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Respiratory Activities of *In Vitro* Grown *Mycobacterium lepraemurium*[1]

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Since the discovery of *Mycobacterium lepraemurium* in 1903 by Stefansky ([32]) as the etiologic agent of murine leprosy, numerous attempts have been made for its *in vitro* cultivation. Although some investigators claimed *in vitro* cultivation of this mycobacterium, their cultures failed to produce the disease in experimental animals. These cultures were not, therefore, considered to be the causative agents of murine leprosy. This microorganism remained noncultivated and an obligate parasite until 1970 when Ogawa and Motomura ([25]) reported the growth of *M. lepraemurium* in the form of very slow-growing, small colonies on 1% Ogawa egg-yolk medium. Mori ([22]) was the first to confirm these findings. Recently, Pattyn and Portaels ([28]) reported *in vitro* growth of murine leprosy bacilli in the form of distinct colonies on Ogawa egg-yolk medium. The yield of *in vitro* grown cells in the form of colonies is very low. On the other hand, large amounts of cells are required for respiratory and other metabolic studies. More recently, using a bacterial mass inoculation technique, we have shown that an adequate amount of *M. lepraemurium* cells can be obtained on Ogawa egg-yolk medium for such studies ([12]).

Using manometric techniques, Gray ([10]) studied the respiratory metabolism of *in vivo* grown murine leprosy bacilli and found that none of the several substrates tested was oxidized. However, employing the same techniques, we have shown that whole cells of *M. lepraemurium* derived from rat lepromata catalyzed the oxidation of some substrates ([15]). It may be argued that the utilization of exogenous substrates may be caused by the host tissues present, although in very small amounts, in the purified cell suspensions used during these studies. Since *M. lepraemurium* now can be cultivated *in vitro* and sufficient amounts of cells can be obtained, the present study was undertaken to determine the respiratory capability of *in vitro* grown murine leprosy bacilli.

MATERIALS AND METHODS

Growth of *M. lepraemurium*. The Hawaiian and Keishicho strains of *M. lepraemurium* used in these studies were cultivated on Ogawa egg-yolk medium as described previously ([12]). Successive subcultures were transferred every 45–50 days of incubation and cells from the sixth subcultures of both strains were used for respiratory studies.

Preparation of cell suspensions. *M. lepraemurium* cells were removed from the surface of Ogawa egg-yolk medium and suspended in 0.05 M potassium phosphate buffer, pH 7.2. They were homogenized thoroughly in a Potter-Elvehjem homogenizer and the suspension was centrifuged at 10,000 × *g* for 10 min at 4°C in a Beckman J-21B centrifuge. The supernatant was discarded and the cells were washed twice more and resuspended in the same buffer. The final cell suspension to be used for respiratory studies was standardized turbidimetrically using a 540 nm filter, so that a 1:10 dilution gave a Klett unit reading of 400 which corresponded to about 20 mg of cells (dry weight) per ml. Dry weights of cell suspensions were determined by drying 1 ml samples of cell suspensions to constant dry weight in an air oven at 105°C.

Preparation of cell-free extracts. After washing the cells twice with buffer, they were suspended (1:4 w/v) in a sonication medium containing 0.1 M tris (hydroxymethyl) aminomethane buffer, 0.3 M sucrose, 0.005 M ethylenediamine-tetraacetic acid (EDTA-Na_2), and 0.02 M $MgCl_2$. The pH was adjusted to 7.2 with HCl. The cell suspensions were passed three times through a chilled Aminco French pressure cell at 18,000 psi

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(1 psi = 703.069 kg/M^2). The homogenate was centrifuged at 12,000 × *g* for 30 min at 4°C to remove unbroken cells and cell debris. The resulting supernatant was used as the cell-free extracts.

Measurements of oxidation of substrates. The Warburg manometric technique as described by Umbreit, *et al.* ([36]) was used to measure the oxygen uptake of bacillary suspensions. All experiments were carried out at 30°C with air as the gas phase. Each flask contained 1 ml of cell suspension and 50 mM potassium phosphate buffer, pH 7.2 in the main compartment and 20 μmol of substrate (pH 7.0) in the side arm. When yeast extract served as the substrate, 0.2 ml of 10% solution was used. To absorb CO_2, 0.2 ml of 20% KOH was added in the center well. The total volume of liquid in each flask was 2 ml. An additional control in all experiments consisted of simultaneous determination of endogenous respiration. After an equilibration period of about 10 min, substrate was mixed with the cell suspension and oxygen uptake was recorded. The experiments were repeated three times.

To measure the oxygen uptake by the cell-free extracts, an oxygen polarograph (model 53, Yellow Spring Instruments Co., Yellow Springs, Ohio, U.S.A.) was used. All the assays were carried out at 30°C in 0.1 M potassium phosphate buffer (pH 7.2) in a total volume of 3 ml. The reaction mixture containing buffer and cell-free extract (1.5 mg protein) was incubated for 3 min and the endogenous rate of oxidation was recorded for at least 2 min. Then about 20 μmol of substrate was added and the rate of oxygen uptake was measured for 10 min during which the rate was linear. The experiments were repeated at least three times and the results expressed as an average value, variations in the multiple assays were less than 5%.

Determination of protein. The protein content of the cell-free extracts was determined by the method of Lowry, *et al.* ([17]). Crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was used as a standard.

Substrates. All the substrates used in this study were of reagent grade and most were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

TABLE 1. *Substrates not oxidized by the cell suspensions of the Keishicho and Hawaiian strains of* M. lepraemurium.

(i) Intermediates of glycolysis
Glucose
Glyceraldehyde-3-phosphate
Phosphoglyceric acid
Pyruvate
Lactate
Acetate
Formate
(ii) Intermediates of the tricarboxylic acid cycle
Oxaloacetate
Citrate
α-Ketoglutarate
Malate
Fumarate
(iii) Fatty acids
Stearate
Oleic acid
(iv) Carbohydrates
Sucrose
Fructose
Mannose
Glucosamine
Sorbitol
Maltose
Ribose
D-xylose
Hyaluronic acid
(v) Sulfur or sulfhydryl-containing compounds
Thiosulfate
Cystine
Thiourea
Mercaptosuccinate
Thioacetate
Monothioglycerol
(vi) Miscellaneous compounds
β-Hydroxybutyrate
Egg albumin
Egg-white
Benzoate
Peptone
Casamino acid

RESULTS

Oxidation of substrates

The conditions and the culture medium for the *in vitro* cultivation of *M. lepraemurium* are rather precise and narrowly selective. Except for the Ogawa egg-yolk medium, the organism did not grow on any other conventional medium. In an effort to improve the Ogawa egg-yolk medium or to find other cultural media, a number of substrates known to be oxidized by other bac-

teria including cultivable mycobacteria were examined as the prospective oxidizable substrates.

Substrates of the glycolysis cycle, tricarboxylic acid cycle, fatty acids, and carbohydrates listed in Table 1 were not oxidized by the whole cell suspensions of both *in vitro* grown Hawaiian and Keishicho strains of *M. lepraemurium*. However, glycerol, bovine serum albumin, and succinate were oxidized at an extremely slow rate and irregular results were obtained. Many compounds such as casamino acids, β-hydroxybutyrate, hyaluronic acid, etc., (Table 1) did not show an increase in the amount of oxygen uptake over the endogenous value. While egg-yolk was inactive, egg-white (ovalbumin) was found to be toxic since in its presence oxygen consumption values lower than the endogenous activity were repeatedly obtained. Although freezing and thawing were found necessary for the oxidation of succinate by *in vivo* grown cells of *M. lepraemurium* ([15]), it was oxidized at the same rate by the frozen or normal suspensions of *in vitro* grown cells used in this study.

Oxidation of yeast extract by whole cell suspensions. The oxidation of yeast extract by *in vitro* grown *M. lepraemurium* is shown in Figure 1. Yeast extract was actively oxidized by the *in vitro* grown Hawaiian (Curve A) and Keishicho (Curve B) strain since its presence caused a marked increase in the amount of oxygen uptake over the endogenous activity. No respiration occurred with boiled cell suspensions in either the absence or presence of yeast extract. When yeast extract was used as an oxidizable substrate, its oxidation was dependent upon the amount of bacilli used and the rate of oxidation was also proportional to the concentration of yeast extract used.

Oxidation of sulfur-containing compounds. No oxygen consumption was observed when all the sulfur-containing compounds shown in Table 1 were used as substrates. However, many other sulfur-

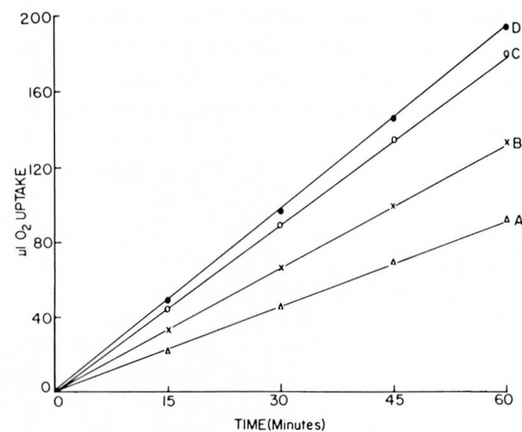

FIG. 2. Oxidation of (A) D-cysteine, (B) L-cysteine, (C) L-cysteine methyl ester, and (D) L-cysteine ethyl ester by whole cell suspensions of the *in vitro* grown Hawaiian strain of *M. lepraemurium*.

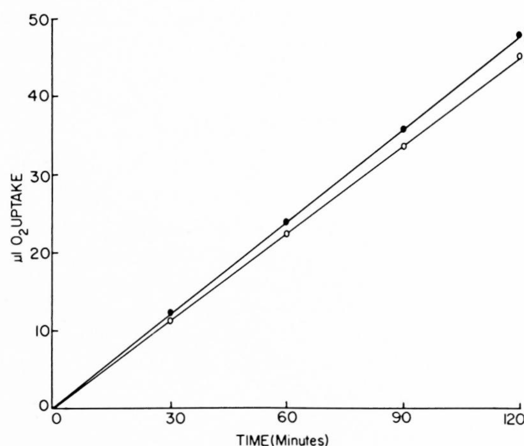

FIG. 1. Oxidation of yeast extract by *in vitro* grown *M. lepraemurium*. Curves A and B show, respectively, exogenous O_2 uptake by whole cell suspensions of the Hawaiian and Keishicho strains of *M. lepraemurium*.

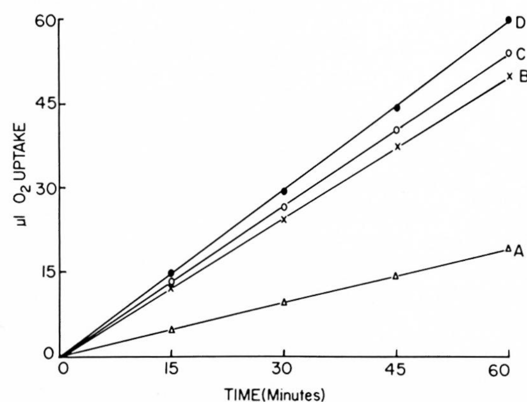

FIG. 3. Oxidation of (B) dithiothritol, (C) dithioerythritol, and (D) DL-penicillamine by whole cell suspensions of the *in vitro* grown Keishicho strain of *M. lepraemurium*. Curve A represents endogenous O_2 uptake.

containing compounds were actively oxidized. Figure 2 shows that D-cysteine (Curve A), and L-cysteine (Curve B) were readily oxidized by cell suspensions of *in vitro* grown Hawaiian strain of *M. lepraemurium.* The same culture oxidized the methyl ester (Curve C) and the ethyl ester (Curve D) of L-cysteine at an extremely fast rate. As shown in Figure 3, cell suspensions of the *in vitro* grown Keishicho strain caused an active oxidation of thiol compounds such as dithiothritol (Curve B), dithioerythritol (Curve C) and DL-penicillamine (Curve D). Likewise, all these compounds were readily oxidized by the cell suspensions prepared from the *in vitro* grown Hawaiian strain of *M. lepraemurium.* To insure that the observations were due to biological respiration, sulfur-containing compounds which were found to be actively oxidized by cell suspensions were incubated at 30°C in the absence of cell suspensions or in the presence of boiled cell suspensions in 0.1 M potassium phosphate buffer, pH 7.2 in the Warburg flasks. Under these conditions no detectable oxygen uptake occurred in 2 hr.

Oxidation of substrates by cell-free extracts

Oxidations of substrates, both those which appeared to be oxidized at a very slow rate as well as those oxidized readily by whole cell suspensions of *in vitro* grown *M. lepraemurium,* were measured polarographically using cell-free extracts. The results are shown in Table 2. Although cell-free preparations failed to oxidize glucose, pyruvate, and the other intermediates of the glycolysis cycle listed in Table 1, glycerol was oxidized at a slow rate. There was no oxygen consumption by cell-free preparations when oxaloacetate and other intermediates of the tricarboxylic acid cycle were used as substrates, but succinate repeatedly showed a slightly higher oxygen uptake than the endogenous value. Fatty acids such as stearic acid and oleic acid were inactive, while Tween 80 was readily oxidized (Table 2) by the cell-free extracts of both the *in vitro* grown strains of *M. lepraemurium.* Cell-free preparations failed to oxidize hyaluronic acid, galactomannan, and the other carbohydrates listed in Table 1. Dithiothritol, dithioerythritol, and DL-penicillamine (Table 2) were actively oxidized. Cell-free extracts of both strains catalyzed oxidation

TABLE 2. *Oxidation of substrates by cell-free extracts of Hawaiian and Keishicho strains of* M. lepraemurium.

Substrates	Exogenous O_2 uptake in atoms/mg protein/hr	
	Hawaiian strain	Keishicho strain
Glycerol	134	180
Glucose	0	0
Pyruvate	0	0
Succinate	68	108
Oxaloacetate	0	0
Tween 80	248	362
Stearate	0	0
Hyaluronic acid	0	0
D-cysteine	2208	3124
L-cysteine	2622	2704
Dithiothritol	490	822
Dithioerythritol	520	872
DL-penicillamine	612	950
Cystine	0	0
Bovine serum albumin	134	232
Ascorbate	552	690

of D-cysteine and L-cysteine at an extremely rapid rate. On the other hand, respiration remained unchanged in the presence of several sulfur- or sulfhydryl-containing compounds such as cystine, thiosulfate, mercaptosuccinate, and monothioglycerol. Bovine serum albumin was oxidized at a slow rate. Addition of ascorbate to the cell-free preparations caused a marked increase in oxygen uptake over the endogenous respiration. The amounts of oxygen consumption of all the substrates by cell-free extracts of the Keishicho strain were always significantly higher than those obtained by cell-free extracts of the Hawaiian strain of *M. lepraemurium.*

DISCUSSION

In our earlier studies ([15]) it was shown that none of the intermediates of the glycolysis cycle and the tricarboxylic acid cycle was oxidized by purified whole cell suspensions of *in vivo* grown *M. lepraemurium* at pH 7.4. However, succinate was oxidized, but only by the cell suspensions frozen for about 1 min at −40°C. Several experiments were carried out at different pH values ranging from 5 to 8, and pH 7.2 was found to be optimal for respiratory activities of *in vitro* grown *M. lepraemurium.* During these studies, our manometric experiments showed that succinate as well as glycerol and

bovine serum albumin were oxidized at an extremely slow rate by cell suspensions prepared from *in vitro* grown *M. lepraemurium*. These substrates were oxidized at the same slow rate by frozen (−40°C for 1 min) whole cell suspensions. Mori ([23]) has shown that glycerol and sodium glutamate were not oxidized by whole cell suspensions of *M. lepraemurium* cultivated on Ogawa egg-yolk medium. Gray ([10]) reported that endogenous respiration of *in vivo* grown *M. lepraemurium* and other mycobacteria was stimulated in the presence of bovine serum albumin. In contrast, whole cell suspensions of *in vivo* grown *M. lepraemurium* used in our earlier studies ([15]) failed to show such a stimulating effect of bovine serum albumin on endogenous or succinate respiration. Although this mycobacterium was cultivated on Ogawa egg-yolk medium, egg-yolk was not oxidized by whole cells or cell-free extracts. Egg-yolk may serve as a growth-promoting substance. Egg-white was found to be toxic for *in vitro* grown *M. lepraemurium*, and Portaels and Pattyn ([29]) have recently shown that egg-white was inhibitory for the growth of this organism.

Yeast extract was slowly oxidized by host-grown *M. lepraemurium* suspensions ([14]). The present studies show that yeast extract is quite readily oxidized by both strains of *M. lepraemurium* grown on Ogawa egg-yolk medium. Yeast extract is commonly used for the growth of many microorganisms and is considered the richest source of growth factors. Smith, *et al.* ([31]) reported that yeast extract was required for growth of *Thermoplasma acidophilum* and the growth promoting activity was found to be proteinaceous in nature. They suggested that yeast extract protects the organism from H^+ concentration and is involved in ion transport. It is possible that the yield of *in vitro* grown *M. lepraemurium* can be increased by the addition of yeast extract to the Ogawa egg-yolk medium.

Whole cell suspensions of *in vitro* grown mycobacteria failed to show oxidation of intermediates of the Kreb's cycle ([7, 9, 11]). However, the same compounds were readily oxidized by cell-free extracts prepared from whole cells of many mycobacteria ([7, 9, 20, 21, 30, 34]). These failures were therefore considered due to impermeability of the cell wall to the substrates. Mori ([23]) reported that the oxygen uptake by cell-free extracts prepared from cultivated *M. lepraemurium* was not stimulated when citrate and α-ketoglutarate were used as substrates. During these studies cell-free extracts prepared from *in vitro* grown murine leprosy bacilli catalyzed an active oxidation of ascorbate and Tween 80 but glycerol, bovine serum albumin, and succinate were oxidized only slowly (Table 2). Portaels and Pattyn ([29]) found that while acetate inhibited growth, Tween 80 and sodium salts of the fatty acids, oleate, caproate, caprylate, and palmitate, had no beneficial effect on the growth of *M. lepraemurium* on egg-yolk medium. As is the case for other mycobacteria ([6, 18, 19, 30, 35]), glycerol is the primary source of carbon in the cultivation of *M. lepraemurium* on Ogawa egg-yolk medium. Generally there is a correlation between the rate of oxidation of a substrate and the rate of growth of a microorganism in a synthetic medium containing that substrate as the sole source of carbon or energy or both. However, it is not necessarily true that the substrates which are oxidized at a slow rate cannot play an important role in the metabolism of *M. lepraemurium*. For example, succinate was oxidized at a slow rate by the Kirschberg strain of *M. avium* ([26]), but it was found to be an excellent substrate for this mycobacterium ([1]). Since *in vitro* grown *M. lepraemurium* are capable of oxidizing some substrates, it is possible that some of these substrates could be utilized as energy and carbon sources. Ogawa egg-yolk medium is not yet optimal. Our experimental results suggest that oxidizable substrates could be incorporated in the Ogawa egg-yolk medium to further improve the medium for the *in vitro* cultivation of *M. lepraemurium*.

Cultivable mycobacteria have long been known to require sulfur for their growth ([2, 5, 16, 27]). Our results show that a number of sulfhydryl compounds such as D-cysteine, L-cysteine, dithioerythritol, dithiothritol, and DL-penicillamine (Figs. 2 and 3) were readily oxidized by whole cell suspensions as well as by cell-free extracts (Table 2) of *in vitro* grown *M. lepraemurium*. Active oxidation of these compounds by *in vivo* grown murine leprosy bacilli has also been shown previously ([15]). Dhople and

Hanks ([4]) have shown that cysteine is one of the most important compounds in promoting the growth of murine leprosy bacilli. These investigators (personal communication, 1975[3]) also reported a marked increase in the amount of ATP in *M. lepraemurium* after an incubation period of eight weeks in the presence of thioglycolate, cysteine, and reduced glutathione in the Nakamura NC-5 medium ([24]). They concluded that the sulfhydryl compounds containing a carboxyl group are essential for growth and suggested that such compounds induce a low oxidation-reduction potential and participate in the metabolism of this mycobacterium. During these studies it was observed that oxidations of NADH and succinate by cell-free extracts of *in vitro* grown *M. lepraemurium* were completely inhibited by the thiol-binding agents such as p-hydroxymercuribenzoate and N-ethylmaleimide (results not shown). These results suggest that sulfhydryl compounds are involved in the electron transport system and play an important role in the metabolism of this mycobacterium. This conclusion is further supported by our recent experimental data ([13]) which show that both NADH and succinate oxidation as well as the coupled ATP formation were markedly inhibited by the thiol-binding agents.

M. lepraemurium is a very slow growing mycobacterium and primary cultures on Ogawa egg-yolk medium can be obtained in about 90 days ([12]) in only 84% of the tubes. *M. leprae* and *M. lepraemurium* have been considered as microaerophilic ([33]). Our very recent studies ([12]) indicated that lower oxygen tension is required for the multiplication of murine leprosy bacilli. It is known ([8]) that sulfhydryl compounds can react with oxygen to form water; they can also react with free radicals, such as superoxide anion or the hydroxyl anion, and thus neutralize the toxicity of oxygen. It is possible that sulfhydryl compounds may act as protective agents for SH-groups required by the microorganisms. Thus far no attempts have been made to investigate the effects of sulfhydryl compounds on the *in vitro* cultivation of *M. lepraemurium* on Ogawa egg-yolk medium. It is quite likely that the *in vitro* cultivation of *M. lepraemurium* on Ogawa egg-yolk medium in the presence of sulfhydryl compounds can be achieved with 100% efficiency, the time period can significantly be reduced, and the yield of cells can further be improved.

SUMMARY

Mycobacterium lepraemurium was cultivated on Ogawa egg-yolk medium and its respiratory activities using several substrates were investigated. Glycerol and succinate were oxidized at a slow rate by the cell-free extracts prepared from *in vitro* grown Hawaiian and Keishicho strains of *M. lepraemurium.* None of the other intermediates of the glycolysis cycle as well as of the tricarboxylic acid cycle was oxidized by the whole cell suspensions or cell-free extracts. Likewise, many sulfur compounds such as cystine, mercaptosuccinate, monothioglycerol, thioacetate, etc., were inactive. However, sulfhydryl compounds such as L-cysteine, D-cysteine, DL-cysteine, dithioerythritol, dithiothritol, and DL-penicillamine were actively oxidized. Yeast extract was also readily oxidized by cell suspensions of *in vitro* grown *M. lepraemurium.* Tween 80 was very poorly oxidized by whole cell suspensions but the cell-free preparations catalyzed an active oxidation of Tween 80. While bovine serum albumin was oxidized at a slow rate by cell-free extracts, egg albumin was inactive. The thiol-binding agents, p-hydroxymercuribenzoate and N-ethylmaleimide were effective inhibitors of succinate and NADH oxidation, thus indicating the involvement of sulfhydryl compounds in the metabolism of *M. lepraemurium.*

RESUMEN

Se cultivó el *Mycobacterium lepraemurium* en el medio de Ogawa con yema de huevo y se estudiaron sus actividades respiratorias usando varios substratos. Los extractos libres de células preparados a partir de los cultivos *in vitro* de las cepas Hawaii y Keishicho de *M. lepraemurium* oxidaron lentamente al glicerol y al succinato. Ninguno de los otros intermediarios de los ciclos glicolítico o de los ácidos tricarboxílicos fue oxidado por las suspensiones de células totales o por los extractos libres de células. De igual manera, muchos compuestos azufrados tales como la cistina, el mercaptosuccinato, el monotioglicerol, el tioacetato, etc., también resultaron inactivos. Sin embargo, otros compuestos con grupos sulfhidrilo como la L-cisteína, la

D-cisteína, la DL-cisteína, el ditioeritritol, el ditiotritol y la DL-penicilamina, fueron oxidados muy activamente. Los extractos de levadura también fueron activamente oxidados por las suspensiones del *M. lepraemurium* crecido *in vitro*. Aunque el Tween 80 fue pobremente oxidado por las suspensiones totales de células, los extractos libres de células catalizaron su activa oxidación. Por otro lado, mientras que la albúmina sérica bovina fue oxidada por los extractos celulares en bajo grado, la albúmina de huevo fue inactiva. Los agentes que interaccionan con grupos tiol, p-hidroximercuribenzoato y N-etilmaleimida, fueron inhibidores muy efectivos de la oxidación del succinato y del NADH indicando así la participación de compuestos sulfhidrílicos en el metabolismo del *M. lepraemurium*.

RÉSUMÉ

Mycobacterium lepraemurium a été cultivé sur le milieu d'Ogawa; son activité respiratoire a été explorée en utilisant plusieurs substrats. On a constaté que le glycerol et le succinate étaient oxydés lentement par des extraits sans cellules préparées à partir des souches Hawaii et Keishicho de *M. lepraemurium* cultivé *in vitro*. Aucun des autres intermédiaires tant du cycle glycolytique que du cycle de l'acide tricarboxylique n'étaient oxydés par des suspensions de cellules entières ou par des extraits libres de cellules. De même, de nombreux composés sulfurés étaient inactifs; tel était le cas pour la cystine, le mercaptosuccinate, le monothioglycerol, le thioacetate, et d'autres. Toutefois, des composés hydrosulfurés tels que la L-cysteine, la D-cysteine, la DL-cysteine, le dithioerythritol, le dithiothritol, et la DL-penicillamine étaient activement oxydés. Un extrait de levure était également facilement oxydé par des suspensions de cellules de *M. lepraemurium* cultivées *in vitro*. Le Tween 80 était très faiblement oxydé par des suspensions de cellules entières, mais par contre, des préparations sans cellules catalysaient une oxydation active de ce produit. Alors que l'albumine sérique de boeuf était oxydée à un rythme lent par des extraits sans cellules, l'albumine de l'oeuf était inactive. Le p-hydroxymercuribenzoate et la N-ethylmaleimide, des agents chélateurs du soufre, se sont révélés, des inhibiteurs efficaces du succinate et de l'oxydation par le NADH, ce qui révèle une intervention de composés hydrosulfurés dans le métabolisme de *M. lepraemurium*.

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