BIKEN'S JOURNAL 4, 225-233 (1961)

Carbohydrate Metabolism of the Murine Leprosy Bacillus I. Studies on the Respiratory Enzyme System, especially the Diaphorase and Malic Dehydrogenase Activities

TATSUO MORI, KENJI KOSAKA AND TONETARO ITO

Department of Leprosy, Research Institute for Microbial Diseases, Osaka University, Osaka
(Received for publication, October 16, 1961)

SUMMARY

1) The murine leprosy bacillus has diphosphopyridine nucleotide and diaphorase I and II activities.
2) Whole cells of the murine leprosy bacillus do not utilize externally added reduced diphosphopyridine nucleotide or reduced triphosphopyridine nucleotide.
3) Reduced diphosphopyridine nucleotide is scarcely oxidized by an extract of ground murine leprosy bacilli.
4) There is malic dehydrogenase activity in extracts of ground murine leprosy bacilli but triosephosphate dehydrogenase and succinic dehydrogenase activities were not found.

INTRODUCTION

Determination of the carbohydrate sources utilized by the murine leprosy bacillus is important basic knowledge for the cultivation of this microorganisms, but until now studies have been unsuccessful. Using the Warburg method Gray (1952) studied the effect of numerous carbohydrates with murine leprosy bacilli isolated from infected tissues and stated that almost no oxidation took place. Ito and Sonoda (1958) confirmed this similar in experiments and found that a substance accelerating respiration is present in dried yeast extracts and infected tissues. Tamemasa and Tsutsumi (1958) examined the dehydrogenase reaction of numerous carbohydrates under anaerobic conditions using the Thunberg tube and reported that except with certain substrates only negative results were obtained.

The authors examined the respiratory enzyme system especially the diaphorase activity, cytochrome system and malic dehydrogenase using intact bacilli and extracts of ground bacteria. The interesting results are presented here.

EXPERIMENTAL MATERIAL AND METHOD

Murine leprosy bacillus

The lepromas were collected 4 months after inoculation of the Kumamoto strain into rats. They were homogenized in distilled water. The bacilli fraction was obtained by differential

centrifugation. The fraction was treated with M/8 NaOH and the bacterial sediment was washed repeatedly with distilled water and the bacilli centrifuged (Mori, 1960 and 1961)

Reagents used

Diphosphopyridine nucleotide (DPN) and reduced diphosphopyridine nucleotide (DPNH) were purchased from Sigma Chemical Company.

Reduced triphosphopyridine nucleotide (TPNH) and glyceraldehyde-3-phosphate were obtained from Nutritional Biochemicals Company.

Triphenyltetrazolium chloride (TTC) was a Merck preparation.

The other reagents were compounds purchased locally.

Alcohol dehydrogenase

This was purified from baker's yeast by Racker's method. (1950)

Diaphorase I

This was prepared from bovine heart by Straub's method (1939)

Determination of Dehydrogenase

Thunberg tubes were used with TTC as the electron acceptor. After preincubation for 15 minutes under anaerobic conditions at reduced pressure, the substrate was added and the tubes incubated for 60 minutes at 37°C. The red color of the formazan formed was measured macroscopically.

EXPERIMENTAL RESULTS

Purity of the alcohol dehydrogenase and diaphorase I

In order to show the presence of DPN and diaphorase I activity in the murine leprosy bacillus, alcohol dehydrogenase purified from a baker's yeast extract and diaphorase I purified from a bovine heart extract were used. Prior to the main experiment, these two enzymes were tested to see whether they were sufficiently pure to meet the requirements of the experiment. Column 5 of Table 1 shows the complete system and only this complete system gave strong red coloration. Columns 3 and 4 show systems in which DPN and diaphorase I respectively were omitted. In these systems only a slight red color was observed after 60 minutes and the results were almost negative. This shows that the purified alcohol dehydrogenase and diaphorase I contain almost no DPN and that the alcohol dehydrogenase contains little diaphorase I.

Diaphorase activity and DPN in an extract of ground murine leprosy bacilli

DPN or diaphorase I or both were deleted from the complete system of Table I and replaced by the extract of ground bacteria. The alcohol dehydrogenase activity was then examined. As shown in Table 2, not only the complete system (No.1) but those in which diaphorase I is omitted (No.2), DPN is omitted (No.3) and in which both were omitted (No.4) showed strong coloration. In addition, the controls (Nos. 5, 6 and 7) were negative and controls (Nos. 8 and 9) were weakly positive. The weakly positive results of the latter control experiments can

Table 1. Purity of Alcohol Dehydrogenase and Diaphorase I

Tube No. / Substances added to reaction mixture	1	2	3	4	5	6	7	8
A	+	+	+	+	+	—	—	—
D	—	—	+	—	+	+	+	+
DPN	—	+	—	+	+	—	+	+
EtOH	+	—	+	+	+	+	+	—
TTC	+	+	+	+	+	+	+	+
Buffer	+	+	+	+	+	+	+	+
Coloration after 60 minutes	—	—	±	±	++++	—	—	—

Main Chamber : A. (Alcohol dehydrogenase) 0.1 ml
D. (Diaphorase I) 0.1 ml
DPN 10^{-7} mol 0.1 ml
TTC 10^{-6} mol 0.1 ml
0.1% glycine in M/10 phosphate buffer of pH 7.6 0.5 ml
Side Arm; EtOH (Ethanol) 2×10^{-4} mol 0.1 ml

be explained by the impurity of the diaphorase preparation used, which contained for instance lactic dehydrogenase. Thus some contaminating enzyme in the diaphorase preparation may attack various substrates in the bacilli. From the

Table 2. Diaphorase I Activity and DPN of an Extract of Ground Murine Leprosy Bacilli

Tube No. / Substances added to reaction mixture	1	2	3	4	5	6	7	8	9
A	+	+	+	+	—	—	—	—	—
D	+	—	+	—	—	—	—	+	+
DPN	+	+	—	—	—	—	+	+	+
Bacterial Extract	—	+	+	+	+	+	+	+	+
TTC	+	+	+	+	+	+	+	+	+
EtOH	+	+	+	+	—	+	+	—	—
Buffer	+	+	+	+	+	+	+	+	glycine omitted
Coloration after 60 minutes	++++	+++	+++	++	—	—	—	+	+

Main Chamber : A. (Alcohol dehydrogenase) 0.1 ml
D. (Diaphorase I) 0.1 ml
DPN 10^{-7} mol 0.1 ml
TTC 10^{-6} mol 0.1 ml
0.1% glycine in M/10 phosphate buffer of pH 7.6 0.2 ml
75 mg of ground lyophilized bacteria extracted with 2.4 ml of distilled water, centrifuged at 6,000 rpm for 15 minutes, 0.2 ml of the supernant used. Dry weight ca. 1.1 mg.
Side Arm; EtOH (Ethyl alcohol) 2×10^{-4} mol 0.2 ml

results, it can be seen that DPN and diaphorase I activity are present in the extract.

Diaphorase I and diaphorase II activities in the extract of ground murine leprosy bacilli.

On the assumption that diaphorase II activity would be present in the presence of diaphorase I activity, synthetic DPNH and TPNH were used as substrates instead of enzymically produced DPNH and TPNH. TTC was used as the electron acceptor, and the diaphorase I and II activities in an extract of ground bacteria was examined. Table 3 shows clearly that both enzymes were present.

Table 3. Diaphorase I and II Activities of an Extract of Ground Murine Leprosy Bacilli

Addition to reaction mixture \ Tube No.	1	2	3	4	5
Bacterial Extract	+	+	+	−	−
Buffer	+	+	+	+	+
TTC	+	+	+	+	+
DPNH	−	+	−	+	−
TPNH	−	−	+	−	+
Coloration after 60 minutes	±	++++	++++	−	−

Main Chamber ;	M/10 phosphate buffer pH 7.0	0.6 ml
	TTC 10^{-5} mol	0.1 ml
	100mg of ground lyophilized bacteria extracted with 2 ml of distilled water, centrifuged at 6,000 rpm for 15 minutes, 0.1 ml of the supernant used.	
	Dry weight ca. 1.42 mg.	
Side Arm;	DPNH 10^{-5} mol	0.2 ml
	TPNH 10^{-5} mol	0.2 ml

Diaphorase I and diaphorase II activities of whole murine leprosy bacilli

The activities of diaphorases I and II of fresh and lyophylized whole murine leprosy bacilli were examined. As shown in Table 4, the DPNH and TPNH added are not utilized.

Oxidation of DPNH by an extract of ground murine leprosy bacilli

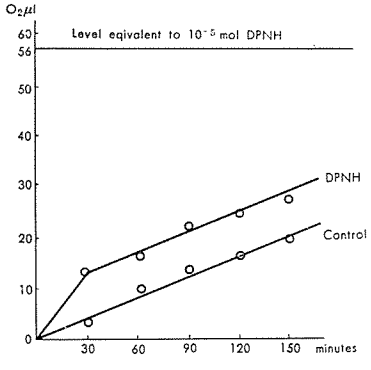
The electron of DPNH is donated to TTC by the action of Diaphorase I. Using the Warburg method, a study was made of whether the electron of DPNH could be passed to oxygen with the enzyme via the electron transmitting system in the extract of ground bacteria, without resort to TTC. The experimental conditions are given in the lower part of Fig. 1. DPNH was omitted in the control. As can be seen in Fig. 1, there was a somewhat greater absorption of O_2 than in the control, but this was far less than the calculated amount of O_2

Table 4. Diaphorase I and II Activities of Whole Murine Leprosy Bacillus

Addition \ Tube No.	1	2	3	4	5	6
Buffer	+	+	+	+	+	+
TTC	+	+	+	+	+	+
DPNH	−	+	−	−	+	−
TPNH	−	−	+	−	−	+
Live Bact.	+	+	+	−	−	−
Lyophilized Bact.	−	−	−	+	+	+
Coloration after 60 minutes	+	+	+	+	+	+

Main Chamber :	M/10 phosphate buffer pH 7.0		0.5 ml
	TTC 10^{-5} mol		0.1 ml
	Bacterial suspension		0.2 ml
	Live bact.	6.5 mg	
	Lyophilized bact.	5 mg	
Side Arm :	DPNH 10^{-5} mol		0.1 ml
	TPNH 10^{-5} mol		0.1 ml

required for complete oxidation of the DPNH. Thus the attempt to clarify electron transmission in the extract was unsuccessful. Spectroscopic observations on a turbid suspension of murine leprosy bacillus did not demonstrate the existence of cytochromes. In considering the results of spectroscopic studies and those of the DPNH oxidation, it can be assumed that the cytochrome system is not present in murine leprosy bacilli. (This will be discussed further in a later paper).



Main Chamber : M/10 phosphate buffer pH 7.0 1.0 ml
Bact. Extract — 150 mg of lyophilized bacteria ground, extracted with 4 ml of distilled water, centrifuged at 6000 rpm for 15 minutes and 1.0 ml of the supernant used. Dry weight ca. 12 mg
Central Well : 20 % KOH 0.2 ml
Side Arm : DPNH 10^{-5} mol 0.5 ml
Shaken at 38°C

Fig: 1 Oxidation of DPNH by Extract of Ground Murine Leprosy Bacilli

Dehydrogenase activity of an extract of ground murine leprosy bacilli with various types of substrate.

Diaphorases I and II are present in the ground bacterial extract and it was assumed that DPNH and TPNH are produced by the action of dehydrogenase

Table 5. Dehydrogense Activity of Extract of Ground Murine Leprosy Bacilli with Various Substrates

Substrate	Buffer	TTC	Bact. Extract	Coloration after 60 minutes
—	+	+	+	—
Citrate	+	+	+	—
α-Ketoglutarate	+	+	+	—
Succinate	+	+	+	—
Pyruvate	+	+	+	—
Acetate	+	+	+	±
Malate	+	+	+	+
Glucose	+	+	+	—
Ribose	+	+	+	—
Glycerol	+	+	+	—
Lactate	+	+	+	—
Glycer-aldehyde-3-p	+	+	+	—

Main Chamber : M/10 phosphate buffer pH 7.0 0.5 ml
TTC 10^{-5} mol 0.1 ml
150 mg of lyophilized bacteria ground well, extracted with 4 ml of distilled water, centrifuged at 6,000 rpm for 15 minutes and 0.1 ml of the supernant used. Dry weight ca. 1.2 mg.
Side Arm : Substrate 10^{-5} mol 0.1 ml

Table 6. Malic Dehydrogenase and Triosephosphate Dehydrogenase in Extract of Ground Murine Leprosy Bacilli

Tube No. / Additions	1	2	3	4	5	6
Bact. Extract	+	+	+	—	—	—
Buffer	+	+	+	+	+	+
Diaphorase	+	+	+	+	+	+
TTC	+	+	+	+	+	+
DPN	+	+	+	+	+	+
Substrate	—	malate	glyceraldehyde -3-p	malate	glyceraldehyde -3-p	—
Coloration after 60 minutes	±	++++	—	—	—	—

Main Chamber ; M/10 phosphate buffer pH 7.0 0.9 ml
DPN 10^{-7} mol 0.1 ml
TTC 10^{-5} mol 0.1 ml
Diaphorase 0.1 ml
Bact. Extract-100 mg of lyophilized bacteria ground, extracted with 2 ml of distilled water centrifuged at 6,000 rpm for 15 minutes and 0.1 ml of the supernant used.
Dry weight ca. 1.42 mg.
Side Arm ; Substrate 10^{-5} mol 0.1 ml

on some substrate in the bacteria. The dehydrogenase activity against the 11 carbohydrate substrates shown in Table 5 was examined using TTC as the electron acceptor. Positive results were obtained only for malic dehydrogenase activity.

Malic dehydrogenase and triosephosphate dehydrogenase in an extract of ground murine leprosy bacilli.

The malic dehydrogenase activity in Table 5 was extremely weak. When DPN and purified bovine heart diaphorase I were added, the activity became very strong as shown in Table 6, column 2. As malic acid is one of the substrates of the TCA cycle, a similar experiment was conducted using glyceraldehyde-3-phosphate, a substrate involving the DPN of glycolysis. But as can be seen in column 3, the results were negative.

Malic dehydrogenase activity of whole murine leprosy bacilli

Though malic dehydrogenase activity is found in extracts of bacteria, it could not be demonstrated in intact bacilli, as shown in Table 7. If malic acid were used by the intact bacilli, it could be used as an energy source in the culture medium.

Table 7. Malic Dehydrogenase Activity of Whole Murine Leprosy Bacillus

Tube No.	1	2
Bact. Suspension	+	+
Buffer	+	+
TTC	+	+
Malate	−	+
Coloration after 60 minutes	+	+
* O.D. 480 mμ	0.05	0.05

Main Chamger :	M/10 phosphate buffer pH 7.0	0.5 ml
	TTC 2×10^{-5} mol	0.2 ml
	Bact. Suspension 5 mg of lyophilized bact.	
Side Arm ;	Malate 3×10^{-5} mol	0.3 ml

* The Formazan produced was extracted with 5 ml of ethyl acetate and measured colorimetrically in a photo-electric spectrophotometer (Hitachi) at 480 mμ.

DISCUSSION

The electrons, produced by dehydrogenation of a substrate involving coenzyme I or II (DPN or TPN), are transmitted to oxygen through several intermediate steps. In order for this to happen, the intermediate enzyme systems must all be active. In the case of the murine leprosy bacillus, which has hardly any dehydrogenase or oxidase activity with carbohydrates, it is necessary to see if some

enzyme system is absent or if there is no enzyme activity at all.

The failure to demonstrate electron transport with any substrate except malate (Table 5), although diaphorase and DPN were found, can only be explained by assuming that the dehydrogenases of these substrates are not present in the murine leprosy bacillus. It is very interesting that malic dehydrogenase activity was detected in the crude extract and malate can serve as an energy source for the bacilli.

In experiments on oxygen consumption of extracts of ground bacilli in the presence of DPNH, the electron transmitters operating between diaphorases and oxygen were studies, as shown in Fig. 1. From the negative results of the experiment, it can be assumed that the electron transmitters are not operating between diaphorases and oxygen. It is also believed that this is one of the probable causes of the negative results in experiments on the respiration of murine leprosy bacilli with various carbohydrate substrates.

In this study the results of enzyme assays of the extract of ground bacilli were always compared to those of the bacterial suspension. The enzymes found only in the extract were regarded as being specific for the bacilli. However the criticism might be raised that it is possible that these are not specialized enzymes of this bacilli, because the al cali-treated, contaminating debris of the host cells in lepromas did not liberate any active enzymes after being ground. As it was very difficult to isolate only the contaminating debris of the host cells in lepromas for a control experiment to exclude this criticism, incomplete Freund's adjuvant was injected subcutaneously into rats and the resulting granulomatous tissues were obtained. The tissues and testises of uninfected rats were treated in the same way to isolate the murine leprosy bacillus. Before al cali-treatment, autoclaved BCG bacilli were added to the sample as carrier. The material obtained was studied in the same manner as described above. No diaphorase or malic dehydrogenase activity could be found in the ground extract of this material. Therefore the enzyme activities in the ground extract of the murine leprosy bacillus can be regarded as specific to the bacillus.

The respiratory system in the murine leprosy bacillus is believed to follow the path way described here. DPNH is synthesized by malic dehydrogenase and as the respiratory system is obstructed here, the hydrogen of DPNH will be accepted by some other substrate.

Heretofore, the murine leprosy bacillus was considered to proliferate only in living organisms and more specifically, only in the living cell and that an intermediate metabolite or coenzyme of the host cell was utilized for its proliferation. However it was found that metabolic coenzymes such as DPNH and TPNH were not utilized by the bacillus and it is considered that such complex metabolic intermediates are not utilized. Even externally added malic acid is not utilized by the whole murine leprosy bacillus. The bacillus may posses some specific

absorption ability for a particular substance which can only be utilized *in vivo*. It has been shown that the murine leprosy bacillus obtains energy from malic acid and there is a strong possibility that this malic acid is synthesized in the cell from some absorbable carbohydrate source. This, however, requires further study.

REFERENCES

Gray, C. T. (1952). The respiratory metabolism of murine leprosy bacilli. *J. Bacteriol.* **64**, 305-313

Ito, T. and Sonoda, R. (1958). Biochemical studies on the murine leprosy bacillus. II. Respiration and respiration accelerating substances. *Biken's J.* **1,** 157-165.

Mori, T., Kosaka, K. and Ito, T. (1960). Detection by nile blue staining of tissue contaminants in murine leprosy bacilli preparation. *Biken's J.* **3,** 261-263

Mori, T., Kosaka, K. and Ito, T. (1961). Collection of murine leprosy bacillus. *Jap. J. Bact.* **16,** 808-813 in press (in Japanese only).

Racker, E. (1950). Crystalline alcohol dehydrogenase from baker's yeast. *J. Biol. Chem.* **184,** 313-319.

Straub, F. B. (1939). Isolation and properties of a flavoprotein from heart muscle tissue. *Biochem. J.* **33,** 787-792.

Tamemasa, O. and Tsutsumi, S. (1958). On the metabolism of Mycobacteria. 1. Dehydrogenase activity of Mycobacterium leprae murium. *Japan. J. Exptl. Med.* **28,** 183-197