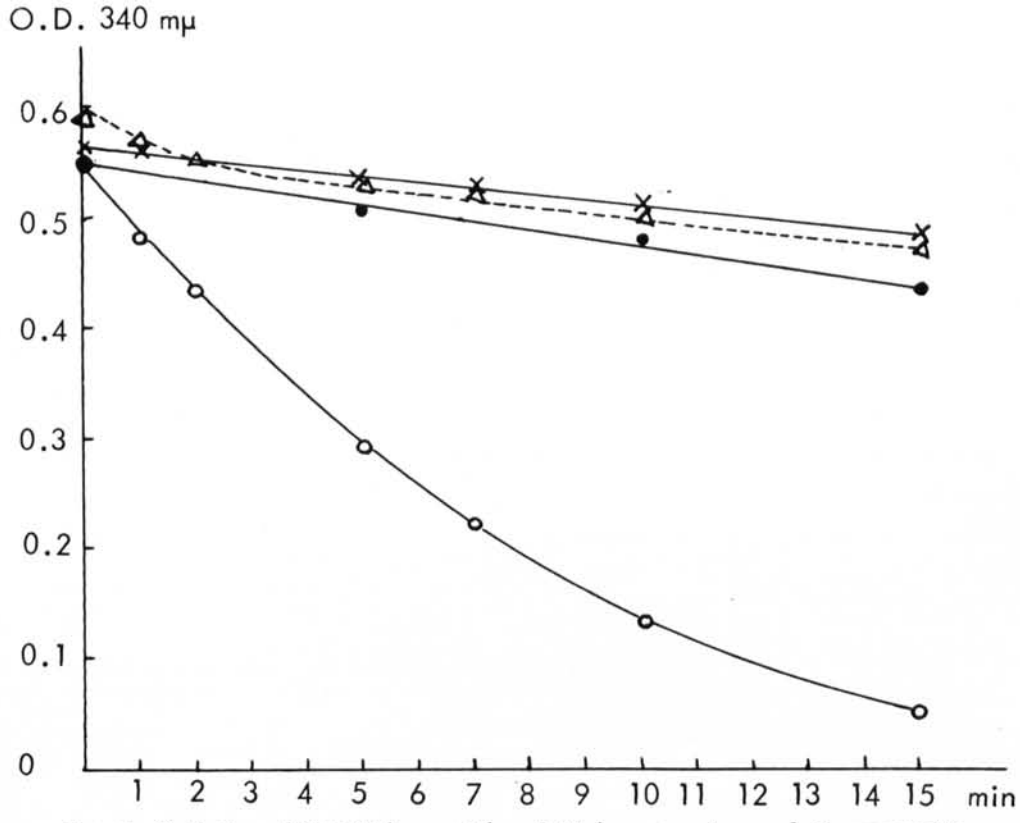

FIG. 1. Oxidation of NADH by particles of *M. lepraemurium* and *M. smegmatis*.

•-• 0.025 ml phosphate buffer, 2.5 ml NADH cytochrome reductase, 1 ml NADH 1.3 x 10^{-7}mols.

Δ-Δ 0.025 ml particle suspension (dry weight 5.2 mg/ml; protein 0.88 mg/ml) from *M. lepraemurium* incubated in Hart-Valentine medium for one week with same additives as above.

x-x 0.025 ml particle suspension (dry weight 4.6 mg/ml; protein 0.78 mg/ml) from *M. lepraemurium* with same additives as above.

o-o 0.025 ml particle suspension (dry weight 2.5 mg/ml; protein 0.48 mg/ml) from *M smegmatis* with same additives as above.

NADH-cytochrome reductase (protein contents 0.12 mg/ml) was prepared from particle free supernatant of *M. smegmatis* as the fraction between one-third and two-thirds by ammonium fractionation. NADH-cytochrome reductase and bacillary particle were diluted with M/20 phosphate buffer, pH 7.0.

average length of 100 organisms selected at random. As shown in Figure 1, NADH is promptly oxidized by the particles of *M. smegmatis* but this NADH oxidizing activity was almost nil with the particle fraction of *M. lepraemurium* (Osaka No. 1 strain) which had become elongated on incubation for three weeks. The findings suggest that no adaptive formation of NADH oxidizing enzyme system is seen in aerobic incubation of *M. lepraemurium*.

Malate-vitamin K_3 reductase. Asano and Brodie [1] have reported that in *M. phlei*, malate is oxidized by flavin enzyme via vitamin K. Kusaka and Sato [12, 13] reported that no cytochrome was detected in *M. lepraemurium*. If, however, the terminal respiration in *M. lepraemurium* takes

place by the activity of flavin enzyme, the reason for the oxygen consumption by endogenous respiration could be explained. As shown in Figure 2, malate is oxidized via vitamin K_3, but citrate, succinate and α-ketoglutarate were not oxidized by this system as may be seen in Figure 3. Malate-vitamin K_3 reductase is a specific enzyme for malate, and oxidation of other substrates by this terminal electron transport system is not possible.

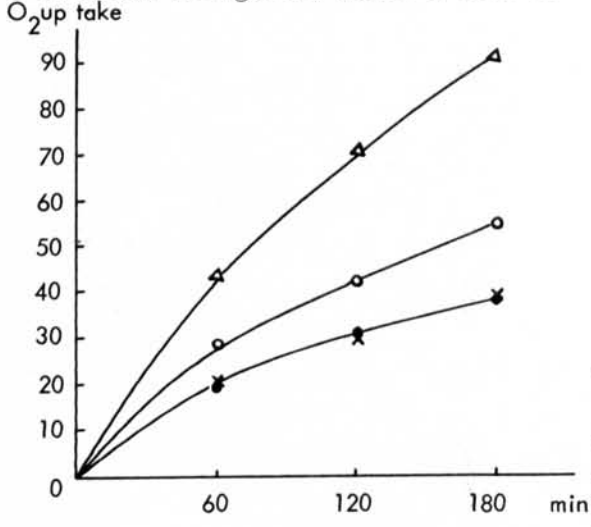

FIG. 2. Oxidation of malate with malate-vitamin K_3 reductase by *M. lepraemurium*.

•-• Control: main chamber, 1 ml of cell-free extract with added NAD 10^{-6}mols, 1 ml M/10 phosphate buffer, pH 7.4; side arm, 0.5 ml distilled water.

x-x Vitamin K_3: main chamber, 1 ml of cell-free extract with added NAD 10^{-6}mols, 1 ml M/10 p.b. pH 7.4 saturated with vitamine K_3; side arm, 0.5 ml distilled water.

o-o Malate: main chamber, same as the above control; side arm, 0.5 ml of malate 10^{-6} mols.

Δ-Δ Malate and vitamin K_3: main chamber, same as above vitamin K_3; side arm, 0.5 ml of malate 10^{-6}mols.

0.5 gm of lyophilized *M. lepraemurium* were ground and extracted with 5 ml of M/20 phosphate buffer, pH 7.4. The supernatant of 9,000 rpm centrifugation was used as enzyme. Protein content of enzyme 1 ml was 7.3 mg by the phenol method.

NADH-cytochrome C reductase. Chino [4] investigated the cytochrome system in the Bombyx silkworm egg at one day of incubation and isolated cytochrome B_5 which has an affinity for lipids. He and Isono [9] reported that the presence of 3-hydroxykynurenine is required for NADH-cytochrome C reductase activity of the supernatant fraction and in the absence of 3-hydroxykynurenine, oxidation of NADH does not take place. It was believed that some unknown substances, which play a role in electron transport, may be present in *M. lepraemurium*. Table 2 shows the reduction of cytochrome C of horse heart muscle by NADH. Though cytochrome C is not found in *M. lepraemurium*, the cell-free extract of *M. lepraemurium* shows NADH-cytochrome C reductase activity.

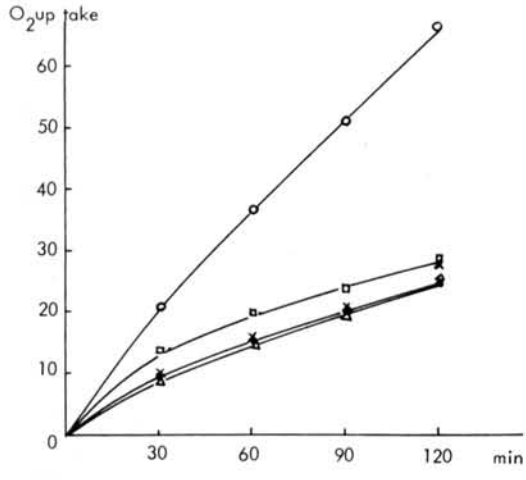

FIG. 3. Terminal respiration of *M. lepraemurium* via vitamin K_3.

• Vitamin K_3
Δ Vitamin K_3, α-ketoglutarate
x Vitamin K_3, succinate
□ Vitamin K_3, citrate
o Vitamin K_3, malate

The manometric procedure was the same as in experiments of Figure 2, except for the addition of vitamin K_3 to ground extract. 0.5 gm of lyophylized *M. lepraemurium* were ground with 7 mg of vitamin K_3, and extracted with 8 ml of M/20 phosphate buffer, pH 7.4. The supernatant of 9,000 rpm centrifugation was used as enzyme. Protein content of enzyme 1 ml was 4.7 mg. All substrates concentration were 5 x 10^{-6}mols.

QO_2 and RQ of the endogenous respiration of *M. lepraemurium*. QO_2 (quantity of consumed oxygen μl/mg, hr) and RQ (respiratory quotient) of *M. lepraemurium*

TABLE 2. *NADH-cytochrome C reductase activity of* M. lepraemurium.

		Experimental	Control
particle free extract		2.5 ml	2.5 ml
cytochrome C 1.5 mg[a]		0.3 ml	0.3 ml
ethyl alcohol		0.1 ml	0.1 ml
NAD 3×10^{-7} mols		0.1 ml	0.1 ml
alcohol dehydrogenase[b]		0.02 ml	—
Reduced cytochrome C	O.D. at 550 mμ	0.33	0
	quantity /mg	0.75	

[a] Crystalline cytochrome C from horse heart 5 mg/ml.
[b] Crystalline alcohol dehydrogenase from yeast 0.5 mg/ml.

1.5 gm wet weight of *M. lepraemurium* were ground with glass powder and extracted with 30 ml of M/100, pH 7.0 phosphate buffer added as 0.25 M sucrose. The supernatant of 40,000 rpm centrifugation was dialyzed overnight against phosphate buffer, pH 7.0. The clear solution was used as enzyme. The enzyme solution contained 4.04 mg/ml protein by the phenol method. Incubation was carried out at 37 °C for 30 minutes.

TABLE 3. *Respiratory quotient and QO_2 of* M. lepraemurium *and Jucho bacillus.*

	M. lepraemurium[a]	Jucho bacillus[b]
$RQ = CO_2/O_2$	0.87	0.91
$QO_2 = O_2\mu l$/mg hr	4 μl	20 μl

[a] Dry weight, 30 mg of *M. lepraemurium.*
[b] Dry weight, 8.3 mg of Jucho bacilli.

were compared to that of *M. smegmatis*, Jucho strain. As noted in Table 3, the QO_2 of *M. lepraemurium* is smaller than that of *M. smegmatis*, however, the RQ of *M. smegmatis*, which can be cultivated and possesses cytochromes, is 0.91 and close to the RQ of 0.87 of *M. lepraemurium*. If amino acids are oxidized by these bacilli, the electron transport should proceed smoothly in *M. lepraemurium* in the same way as in organisms which possess cytochromes.

Cytochromes of *M. lepraemurium*. Kusaka, Sato *et al* [12, 13] presented a detailed report indicating that cytochromes are not detected in *M. lepraemurium*. Therefore, these studies were made under

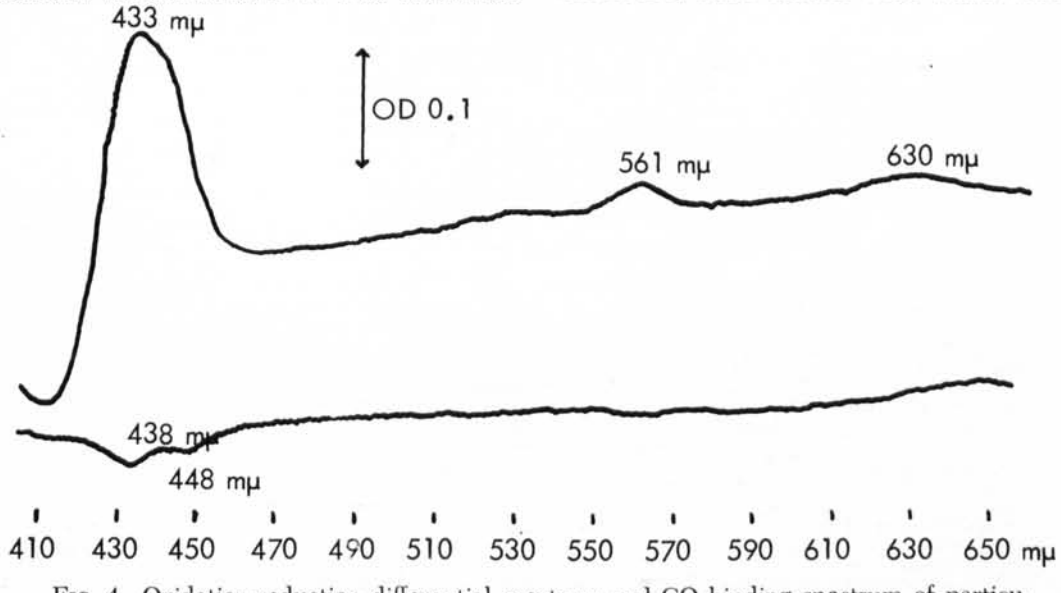

FIG. 4. Oxidation-reduction differential spectrum and CO binding spectrum of particulate fraction of *M. lepraemurium*.

Particulate fraction (dry weight 5.3 mg/ml, protein 0.901 mg/ml) was suspended in M/10 phosphate buffer, pH 7.0, containing 1% sodium desoxycholate. Cary Model 14 auto-recording spectrophotometer was used.

the assumption that this organism lacked cytochromes. The presence of non-heme iron protein with an electron spin resonance signal at $gi = 1.94$ ([2]) in the particles of *M. lepraemurium* was examined but was extremely weak. Since there is a possibility that the cytochromes of *M. lepraemurium* were separated in the reduced state, ferricyanide was added for oxidation, and hydrosulfide added for reduction and the difference spectra examined. Two cytochromes with absorption at 561 mμ and 630 mμ, respectively, were found. As shown in Figure 4, there was no need to add ferricyanide since the cytochromes of the particle fraction of *M. lepraemurium* are in the oxidized form and can be readily reduced with hydrosulfite. A B_1-type cytochrome with an absorption peak at 561 mμ and Soret peak at 433 mμ and an A_2-type cytochrome with an absorption peak at 630 mμ and Soret shoulder at about 440 mμ were detected. The CO-difference spectrum showed a peak at 438 mμ and a trough at 448 mμ. The peak and trough may be due to a CO-binding A_2-type cytochrome ([3]).

Reduction of B_1 and A_2-type cytochromes by reducing vitamin K_3. The presence of a terminal respiration system by malate-vitamin K_3 reductase in *M. lepraemurium* has already been described. In *in vitro* experiments, oxygen uptake by the auto-oxidation of reduced vitamin K_3 at pH 7.4 was observed but it is believed that under physiological conditions this is related to oxygen by way of B_1 and A_2-type cytochromes. Figure 5 shows the reduction of B_1 and A_2-type cytochromes by the reduced vitamin K_3.

Reduction of B_1 and A_2-type cytochromes by tetrahydropteridine and reoxidation. Acid-fast bacilli contain large amounts of pteridine compounds ([11]), and under the assumption that this was related to the respiratory system, B_1 and A_2-type cytochromes were reduced with 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8,-tetrahydro-

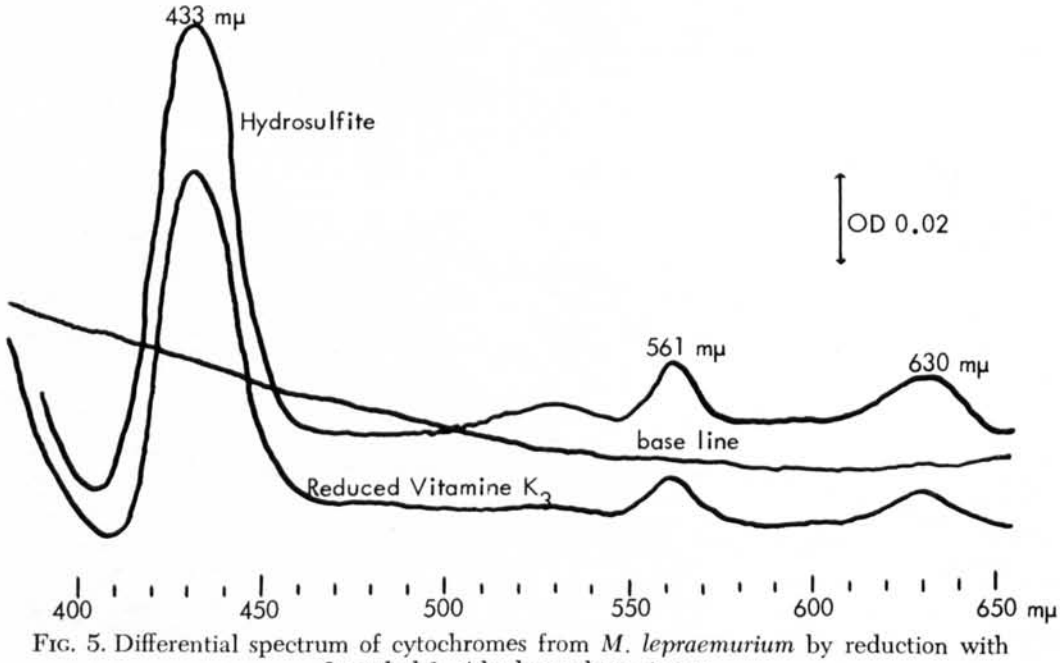

FIG. 5. Differential spectrum of cytochromes from *M. lepraemurium* by reduction with 2-methyl-1, 4-hydronaphtoquinone.

Particulate fraction (dry weight 12.2 mg/ml, protein 2.07 mg/ml) was suspended in M/10 phosphate buffer, pH 7.0. A 1.25 ml sample of particle suspension was placed in 4 mm wide photometer cells. Powdered reduced vitamin K_3 (2-methyl-1, 4-hydronaphtoquinone) 10^{-5}mols was added to the side tube of the vacuum photometer cell.

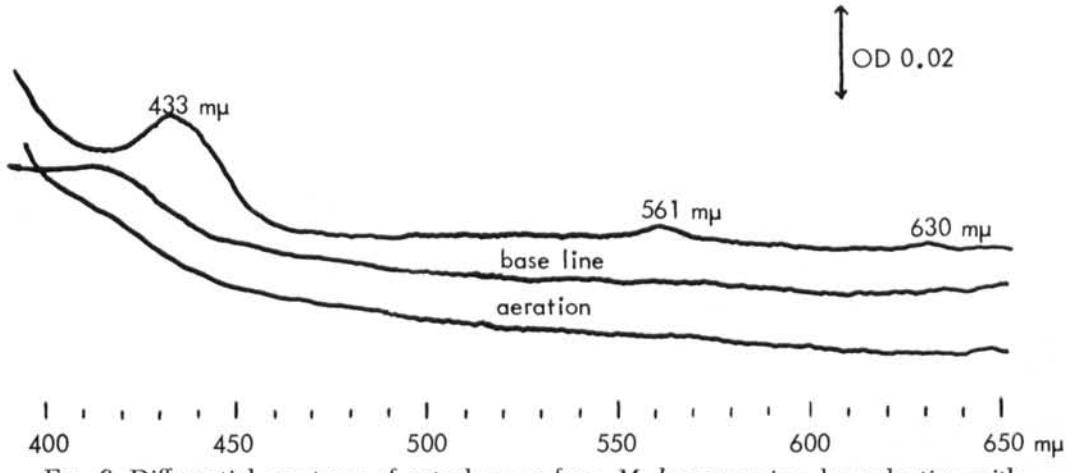

FIG. 6. Differential spectrum of cytochromes from *M. lepraemurium* by reduction with 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

Particulate fraction (dry weight 5.3 mg/ml, protein 0.90 mg/ml) was suspended in the supernatant (dry weight 2.3 mg/ml, protein 0.50 mg/ml). A 1.25 ml sample of particle suspension was placed in a 4 mm wide photometer cell. Powdered 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine, 10^{-5}mols, was added to side tube of vacuum photometer cell.

pteridine and 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine and then reoxidized by aeration. The findings are shown in Figures 6 and 7.

Reduction of B_1 and A_2-type cytochromes by NADH and NADPH. Since the presence of B_1 and A_2-type cytochromes became apparent, several attempts were made to re-reduce the B_1 and A_2-type cytochromes with NADH and NADPH but these were unsuccessful at first. This was due to the use of *M. lepraemurium,* which had been preserved by lyophilization as noted in Figure 14. In the present study, organisms

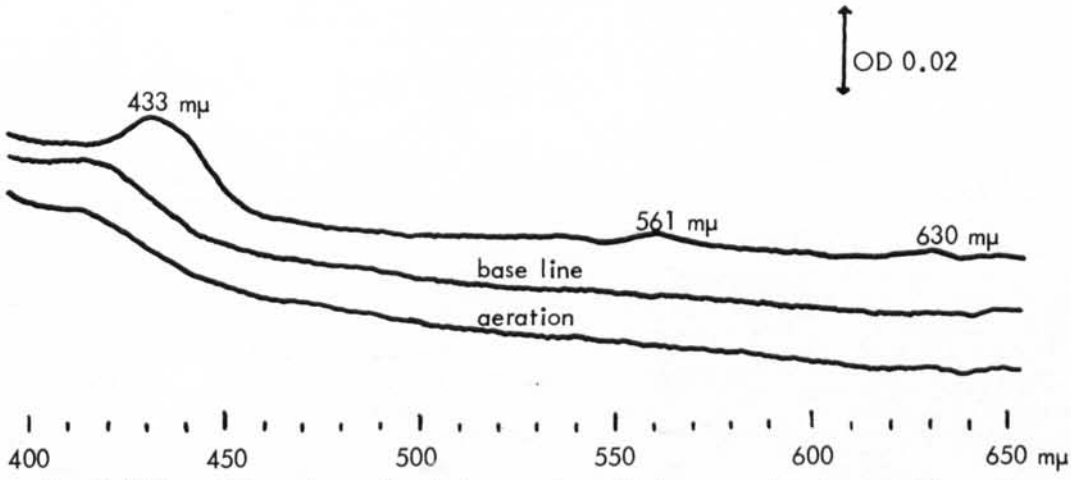

FIG. 7. Differential spectrum of cytochromes from *M. lepraemurium* by reduction with 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine.

Experimental conditions were the same as for Figure 6 except for the use of 2-amino-4-hydroxy-6-methyl tetrahydropteridine 10^{-5}mols.

collected on the previous day were ground and extracted, the particulate fraction separated and reduction of B_1 and A_2-type cytochromes with NADH and NADPH was attempted, using the supernatant as NADH-cytochrome reductase as a precaution against the lack of enzyme activity in particulate fraction. Figures 8 and 9 show the reduction spectra.

Inhibition of B_1 and A_2-type cytochromes reduction by potassium cyanide and sodium azide and reoxidation. The B_1 and A_2-type cytochromes were reduced with 2-amino-4-hydroxy-dimethyl-tetrahydropteridine. As noted in Figures 10 and 11, reduction of B_1-type cytochrome is not inhibited by KCN and NaN_3 and reduction of A_2-type cytochrome is slightly inhibited by KCN, the peak of the reduced B_1-type cytochrome promptly dissappears on aeration. This indicates that B_1-type cytochrome may be auto-oxidative.

Action of pteridine compound in the electron transport system of *M. lepraemurium*. Detailed analyses with the purified enzyme are necessary in order to verify that a pteridine carrier functions between NADH and the cytochrome chain. However since it is very difficult to obtain sufficient bacillary particles, we used an indirect method to study the pteridine interaction with the terminal respiration chain of *M. lepraemurium*. Since 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (PtH_4) is a strong reducing agent, the cytochromes of *M. lepraemurium* may be reduced chemically by PtH_4 similarly to hydrosulfite. Hydrosulfite rapidly reduces cytochromes, but PtH_4 takes time to reduce them, as may be seen in Figure 12. Using particles prepared from lyophilized *M. lepraemurium* preserved in a refrigerator more than six months, hydrosulfite can reduce the B_1 type cytochrome but PtH_4 and NADH cannot (Figures 13 and 14). These findings suggest that the reduction of cytochromes with PtH_4 may depend on some enzyme action. As the pteridine compound was decomposed by ultraviolet light irradiation, we used a particle suspension irradiated with ultraviolet light. As seen in Figures 15 and 16, the cytochromes were

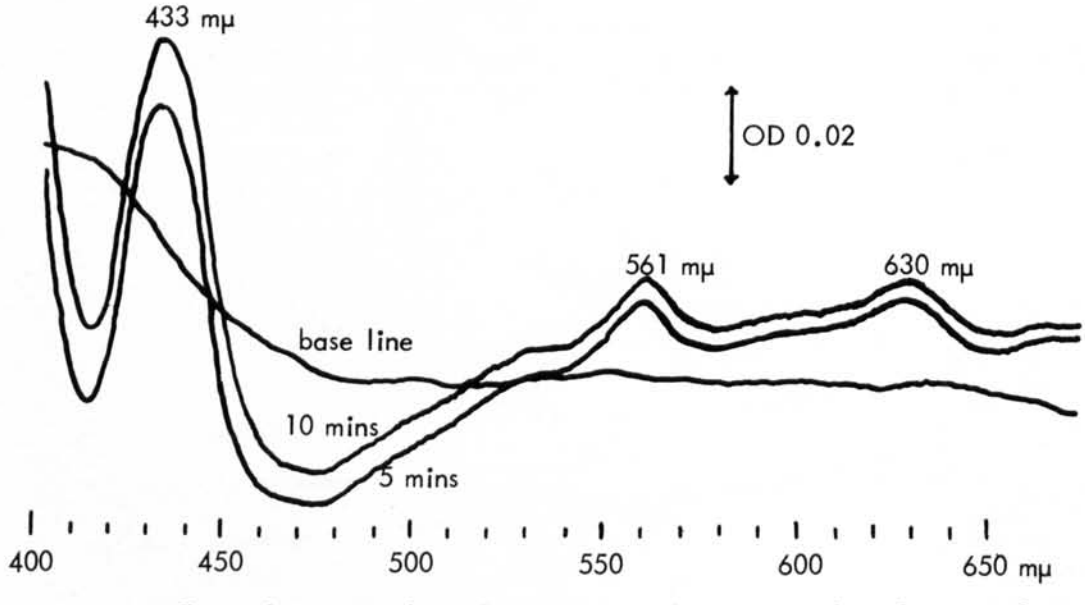

FIG. 8. Differential spectrum of cytochromes from *M. lepraemurium* by reduction with NADH.

Particulate fraction (dry weight 11.8 mg/ml, protein 2.0 mg/ml) was suspended in the supernatant (dry weight 5.5 mg/ml, protein 1.2 mg/ml). A 1.25 ml sample of particle suspension was placed in a 4 mm wide photometer cell. Powdered NADH, 10^{-5}mols, was added to side tube of vacuum photometer cell.

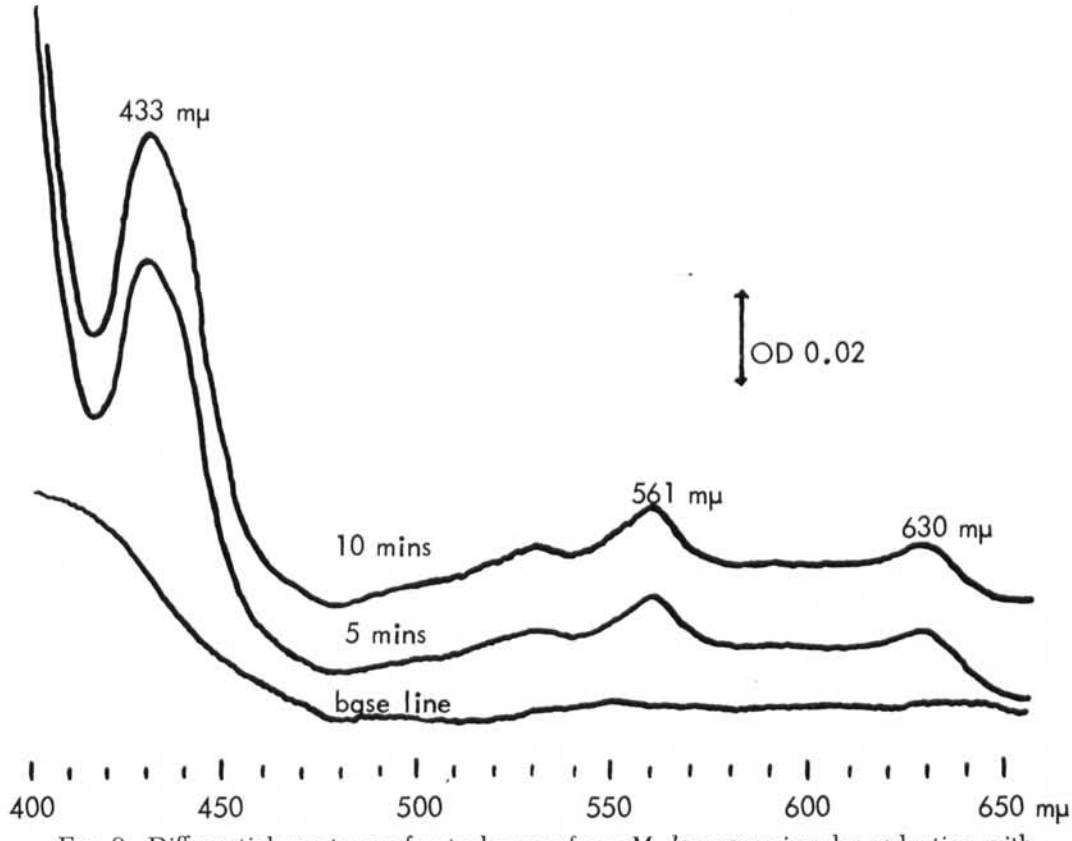

FIG. 9. Differential spectrum of cytochromes from *M. lepraemurium* by reduction with NADPH.

Experimental conditions were the same as for Figure 8 except for the use of NADPH, 10^{-5}mols.

not reduced with NADH but were reduced with PtH_4. This finding suggests that a B_1-type cytochrome may be reduced via reduced pteridine compound and NADH cannot reduce directly the B_1-type component. We attempted reconstitution of the reducing system by the addition of a small amount of PtH_4, but little effect of PtH_4 was observed and the results were not clear (Figure 16).

DISCUSSION

Kusaka and Sato ([12, 13]) reported failure to detect any cytochromes in *M. lepraemurium* supplied by the authors. However, the isolation of *M. lepraemurium* in this report differs from Kusaka's method. We could not detect cytochromes of *M. lepraemurium* in the previous report ([14]) because of insufficient organisms. As the isolation method of *M. lepraemurium* from leproma was improved to remove host tissue components ([16]), contamination with host tissue cytochromes was negligible.

In the study of the enzyme system of the TCA cycle in *M. lepraemurium* ([15]), phenazine methosulfate was added as the auto-oxidative dye in the terminal electron transport system at the step of dehydrogenase reaction. This dye acts as an electron acceptor of succinate dehydrogenase and also spontaneously oxidizes NADH and NADPH. The B_1 and A_2-type cytochromes are present in *M. lepraemurium* as the terminal electron transport system, but the addition of artificial dye was indispensable to complete the oxidation of substrates. On the other hand, the cytochromes can be reduced with NADH in particulate fractions and reoxidized by aeration. This is paradoxical. Tetrahydropteridine compounds may possibly function between

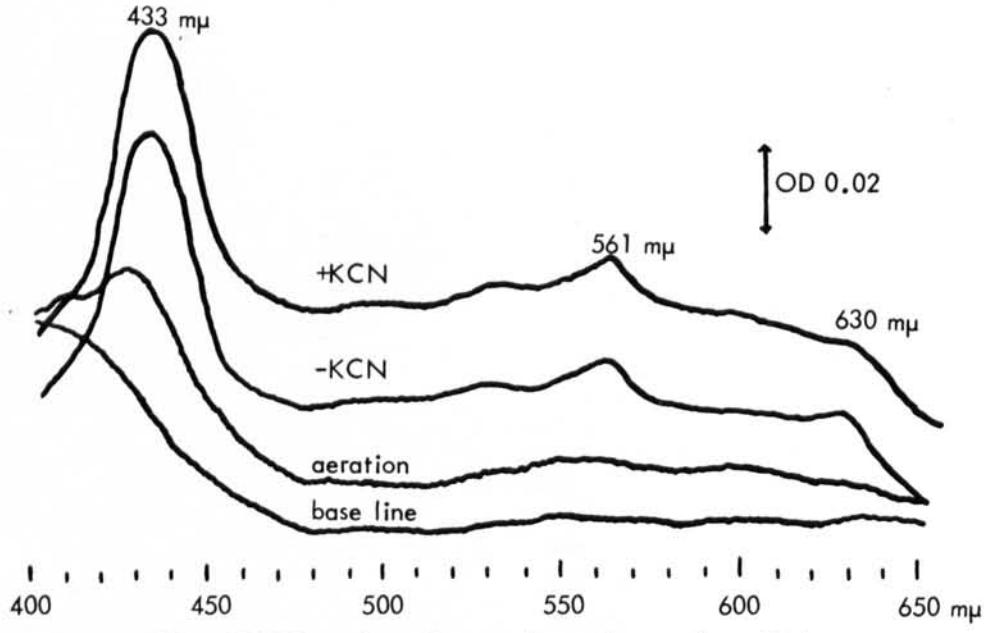

FIG. 10. Effect of KCN on the reduction of cytochromes from *M. lepraemurium*.

Particulate fraction (dry weight 11.8 mg/ml, protein 2.0 mg/ml) was suspended in the supernatant (dry weight 5.5 mg/ml, protein 1.2 mg/ml). A 1.0 ml sample of particle suspension was placed in 4 mm wide photometer cells. Powdered 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine 10^{-5}mols were added to the side tube of the vacuum photometer cell, and the cytochromes were reduced with it. KCN solution (0.25 ml) was added to the particle suspension to yield a 10^{-3} final concentration.

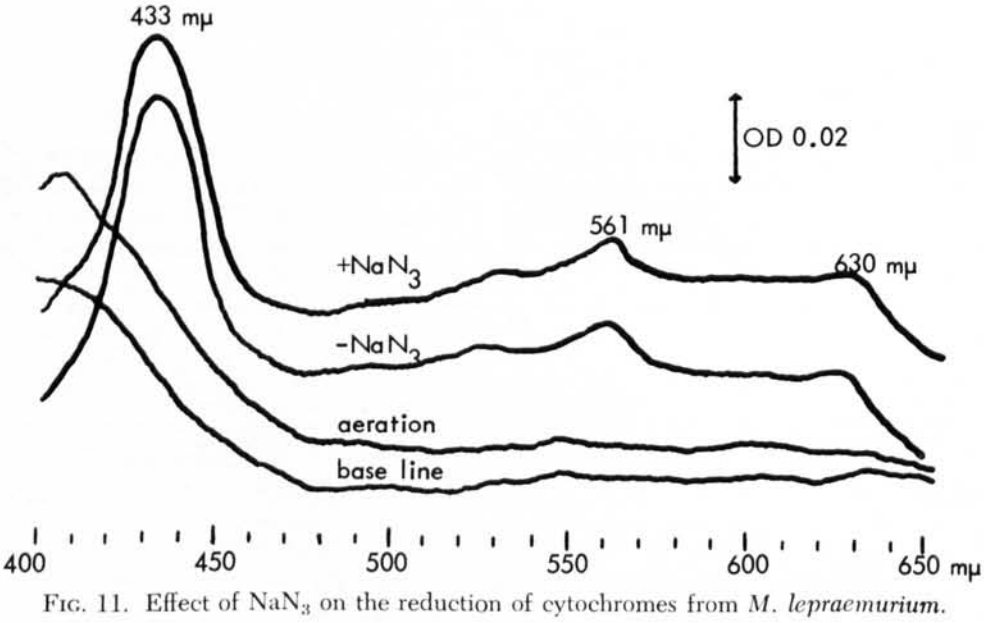

FIG. 11. Effect of NaN_3 on the reduction of cytochromes from *M. lepraemurium*.

Experimental conditions were the same as for Figure 10 except for the addition of NaN_3 in place of KCN.

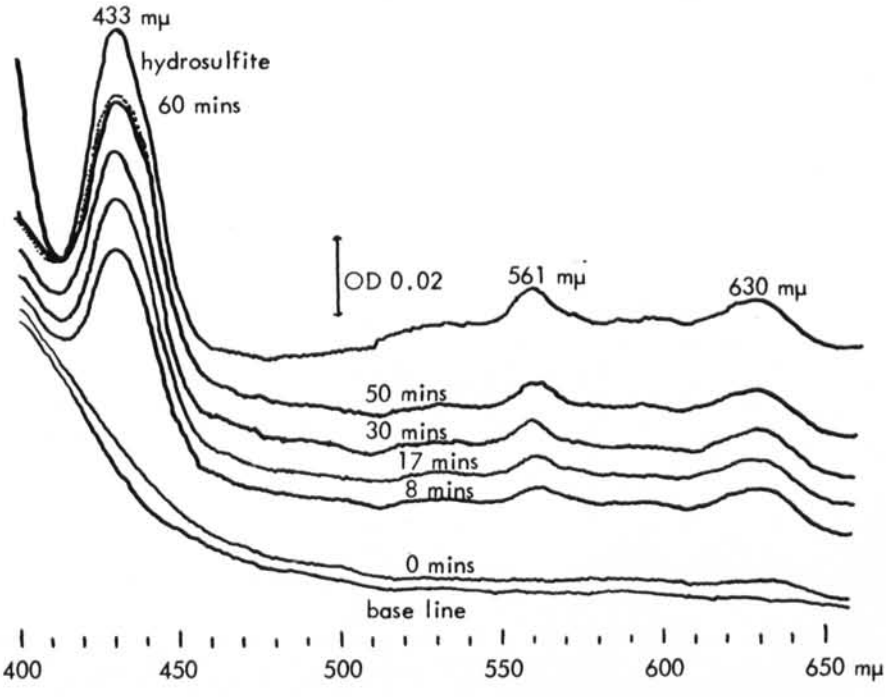

FIG. 12. Time course of the reduction of cytochromes with 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8,-tetrahydropteridine.

Particulate fraction (dry weight 13.2 mg/ml, protein 2.24 mg/ml) was suspended in 0.25 M sucrose, M/10 phosphate buffer, pH 7.0. Reduction of cytochromes with dimethyl tetrahydropteridine was done by the same method as for Figure 6.

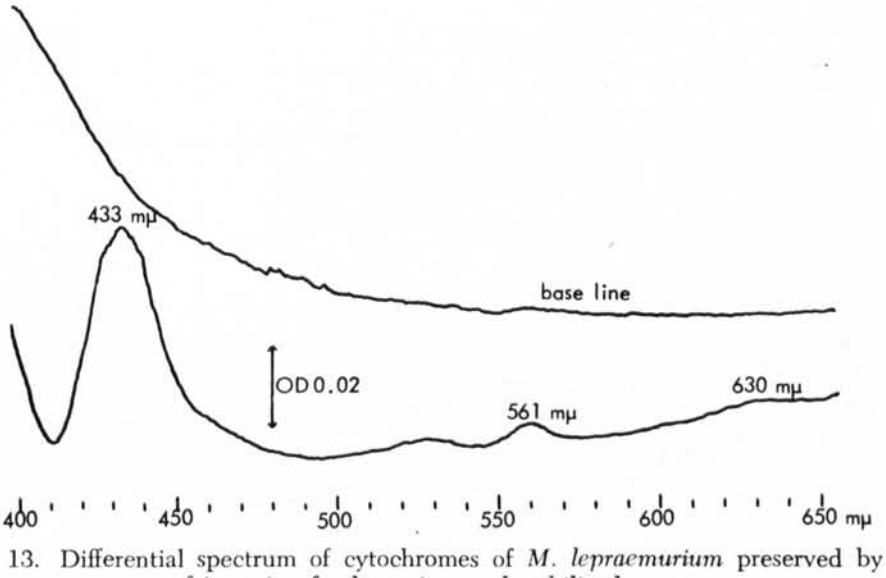

FIG. 13. Differential spectrum of cytochromes of *M. lepraemurium* preserved by refrigeration for long time as lyophilized state.

Particulate fraction was washed with 0.25 M sucrose phosphate buffer, pH 7.0, by ultracentrifugation. Particles (dry weight 13.1 mg/ml, protein 2.20 mg/ml) were suspended in 0.25 M sucrose M/10 phosphate buffer, pH 7.0. The particle suspension (3.5 ml) was placed in a 10 mm wide cuvette. A small amount of powdered hydrosulfite was added to one particle suspension and the cytochromes were reduced.

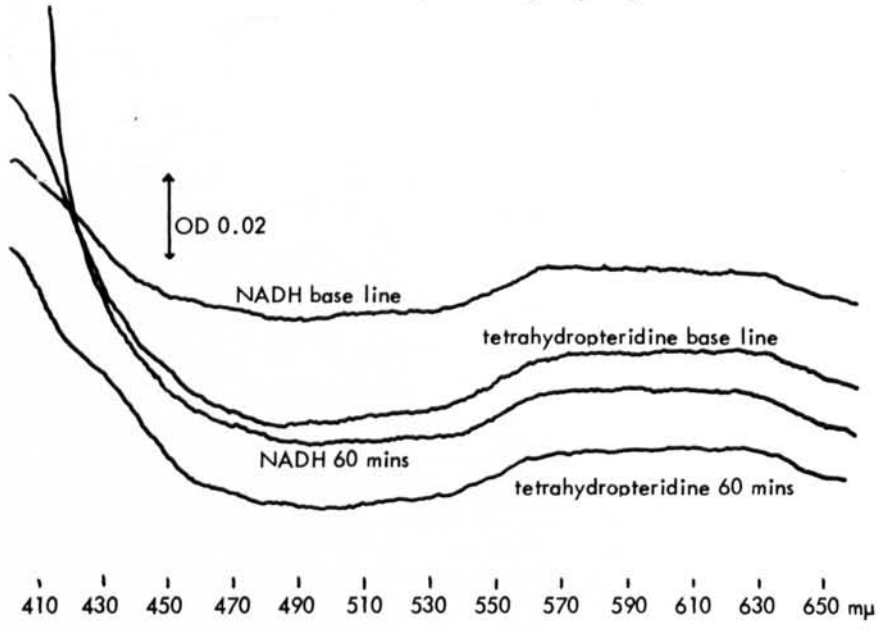

FIG. 14. Reduction of cytochromes of *M. lepraemurium* preserved by refrigeration for long time as lyophilized state.

Particulate fraction was same as for Figure 13. Reduction of cytochromes with NADH and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine was carried out by the same method as for Figures 6 and 8.

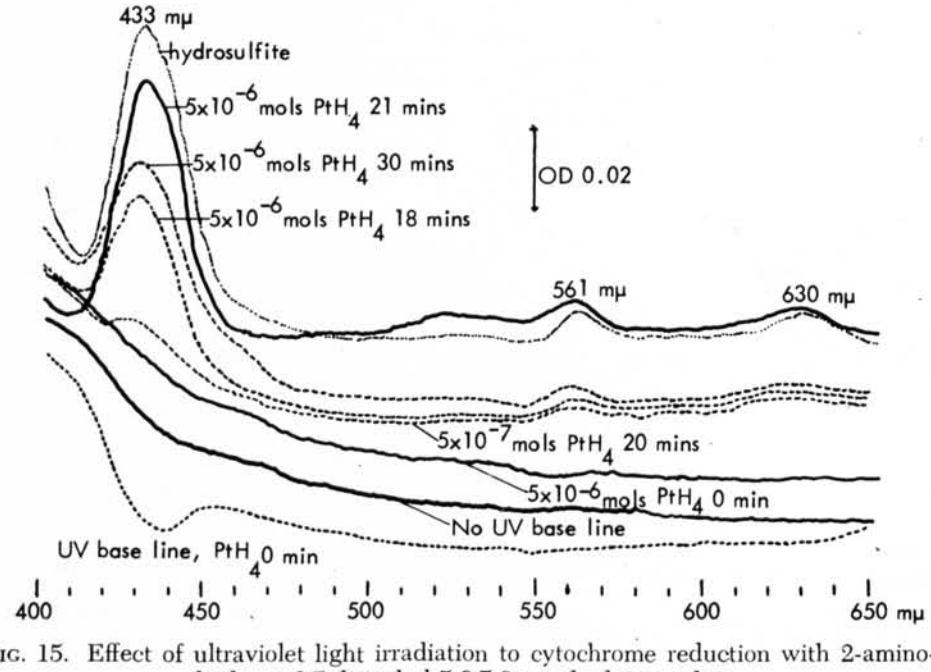

FIG. 15. Effect of ultraviolet light irradiation to cytochrome reduction with 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

Particle fraction was prepared from fresh wet *M. lepraemurium* by the same method as for Figure 13. The particulate fraction (dry weight 3.8 mg/ml, protein 0.66 mg/ml) was suspended in 0.25 M sucrose, M/10 phosphate buffer, pH 7.0. Reduction of cytochromes with 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine was done by the same method as for Figure 6.

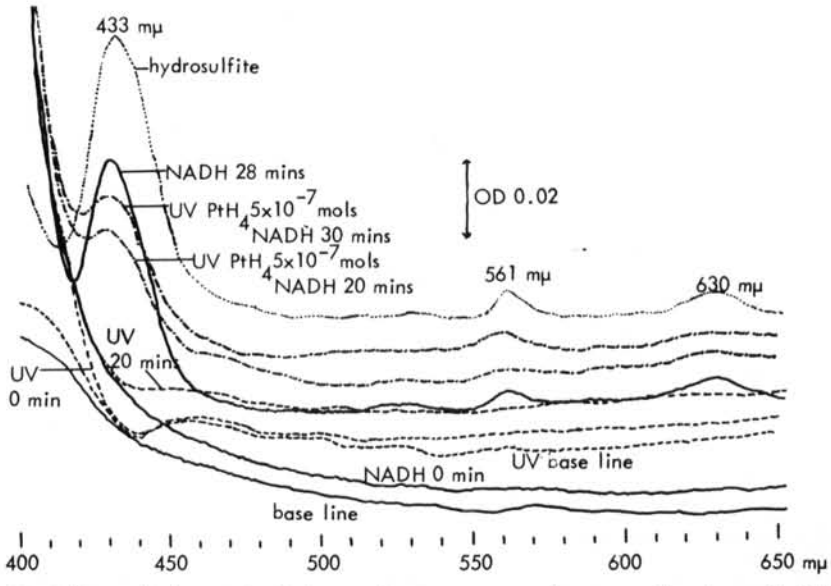

FIG. 16. Effect of ultraviolet light irradiation on cytochrome reduction with NADH.

All experimental conditions were the same as for Figure 15 except for the use of NADH.

NADH and the cytochromes and are unstable in oxygen and easily inactivated like the phenylalanine hydroxylase cofactor of Kaufman ([10]). However, if cytochromes of particle fractions collected under aerobic conditions can be reduced with NADH, then the unknown pteridine compounds are probably active. If we accept the hypothesis that tetrahydropteridinecytochrome reductase might be inhibited by oxygen, we can answer the paradox and explain the difficulty of cultivating *M. lepraemurium*. Presumably, cytochrome enzyme systems are active in the whole cell and endogenous respiration can not be explained by the above hypothesis. These conflicting facts may be elucidated by the degradation of cytochrome enzyme systems due to disintegration of the whole cell enzyme system on breaking up of the cell. Oxygen consumption corresponding to the added substrates was not seen in whole cells by manometric methods despite a fairly constant amount of endogenous respiration. This phenomenon may be due to substrate impermeability. In a subsequent report it will be noted that *in vivo* grown *M. lepraemurium* probably do not have transport mechanisms necessary to some substrates when grown *in vivo*.

Though the detailed properties of the B_1-type cytochromes of *M. lepraemurium* must await purification, they may resemble those of cultivable *Proteus vulgaris* ([19]), *E. coli* ([5, 6]) and Diphtheria bacillus ([17]) as judged from the position of the absorption peaks.

NADH-cytochrome C reductase activity is detected in cell-free extracts of *M. lepraemurium*, though cytochrome C is not found in it. This may suggest that the electron of NADH can be transferred to an electron acceptor which possesses an oxidation-reduction potential identical to that of NADH-triphenylterazolium chloride reductase.

SUMMARY

Cytochromes of the B_1 and A_2-types are present in the terminal electron transport system of *Mycobacterium lepraemurium*. A Soret zone absorption peak at 433 mμ and α Zone at 561 mμ is shown by the B_1-type cytochrome while the A_2-type cytochrome has a Soret shoulder at 440 mμ and absorption peak at 630 mμ. In the co-difference spectrum, A_2-type cytochrome is observed as a trough at 448 mμ and a peak at 438 mμ. Reduction of A_2-type cytochrome is somewhat inhibited by potassium cyanide but

not by sodium azide. Reduction of B_1-type cytochrome is not inhibited by either potassium cyanide or sodium azide. Both B_1 and A_2-type cytochromes are auto-oxidative and linked to oxygen. Cytochromes of the B_1 and A_2-types are reduced by reduced vitamin K_3 (2-methyl-1-1,4-hydronaphthoquinone), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (PtH_4) and are reoxidized by aeration. The QO_2 of the endogenous respiration of *M. lepraemurium* is 4 μl and the respiratory quotient is 0.87. Cytochrome C is not found in *M. lepraemurium* but NADH-cytochrome C reductase activity is present.

Comparative examination of the oxidation of NADH by the particulate fractions of *M. lepraemurium* and *M. smegmatis* reveals that the former have a much lesser activity. Even *M. lepraemurium*, elongated by incubation on Hart-Valentine medium for three weeks under aerobic conditions, shows no speedy oxidative activity on NADH.

Using the particles prepared from lyophilized *M. lepraemurium* preserved in a refrigerator more than 6 months, hydrosulfite can reduce the B_1-type cytochrome, but PtH_4 and NADH can not. When a particle suspension irradiated with ultraviolet light is used, the cytochromes are not reduced with NADH but are reduced with PtH_4. These findings suggest that pteridine compounds probably interpose between NADH and the cytochromes.

RESUMEN

En el sistema terminal de transporte de electrones del *Mycobacterium lepraemurium* se encuentran citocromos de los tipos B_1 y A_2. El citocromo tipo B_1 muestra un pico de absorción en la zona de Soret a 433 mμ y una zona alfa a 561 mμ, mientras que el citocromo tipo A_2 tiene una pequeña inflección de Soret a 440 mμ y un pico de absorción a 630 mμ. En el espectro diferencial C), se observa un citocromo tipo a que aparece como una depresión a 448 mμ y un pico a 438 mμ. La reducción del citocromo tipo A_2 se inhibe ligeramente por el cianuro de potasio pero no por la azida de sodio. La reducción del citocromo tipo B_1 no es inhibida ni por el cianuro de potasio ni por la azida de sodio. Tanto el citocromo tipo B_1 como el tipo A_2 son auto-oxidantes y están ligados a oxígeno. Los citocromos de tipo B_1 y A_2 son reducidos por Vitamina K reducida (2-metil-1,4-hidronaftoquinona), nicotinamida adenina nucleótido di (NADH) y trifosfato reducido, 2-amino-4-hidroxi-6-metil-5,6,7,8-tetrahidropteridina y 2-amino-4-hidroxi-6,7-dimetil-5,6,7,8-tetrahidropteridina (PtH_4) y son re-oxidados por aireación. El QO_2 de la respiración endógena del *M. lepraemurium* es 4 μl y el cuociente respiratorio es 0,87. El *M. lepraemurium* no contiene citocromo C pero si se observa actividad NADH-citocromo C reductasa.

Un examen comparativo de la oxidación de NADH por las fracciones paraticuladas de *M. lepraemurium* y *M. smegmatis* revela que el primero tiene mucho menos actividad. Aún el *M. lepraemurium* elongado por inucubación en el medio de Hart-Valentine durante tres semanas bajo condiciones aeróbicas no muestra actividad oxidativa rápida sobre NADH.

Utilizando partículas obtenidas de *M. lepraemurium* liofilizado y mantenido en refrigerador durante un largo tiempo, el hidrosulfito puede reducir el citocromo tipo B_1, pero el PtH_4 y la NADH no lo hacen. Cuando se utiliza una suspensión de partículas irradiadas con luz ultravioleta, los citrocromos no son reducidos con NADH pero si se reducen con PtH_4. Estos hallazgos sugieren que los compuestos de pteridina probablemente se interponen entre NADH y los citocromos.

RÉSUMÉ

Des cytochromes du type B_1 et du type A_2 existent dans le système de transport terminal de *Mycobacterium leprae*. Le cytochrome de type B_1 présente un pic dans la zone d'absorption de Soret a 433 mμ et dans la zone alpha à 561mμ: par contre, le cytochrome de type A_2 présente une élévation plus aplatie à 440 mμ dans la zone de Soret, et un pic d'absorption à 630 mμ. Dans le spectre de différence C), le cytochrome de type a est observé sous forme d'un passage sans élevation à 448 mμ (tough) et d'un pic à 438 mμ. La réduction du cytochrome de type A_2 est dans une certaine mesure inhibé par le cyanure de potassium mais non par l'azide de sodium. La réduction du cytochrome de type B_1 n'est inhibée ni par le cyanure de potassium ni par l'azide de sodium. Les cytochromes du type B_1 et du type A_2 sont l'un et l'autre auto-oxydants et liés à l'oxygène. Ces cytochromes sont réduits par la vitamine K3 réduite (2-méthyl-1,4-hydronaphthoquinone), par

le triphosphate réduit de l'adénine nicotinamide nucléotide (NAPH) et par le triphosphate réduit de la nicotinamide adénine nucléotide, par le 2-amino-4-hydroxy-6-menthyl-5,6,7,8-tetrahydroptéridine, et par la 2-amino-4-hydroxy-6,7-diméthyl-5,6,7,8,-tetrahydroptéridine (PtH4); ils sont réoxydés par exposition à l'air. Le QO_2 de da respiration endogène de *M. lepraemurium* se situe à 4 μl et le quotient respiratoire est de 0,87. Le cytochrome C n'est pas mis en évidence dans *M. lepraemurium*, mais une activité de la réductase du NADH-cytochrome C est présente.

L'examen comparatif de l'oxydation du NADH par les fractions particulées de *M. lepraemurium* et de *M. smegmatis* révèle que le premier de ces organismes témoigne d'une activité bien moindre. Même *M. lepraemurium* présentant une élongation à la suite d'une incubation pendant 3 semaines dans le milieu de Hart-Valentine en conditions aérobies, ne présente pas d'activité oxydante rapide sur le NADH.

Lorsqu'on utilise des particules préparées à partir de *M. lepraemurium* lyophilisé, conservées en réfrigérateur durant une longue période, l'hydrosulfite peut réduire le cytochrome de type B_1, alors que le PtH4 et NADH ne témoignent pas de cette action. Quand on étudie une suspension de particules, irradiée par la lumière ultraviolette, on constate que les cytochromes ne sont pas réduits en présence de NADH, mais qu'ils le sont en présence de PtH4. Ces observations suggèrent que des composés de la ptéridine s'interposent vraisemblablement entre le NADH et les cytochromes.

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