BIKEN'S JOURNAL 1, 166-172 (1958)

Biochemical Studies on the Murine Leprosy Bacillus

III. Enzymic Activity

TONETARO ITO AND RYUZO SONODA

Department of Leprosy, Research Institute for Microbial Diseases, Osaka University and Department of Dermatology, Osaka University Medical School

(Received for publication, November 25, 1958)

SUMMARY

1) Enzyme activity of *Mycobacterium leprae murium* was examined in asparagine and aspartic acid, and it was found that asparagine is converted to aspartic acid with freeing of ammonia. It was also shown by studies with the Friedman-Haugen method that transamination takes places between aspartic acid and α-ketoglutaric acid to give oxalacetic acid. So murine leprosy bacillus was found to possess asparaginase and transaminase.

2) The presence of catalase and peroxydase of *Mycobacterium leprae murium* was substantiated but nitrate reductase and amylase were not found.

3) The attempt was made to adapt *Mycobacterium leprae murium* to several organic acids, but results suggestive of production or existence of adaptive enzymes were not obtained by incubation for 1 hour.

INTRODUCTION

The results of studies on substances which accelerate the respiration of murine leprosy bacilli have been reported in a previous paper and in the present report, the enzymatic activity of the bacillus was investigated. There are few studies on the enzymatic activity of the murine leprosy bacilli and only 2 or 3 are found on review of the literature. Terada et al. (1936) conducted studies with murine leprosy bacilli collected by centrifugation of leproma and reported that catalase and lypase were present but oxydase, and oxydative enzymes of glucose, galactose and levurose could not be found. Toda and Urabe (1936) conducted a similar study and confirmed the presence of catalase. Yoshinaga (1943) has shown that a minute quantity of lecithinase is also present. Recently Gray (1952) has examined the effect of more than 50 different substrates, including serum albumin, on the respiration of the murine leprosy bacillus and reported that serum albumin has a respiration accelerating action. But as an increase in ammonia which should occur on breakdown of the substrate was not found, it was suggested that this was due to an enzymatic action of the bacillus.

As stated in Report II, the respiration of the murine leprosy bacillus was intensified by asparagine. (Ito, T., Sonoda, R.; 1958) On the basis of this, studies were conducted as to whether this bacillus possesses an asparagine decomposing enzyme. Studies were also conducted on several other enzymes and on the adapta-

tion of the bacillus to some organic acids. The results are presented here.

EXPERIMENTAL

The bacilli used in the experiments were isolated from murine leprosy leproma by the method presented in Report I. (Ito, T., Sonoda, R. and Kosaka, K.; 1958)

I. *Asparagine Deamination Activity.*

The presence of asparaginase was examined by reacting murine leprosy bacillary suspension with M/10 asparagine in a Warburg manometer at 37°C for 2 hours under shaking, then measuring ammonia quantitatively with Conway's unit. (1950)

Method: Two Warburg vessels (A and B) were prepared so that each contained the following.

A

Bacillary suspension	1.0ml	(Main chamber)
M/5 phosphate buffer (pH8.0)	0.5ml	
M/10 asparagine	0.2ml	(Side arm a_1)
50% sulfuric acid	0.3ml	(Side arm a_2)

B

Bacillary suspension	1.0ml	(Main chamber)
M/5 phosphate buffer (pH8.0)	0.5ml	
M/10 asparagine	0.2ml	(Side arm b_1)
50% sulfuric acid	0.3ml	(Side arm b_2)

In vessel A, the contents of side a_1 were combined from the first while that of side arm b_1 of tube B was not and the vessels shaken for 2 hours at 37°C. After shaking, the 50% sulfuric acid solution in side arm a_2 of vessel A was tipped in and the ammonia evolved, caught as the ammonium sulfate. In vessel B, the 50% sulfuric acid in side arm b_2 was tipped in and then the contents of side arm b_1 added. This was the control.

Two Conway's units (I and II) were prepared and the following placed in the inner and outer dishes respectively.

	Inner Dish	Outer Dish
I.	2cc of N/100 H_2SO_4	2cc of content of A, then 1cc of 50% NaOH
II.	2cc of N/100 H_2SO_4	2cc of content of B, then 1cc of 50% NaOH

II was the control. In the above procedure, the lid must be fastened promptly and tightly after addition of the NaOH, before the sample and NaOH can come into contact and mix. After leaving overnight at room temperatur, the ammonia which has been transfered to the inner dish is colorized with Nessler's reagent and measured with the spectrophotometer.

Results: As shown in Table 1, the evolution of ammonia is significantly higher compared to the control when asparagine is used as the substrate, proving the presence of asparaginase.

Table 1. Ammonia Production from Asparagine by Murine Leprosy Bacilli

Experiment No.	No. 1	No. 2	No. 3
Reaction Time (min.)	120	120	120
Temperature	37°C	37°C	37°C
pH	8.0	8.0	8.0
A-Sample	4.5 μM	3.4 μM	3.0 μM
B-Sample	2.6 μM	2.6 μM	2.4 μM
Difference	1.9 μM	0.8 μM	0.6 μM

II. *Aspartic acid transamination activity*

Whether transamination between α-ketoglutaric acid and aspartic acid by the action of transaminase of murine leprosy bacilli takes place with the formation of oxaloacetic acid was examined by measurement of pyruvic acid with the Friedmann-Haugen method. (1943)

Method: The following were placed in each of three tubes, A, B and C.

	A	B	C
M/10 α-ketoglutaric acid	0.2ml	0.2ml	——
M/10 aspartic acid	0.2ml	——	0.2ml
M/5 phosphate buffer (pH 8.0)	2.0ml	2.0ml	2.0ml
Bacillary suspension	3.6ml	3.6ml	3.6ml
Distilled water	——	0.2ml	0.2ml

B and C were controls. The tubes were shaken for 2 hours at 37°C after which the reaction was stopped by adding 0.1ml of 50% acetic acid to each. The samples were then sterilized by heating the tubes in boiling water for 5 minutes at 100°C. To A was added 0.2ml of distilled water, to B was added 0.2ml of aspartic acid while 0.2ml of α-ketoglutaric acid was added to C. After centrifugation for 20 minutes at 10,000 r.p.m., the supernatant was taken and the pyruvic acid measured colorimetrically according to the Friedmann-Haugen method. The method is presented below.

1) To 5.0ml portions of A, B and C was added 1ml of previously prepared aniline citrate (equal quantities of aniline and 50% citrate combined), and the mixture shaken for 15 minutes at 37°C.

2) To each tube was then added 0.5ml of 2·4 dinitrophenyl hydrazine reagent (350mg 2·4 dinitrophenyl hydrazine dissolved in 20ml concentrated HCl by heating, distilled water added to make the total volume 100ml) and shaken for 10 minutes at 37°C.

3) Ten ml of xylol was added and the tubes shaken well by hand (about 60 times) and the pyruvic acid which has been formed transfered to the xylol layer.

4) The xylol layer was drawn off and washed by adding 5ml N/2 HCl and shaking.

5) To 5ml of the xylol layer was added 5ml of 10% sodium carbonate and shaken.

6) The sodium carbonate layer was drawn off, 2ml of 4N NaOH added, the mixture shaken and colorized. After leaving for 20-30 minutes, spectrophotometric measurements were carried out.

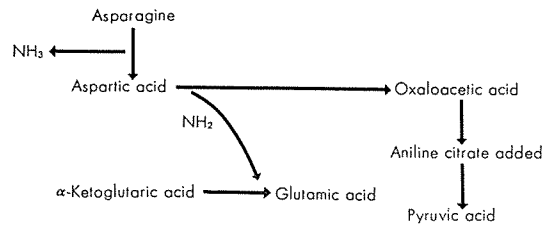
Results: As can be seen in Table 2, the enzymic activity is very strong. Experiments 3 and 4 in Table 2 show the results obtained with bacteria collected after treating with 0.1% trypsin for 1 hour at 37°C. The data suggests that the

Table 2. Transamination between Aspartic acid and α-ketoglutaric Acid

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Method of Collection of Bacilli	Centrifugation Method		Trypsin Method	
Reaction Time (min.)	120	120	120	120
Temperature	37°C	37°C	37°C	37°C
pH	8.0	8.0	8.0	8.0
A. Experimental Sample	27.6γ/ml	29.6γ/ml	17.6γ/ml	13.2γ/ml
B. No Aspartic acid	0.4γ/ml	2.4γ/ml	4.0γ/ml	4.6γ/ml
C. No α-ketoglutaric acid	0.4γ/ml	2.4γ/ml	2.8γ/ml	14.6γ/ml

aspartic acid transaminase of murine leprosy bacilli is destroyed to some extent by treatment with trypsin. Chart I shows the above chemical reactions schematically.

Fig. 1. Scheme of Transamination Process



III. *Catalase activity*

The catalase activity of the murine leprosy bacilli has already been shown by Terada et al. (1956) with the potassium permanganate method and microscopic method and has been substantiated by Toda and Urabe. (1936)

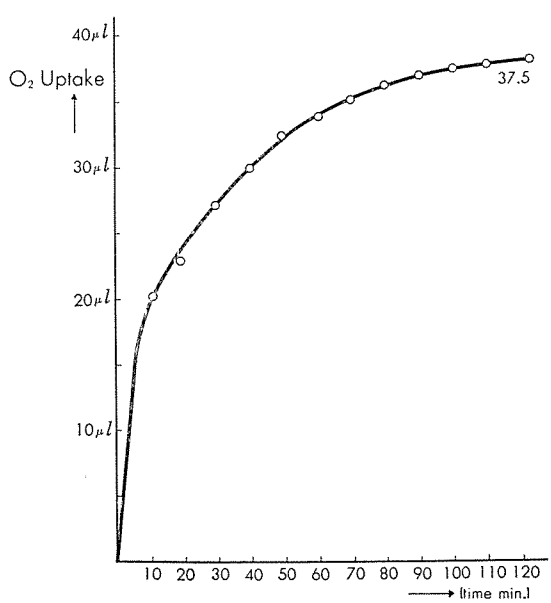
We measured the O_2 evolved quantitatively with the Warburg manometer when H_2O_2 is decomposed by murine leprosy bacilli.

Method: In the main chamber of the Warburg vessel was placed 2.0ml of bacillary suspension, and 1.0ml of M/5 phosphate buffer (pH 7.2) and 0.2ml of 20% KOH was placed in the center well while 0.4ml of 0.03% H_2O_2 was placed in the side arm. This vessel was shaken at 37°C in the water bath and the 2 hours valued read by the direct method.

Experimental Results

As can be seen in Figure 2, O_2 is evolved, showing that murine leprosy bacilli has catalase activity.

Fig. 2. Catalase Activity of Murine Leprosy Bacilli (0.03% H_2O_2 substrate)



IV. Peroxidase Activity

Activity of this enzyme was examined qualitatively on filter paper using orthotolidine.

Experimental Method

1) Small pieces of filter paper were soaked in a 500γ/ml solution of ethylene diamine tetraacetate and dried at room temperature (for the purpose of removing the catalytic action of metals contained in the paper).

2) A drop of bacillary suspension was placed in the center of the dried filter paper.

3) One drop of 10% orthotolidine-glacial acetic solution was then placed in the center of the filter paper.

4) Then 1 drop of 0.15% H_2O_2 was added.

5) When peroxidase is present a bluish-green color becomes apparent.

Results: A bluish-green spot, showing the presence of peroxidase, was obtained.

V. *Nitrate Reductase*

The following method was used to investigate this enzyme.

Method:

Reagents:

a) M/10 $NaNO_3$

b) M/10 phosphate buffer (pH7.0)

c) 1% Sulfanilamide (1g sulfanilamide dissolved in 75ml of distilled water and 25ml of conc. HCl then added)

d) 0.02% N-(1-naphthyl) ethylendiamine hydrochloride

Bacillary Suspension: 40mg/ml suspension in distilled water.

Procedure:

1) In a Tumberg's tube were placed 1.0ml of M/10 $NaNO_3$, 2.5ml of M/10 phosphate buffer and 1.5ml of bacillary suspension. The tube was attached to a vacuum pump and air drawn off.
2) In the control, 1.5ml distilled water was added in place of the baciliary suspension.
3) The mixture was shaken for 3 hours in a 37°C constant temperature room.
4) The reaction was stopped by adding 0.5ml of glacial acetic acid.
5) The mixture was filtered to remove the organisms.
6) To 1.0ml of the filtrate was added 1.0ml of 1% sulfanilamide and 1.0ml of 0.02% N-(1-naphthyl) ethylendiamine hydrochloride.
7) A red color compared to the control is seen when nitrate reducing enzyme is present.

Results: No difference in color was noted compared to the control showing that nitrate reductase was absent.

VI. *Amylase*

Amylase has already been studied by Terada et al. (1936) with the Wohlgemuth method and it has been reported that this enzyme is not present in murine leprosy bacilli. We studied this enzyme by the following method.

Method:

Reagents:

a) 0.4%, 0.2% soluble starch solution

b) M/5 phosphate buffer, pH7.0

c) Iodine solution (1g I, 2g KI,100ml distilled water)

Bacillary suspension: 40mg/ml suspension in distilled water.

Procedure:

1) To 2.0ml of 0.4% and 0.2% soluble starch solution in separate tubes were added 0.5ml M/5 phosphate buffer and 1.5ml of bacillary solution.
2) In the control, the bacillary solution was heated for 30 minutes at 85°C.
3) The mixtures were shaken for 3 hours at 38°C in a water bath.
4) The cells were removed by filtration.
5) To 2.0ml of the filtrate was added 1 drop of iodine solution
6) The purplish blue color which is produced was compared to that of the control.

Results:

No difference was noted between the control and the 0.4% or 0.2% starch solution. In other words, amylase was not present as reported by Terada et al.

VII. *Adaptation of the Murine Leprosy Bacillus to Some Organic Acids.*

The murine leprosy bacillus was adapted to some of the organic acids involved in the TCA cycle. The effect of these organic acids on the respiration of the

adapted bacilli, when used as the substrate, was measured by the direct method with the Warburg manometer. Results suggesting the production or presence of adaptive enzymes towards the organic acids were not obtained.

Method :

Substrate :

(a) Sodium citrate,
(b) succinic acid,
(c) glutamine,
(d) asparagine,
(e) aspartic acid.

Bacillary solution : 40mg/ml suspension in distilled water.

Procedure :

The bacilli were adapted by combining bacillary suspension, M/5 phosphate buffer (pH7.2) and M/10 substrate in a test tube in a ratio of 10 : 4 : 1 and shaking for 1 hour at 37°C.

The mixture was then centrifuged at 10,000 r.p.m. for 20 minutes, the sediment washed several times with distilled water by centrifuging at 10,000 r.p.m. for 20 minutes to remove all the substrate and the final sediment resuspended in distilled water.

The oxygen consumption of the treated bacilli was measured with the Warburg manometer according to the mehod presented in Report II using the various organic acids as the substrate.

Results :

As shown in Table 3, adaptaion phenoemnon was not observed.

Table 3. Adcptatcn of Murine Leprosy Bacilli to Organic Acids

Substrate	Adaptation Phenomenon
M/10 sodium citrate	Negative
M/10 succinic acid	〃
M/10 glutamine	〃
M/10 asparagine	〃
M/10 asparaginic acid	〃

DISCUSSION

Many reports have already been published on the enzyme systems of the tubercle bacillus but studies in this field are few in the case of leprosy and murine leprosy due to the inability of obtaining pure cultures of these organisms.

Hanks and Gray (1951, 1954) have measured the hydrogen transfer capacity of murine leprosy bacilli isolated from the leproma and the effect of various substrates on the internal metabolism of the bacilli is discussed but the enzymic activity related to external metabolism has not been considered.

In a previous experiment it was noted that asparagine accelerated the respiration of the murine leprosy bacillus and on the basis of this, experiments were conducted on the enzymic activity. It was found that asparagine is converted to aspartic acid with the evolvement of ammonia by the action of the bacillus and furthermore transamination takes place between the aspartic acid and α-ketoglutaric acid and oxaloacetic acid is produced.

In other words, the presence of asparaginase and transaminase was confirmed. This finding strengthens our assumption that the murine leprosy bacilli, though weak, possesses a metabolic system similar to other mycobacteria, and is believed to be of value in studies of this type.

Studies were also conducted on catalase, peroxydase, nitrate reductase and amylase. Catalase and peroxydase were proven to be present but the others could not be found. The production of adaptive enzymes by the bacillus against several organic acids related to the TCA cycle was investigated but no enzyme production was found on incubation for 1 hour. Enzyme production however, may take place with a longer period of incubation but this must await further investigation. This shows that external metabolism by murine leprosy bacilli is difficult but in view of the fact that respiration is accelerated by the addition of liver or testis extract to succinic acid which alone had no accelerating effect it is suggested that aspartic acid and oxaloacetic acid may be utilized as a substrate of some coenzyme is added.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Fujinami of the Department of Dermatology, Dr. Nishimura of the Department of Leprosy, Research Institute for Microbial Diseases and Dr. Sakamoto of the Department of Biochemistry for their advice in conducting this study.

REFERENCES

1) Conway, E. J., (1950) "*Microdiffusion Analysis and Volumetric Error*", London, Crosby Lockwood and Son Ltd.

2) Friedeman, T. and G. E. Haugen (1943) Pyruvic Acid II. The Determination of Keto Acid in Blood and Urine. *J. Biol. Chem.* **62,** 529-537.

3) Gray, C. T. (1952) The respiratory Metabolism of Murine Leprosy Bacilli. *J. Bact,* **64,** 305-313.

4) Hanks, J. H. (1951) The Biological Significance of the Hydrogen Transfer Capacity of Murine Leprosy Bacilli. *J. Bact.* **62,** 529-537.

5) Hanks, J. H. and C. T. Gray (1954) The Application of Metabolic Studies to Leprosy Research. *Int. J. Leprosy* **22,** 147-161.

6) Ito, T., R. Sonoda and K. Kosaka (1958) Biochemical Studies on Murine Leprosy Bacilli. (I) collection of Bacilli from Infected Tissue. *Med. J. of Osaka University* **8,** No. 4, 633-638.

7) Ito, T. and R. Sonoda (1958) Biochmical Studies on the Murine Leprosy Bacillus. (II) Respiration and Respiration Accelerating Substances. Biken's J., 157-165.

8) Terada, M. et al. (1936) Studies on Human Leprosy, Murine Leprosy and other Acid-fast Bacilli. Biological Studies on the Human and Murine Leprosy Bacilli. *Tokyo Iji Shinshi* 2978, 1153-1157 ibid 2979, 1228-1230. (in Japanese)

9) Toda and Urabe (1936) Studies on Acid-fast Bacilli Enzymes (VI) Acid-fast Bacilli. *Tokyo Iji Shinshi* 2981, 1361-1364. (in Japanese)

10) Yoshinaga, T. (1943) Lecithinase of Acid-fast Bacilli. *Lepra* **14,** 283-297. (in Japanese)