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Absence of β-Glucuronidase in *Mycobacterium leprae* and Elevation of the Enzyme in Infected Tissues

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β-Glucuronidase activity was determined in mouse footpads infected with *Mycobacterium leprae,* in the leprosy organisms separated from the liver and spleen of experimentally infected armadillos, and in the armadillo tissues. Enzyme assays in the mouse footpads were initiated 1 week after inoculation with *M. leprae* and continued at monthly intervals for 12 months. In the mouse footpads and in the armadillo tissues, *M. leprae* infection resulted in remarkable elevations of β-glucuronidase levels. The leprosy bacilli seemed to be devoid of the enzyme. In its properties like pH optimum, reaction velocity and effect of inhibitors, the activity detected in *M. leprae* resembled the host tissue enzyme rather than bacterial β-glucuronidase; and the activity was found to be superficially adsorbed on the bacilli. It is well established that phagocytes are rich in lysosomal enzymes. Evidently, the increased β-glucuronidase of the infected tissues is not derived from the invading organisms, but from the different types of phagocytic cells infiltrating the tissues.

Introduction

β-Glucuronidase is an important hydrolytic enzyme ubiquitously distributed in animal tissues and in tissue fluids. Phagocytic cells are especially rich in β-glucuronidase. In the mammalian liver, the enzyme is largely associated with lysosomes, and approximately one-third of the activity is distributed in the endoplasmic reticulum. The hydrolase is closely correlated with cellular proliferation and tissue repair; high levels of the enzyme are found in the reproductive and endocrine organs and in tumours. Skin cancers as well as normal skin contain β-glucuronidase. The enzyme has been reported in molluscs and in insects. Among bacteria, *Escherichia coli, Streptococcus pyogenes, Corynebacterium xerose, C. hoffmani* and rumen micro-organisms have been shown to produce β-glucuronidase (Fishman, 1955; Levy and Marsh, 1959). In bacterial infections characterized by intracellular parasitism of the invading organisms, marked elevation of hydrolytic enzymes has been observed (Allen, 1969).

β-Glucuronidase splits off D-glucuronic acid residues from oligosaccharides, thus degrading these complex molecules. In the body, glucuronic acid forms

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conjugates of hormones, drugs and other substances, thus serving a detoxification function; also β-glucuronidase can hydrolyze such conjugates and release the organic compounds (Fishman, 1955). Because of their degradative activities, lysosomal enzymes, including β-glucuronidase, have a major role in the killing and digestion of ingested bacteria (Allen, 1969). In leprosy, as in certain other mycobacterial infections, a paradoxical situation occurs; the bacteria, instead of being destroyed by the phagocytes, seem to be protected in these cells (Goren, 1977; Brown *et al.,* 1969).

No significant elevation of β-glucuronidase was observed in peripheral blood leucocytes of leprosy patients (Avila and Convit, 1970; Garcia-Gonzalez *et al.,* 1977). Increase in acid phosphatase was demonstrated histochemically in histiocytes containing *Mycobacterium leprae* (Brieger and Allen, 1962). Based on histochemical evidence, it was concluded that *M. leprae* possesses β-glucuronidase (Matsuo and Skinsnes, 1974). In the course of our investigations over the past several years on lysosomal enzymes in leprosy, we studied the levels of acid phosphatase and β-glucuronidase in mouse footpads and in tissues of armadillos experimentally infected with *M. leprae.* We also determined β-glucuronidase activity in concentrates of the bacilli separated from infected armadillo tissues. Because acid phosphatase is extremely labile, its assay was found to be unreliable in tissues stored for varying periods of time. As such, acid phosphatase assays were not pursued further. However, β-glucuronidase gave consistent and reproducible results. The data presented in this report show that *M. leprae* purified from infected armadillo tissues apparently does not contain β-glucuronidase; in the mouse footpad, the enzyme activity continues to rise as the infection progresses; and in the infected armadillo tissues, the enzyme levels are elevated 2 to 3 times over that of the uninfected tissues.

Materials and Methods

ANIMALS AND MYCOBACTERIA

Approximately 8-week-old female Swiss mice of the NIH strain were used in the study. The experimental animals were inoculated in the left hind footpads with 1×10^4 *M. leprae.* The *M. leprae* used was a mouse-passage strain originally obtained from the skin biopsy of an untreated lepromatous patient. Control mice were of the same age and sex. Starting at 1 week after inoculation, a few mice (usually 5) were sacrificed at monthly intervals for 12 months. The soft tissues from the left hind footpads of both the experimental and the control animals were collected and stored at −80°C. At the time of assay, the material was thawed out, washed in cold saline, blotted and minced. The tissues were extracted in small amounts of water by grinding in a chilled agate mortar. The extract was centrifuged briefly, to remove the particulate matter. Enzyme determinations were made in the supernatant fraction. Protein was estimated by the method of Lowry *et al.* (1951). Enumeration of *M. leprae* in the mouse footpad was done at 6 months and 12 months, by the method of Hanks *et al.* (1964), modified by Kirchheimer.

Liver and spleen of "normal" and infected armadillos were collected

aseptically at autopsy and were held at 0°C or at −80°C before processing. Ten percent homogenates of these organs were made in water. Suspensions of the leprosy bacilli were prepared as reported before (Prabhakaran *et al.,* 1976) from the liver or spleen of 9-banded armadillos experimentally infected with *M. leprae* (Kirchheimer and Storrs, 1971; Kirchheimer *et al.,* 1972). The bacilli were disrupted by ultrasonic oscillation (Prabhakaran *et al.,* 1973). In an effort to remove superficially adsorbed host-tissue materials from the bacterial concentrate, it was treated with trypsin (final concentration 0.5%), NaOH (0.1 N), sodium dodecyl sulfate (1%), or sodium deoxycholate (0.5%) for 30 min at 0°C, except for trypsin, which was done at 37°C.

Mycobacterium phlei was grown in Proskauer-Beck medium (Youman's modification) at 37°C for 2 weeks. The bacilli were harvested by centrifugation and washed twice with cold saline and once with deionized glass-distilled water.

ENZYMES AND CHEMICALS

Purified *E. coli* β-glucuronidase (Type VII), bovine liver β-glucuronidase (Type B-10), phenolphthalein glucuronic acid (sodium salt) and glycine buffer were purchased from the Sigma Chemical Co., St Louis, MO, USA. Other chemicals used were of the highest purity commercially available.

ENZYME ASSAY

The enzyme assay was done essentially by the standard procedure of Talalay *et al.* (1946). The reaction mixture consisted of the following constituents: 0.1 M acetate buffer (pH 4.7), 0.4 ml; 0.01 M phenolphthalein glucuronic acid, 0.1 ml; sample, 0.5 ml. The sample blank contained 0.5 ml buffer and 0.5 ml sample, and the reagent blank 0.9 ml buffer and 0.1 ml substrate. For *E. coli* β-glucuronidase, 0.075 M phosphate buffer (pH 6.8) was substituted for acetate buffer; 40 units of the enzyme were added. The mycobacterial suspensions used contained $2–5 \times 10^9$ organisms/ml. The final concentration of the inhibitors tested was 0.005 M. After the reaction mixture was incubated at 37°C for 60 min, 1 ml of 5% TCA was added, followed by 2.5 ml of alkaline glycine reagent. Volume was made up to 6 ml with 1.5 ml of water. The reaction mixture was centrifuged and the purple colour of the supernatant fraction was read at 540 nm in a Beckman DU-2 spectrophotometer. The readings were corrected for any absorbance due to the sample or reagent blanks. The amount of phenolphthalein liberated from the substrate by the enzyme was calculated from a standard curve. All reactions were carried out in duplicate and each experiment was done at least 3 times. The results reported are mean values of representative experiments.

Results

MOUSE FOOTPADS

M. leprae inoculated in the mouse footpads multiplied normally; enumeration of the bacilli showed $2.4 \pm 0.18 \times 10^6$ organisms per footpad in 6

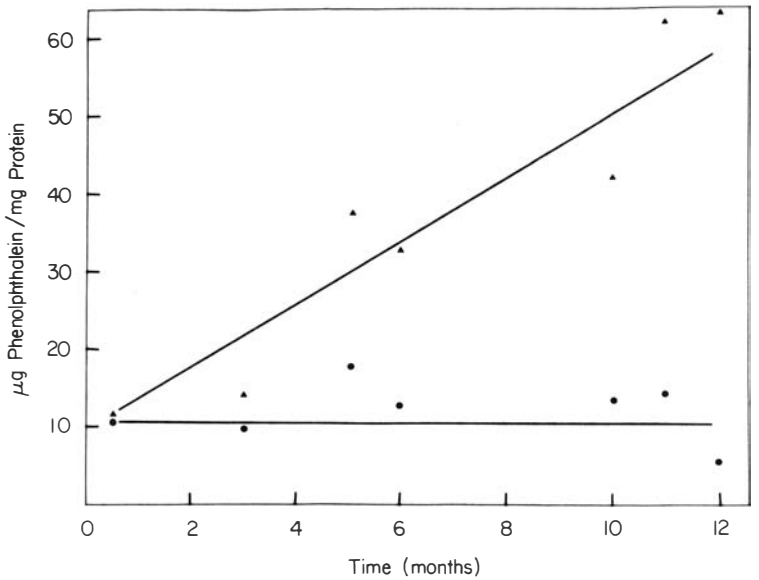

Fig. 1. β-glucuronidase of mouse footpads infected with *M. leprae*. (▲—▲) Experimental; (●—●) control.

months, and $1.8 \pm 0.15 \times 10^7$ in 12 months. β-Glucuronidase activity was assayed in the footpad tissues at monthly intervals for 12 months. To correct for variations in individual assays, the values obtained for both the control and the experimental samples were subjected to regression analysis. The results are presented in Fig. 1. In the uninfected mice, the enzyme activity remained fairly stable throughout the experiment. However, β-glucuronidase continued to rise in the footpads of mice where *M. leprae* multiplied progressively. These results do not indicate whether the higher activity is derived from the bacilli or from the cells that infiltrate the infected footpads.

ARMADILLO TISSUES

β-Glucuronidase was determined in the liver and the spleen tissues of both the infected and the uninfected armadillos. (On the average, the infected organs

TABLE 1

β-Glucuronidase in "normal" and infected spleen tissue of armadillos: μg *of phenolphthalein released/*mg *protein*

Number of animals	"Normal"	Infected
1	32.2	92.1
2	37.7	173.5
3	44.4	110.5
Mean	38.1	125.4

TABLE 2
β-Glucuronidase in "normal" and infected liver tissue of armadillos: µg *of phenolphthalein released/*mg *protein*

Number of animals	"Normal"	Infected
1	77.4	188.9
2	162.0	200.0
3	118.4	286.3
4	130.8	234.4
Mean	122.2	227.4

contained over 10^9 *M. leprae* per g of tissue.) Compared to the controls, the activity in the infected spleen was elevated by over 3 times, and by almost 2 times in the liver (Tables 1 and 2).

M. LEPRAE

It was not clear whether the source of the elevated enzyme activity observed was the invading organisms or the host tissues. To ascertain this, *M. leprae* was separated from both the spleen and the liver tissues and β-glucuronidase activities determined in the bacterial preparations. The preparative procedure involved differential and density-gradient centrifugations in solutions of sucrose and KCl (Prabhakaran *et al.,* 1976). When assayed without further purification, these bacterial suspensions contained β-glucuronidase. When the bacilli were disrupted by ultrasonic oscillation, more enzyme acitivity was not released. Both intact and disrupted organisms gave similar results. When the disrupted suspension was fractionated, the supernatant contained a greater amount of β-glucuronidase than the particulate fraction. Additional washing resulted in further loss of enzyme activity from the bacterial particles (Table 3). Evidently the enzyme is not firmly attached to the bacterial membranes.

TABLE 3
Distribution of β-glucuronidase in M. leprae *disrupted by ultrasonic oscillation:* µg *of phenolphthalein released*

Sample	Unheated	Heated
Unfractionated suspension	47.0	0
Particulate fraction	15.0	0
Supernatant fraction	20.0	0
I wash of particulate fraction	5.0	0

To determine whether the activity was due to enzyme molecules superficially adsorbed on the bacilli, intact *M. leprae* preparations were treated with trypsin, NaOH and 2 detergents. Trypsin-treatment removed over 50% of the activity from the bacilli. NaOH and sodium dodecylsulfate produced total inactivation, and only a residual activity was left on treatment with deoxycholate (Table 4). Probably, β-glucuronidase is not an inherent property of the leprosy bacilli, but is only superficially attached to the organisms.

TABLE 4
Effect of different treatments on β-glucuronidase of M. leprae: μg *of phenolphthalein released*

Treatment	Untreated bacilli	Treated bacilli
Ultrasonic oscillation	29.0	28.0
Trypsin	66.0	37.0
Deoxycholate	46.0	7.0
Sodium dodecyl sulfate	46.0	0
NaOH	65.0	0

TABLE 5
β-Glucuronidase in M. phlei: μg *of phenolphthalein released*

Sample	Unheated	Heated
Intact bacilli	0	0
Disrupted bacilli	0	0

Another mycobacterium tested, *M. phlei,* both intact and disrupted had no β-glucuronidase (Table 5).

pH OPTIMA

In order to characterize the enzyme activity detected in the untreated *M. leprae* suspensions, properties of the enzyme were compared with those of β-glucuronidase of *E. coli* and of mammalian liver. Bacterial and mammalian β-glucuronidases are distinguished from one another by their pH optima. With phenolphthalein glucuronide as substrate, optimal hydrolysis by the mammalian enzyme takes place at pH 4.5–5.2, and by the bacterial enzyme at pH 6–7 (Levy and Marsh, 1959). The pH-activity curve of the enzyme in armadillo liver is shown in Fig. 2. The pH optima for the β-glucuronidase of *E. coli* and of the *M. leprae* suspension are given in Fig. 3. It may be seen that the activity detected in *M. leprae* resembles closely that of the host tissue, rather than the bacterial enzyme.

REACTION VELOCITY AT HIGH SUBSTRATE LEVELS

Increasing substrate concentrations resulted in a pronounced inhibitory effect on *E. coli* β-glucuronidase. No such effect was detected with mammalian β-glucuronidase or with *M. leprae*; the results obtained with armadillo liver and with the leprosy bacilli gave hyperbolic curves. The reaction velocity of the liver enzyme is shown in Fig. 4. The results obtained with *E. coli* and *M. leprae* are presented in Fig. 5. It is evident that the activity in the leprosy bacilli is similar to that of the host tissue enzyme.

Figure 6 represents the double-reciprocal Lineweaver-Burk plot of the reaction velocity of *E. coli* β-glucuronidase. K_m value of the enzyme was found to be 8.0×10^{-5} M. This compares favourably with the K_m of 7.5×10^{-5}M reported for sheep rumen bacteria (Levy and Marsh, 1959),

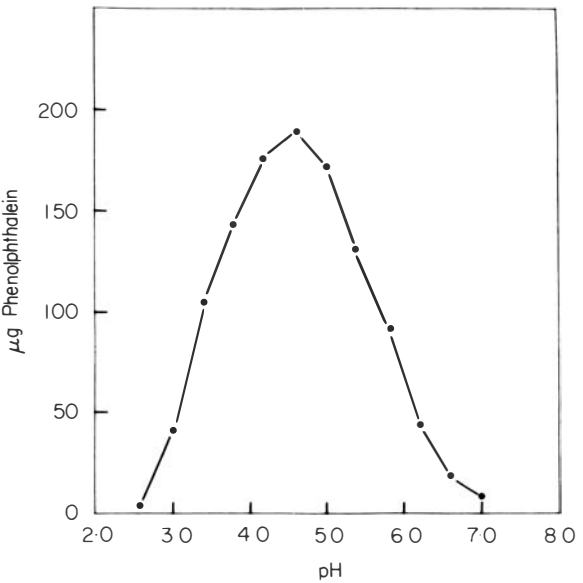

Fig. 2. pH-Activity curve: armadillo liver.

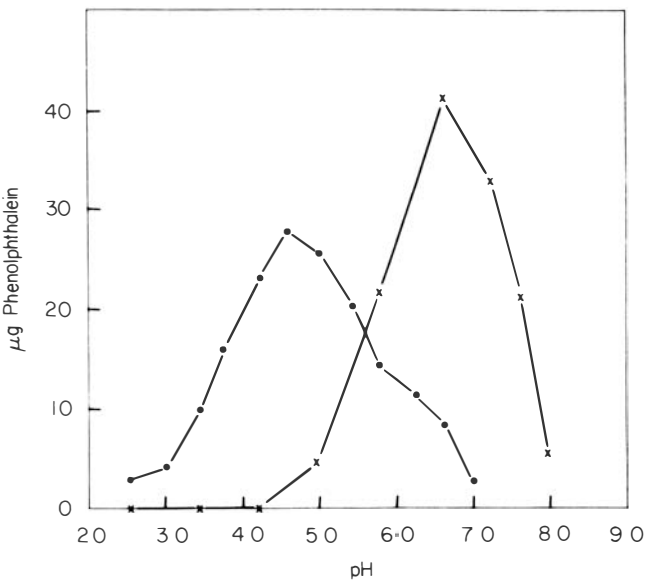

Fig. 3. pH-Activity curve: *M. leprae* and *E. coli*. (●—●) *M. leprae*; (×—×) *E. coli*

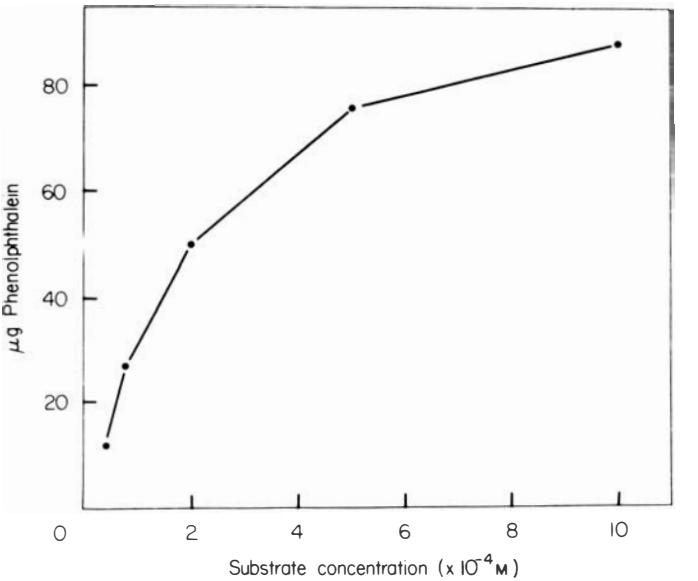

Fig. 4. Reaction velocity at high substrate levels: armadillo liver.

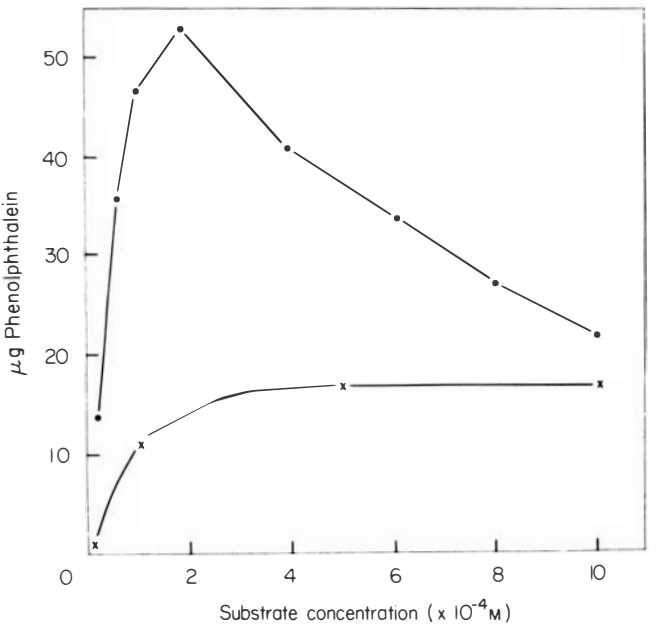

Fig. 5. Reaction velocity at high substrate levels: *M. leprae* and *E. coli*. (✖—✖) *M. leprae*; (●—●) *E. coli*.

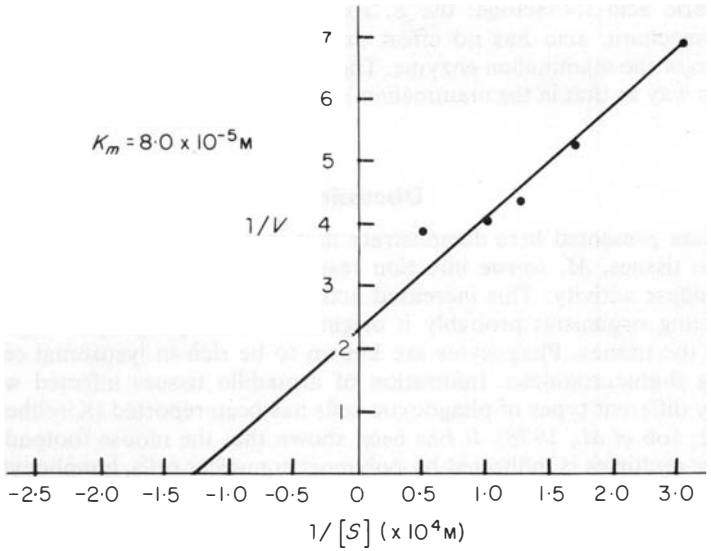

Fig. 6. *E. coli* β-glucuronidase: double-reciprocal plot of substrate concentration *versus* reaction velocity.

confirming the reliability of the assay procedure we adopted for β-glucuronidase. However, it may not be valid to derive such kinetic data with crude preparations like tissue homogenates and the *M. leprae* suspensions.

INHIBITORS

In addition to the above criteria, bacterial and mammalian β-glucuronidases are differentiated by the effects of inhibitors on the enzymes. Contrary to what has been reported recently (Matsuo *et al.*, 1975), ascorbic acid by itself has no inhibitory effect on β-glucoronidase (Levy and Marsh, 1959). We tested 2 specific inhibitors of the enzyme *M. leprae* suspensions, armadillo liver homogenates, and purified bovine liver and *E. coli* β-glucuronidases. The results are given in Table 6. The mammalian enzyme is completely inhibited by

TABLE 6
Effect of inhibitors on β-glucuronidase: μg *of phenolphthalein released*

Sample	D-Saccharic acid-1,4-lactone			Galactosaccharic acid		
	−Inhibitor	+Inhibitor	% Inhibition	−Inhibitor	+Inhibitor	% Inhibition
Armadillo liver	115	0	100	99	26	74
M. leprae	32	0	100	37	5	87
β--glucuronidase (beef liver)	192	0	100	110	11	90
β--glucuronidase (*E. coli*)	56	14	75	59	59	0

D-saccharic acid-1,4-lactone; the *E. coli* enzyme is inhibited only partially. Galactosaccharic acid has no effect on *E. coli,* whereas it shows 70–90% inhibition of the mammalian enzyme. The activity present in *M. leprae* behaves the same way as that in the mammalian liver.

Discussion

The data presented here demonstrate that in mouse footpads as well as in armadillo tissues, *M. leprae* infection results in a significant elevation of β-glucuronidase activity. This increased activity is apparently not derived from the infecting organism; probably it originates in the phagocytic cells which infiltrate the tissues. Phagocytes are known to be rich in lysosomal enzymes including β-glucuronidase. Infiltration of armadillo tissues infected with *M. leprae* by different types of phagocytic cells has been reported (Kirchheimer *et al.,* 1972; Job *et al.,* 1978). It has been shown that the mouse footpad where *M. leprae* multiples is infiltrated by polymorphonuclear cells, lymphocytes and monocytes (Evans *et al.,* 1973).

The activity detected in the *M. leprae* separated from the armadillo tissues seems to be of host-tissue origin. In its properties like pH optima, reaction velocity and effect of inhibitors, the activity in the bacilli resembled β-glucuronidase derived from mammalian tissues rather than the bacterial enzyme. Moreover, the enzyme seemed to be superficially adsorbed on the organisms, and could be easily removed by various treatments. Apparently, β-glucuronidase is not an intrinsic property of *M. leprae.* This conclusion is supported by the finding that another lysosomal enzyme, acid phosphatase, detected in *in vivo*-grown tubercle bacilli is derived from the host cells (Kanai, 1969). The precise role of the lysosomal acid hydrolases in leprosy infection remains to be elucidated.

References

Allen, J. M. (1969). Lysosomes in bacterial infection. *Lysosomes in Biology and Medicine,* Vol. 2, pp. 41–68. J. T. Dingle and H. B. Fell, Eds. North-Holland Publishing Co., Amsterdam.

Avila, J. L. and Convit, J. (1970). Studies on cellular immunity in leprosy. 1. lysosomal enzymes. *Int. J. Lepr.* **38,** 359.

Brieger, E. M. and Allen, J. M. (1962). Cytopathological changes in lepra cells. *Expt Cell Res.* **28,** 438.

Brown, C. A., Draper, P. and D'Arcy Hart, P. (1969) Mycobacteria and lysosomes: a paradox. *Nature* (Lond.) **221,** 658.

Evans, M. J., Newton, H. E. and Levy, L. (1973). Early response of mouse footpads to *Mycobacterium leprae. Infect. Immun.* **7,** 76.

Fishman, W. H. (1955). β-Glucuronidase. *Advances in Enzymol.* **16,** 361.

Garcia-Gonzalez, J. E., Rojas-Espinosa, O. and Estrada-Parra, S. (1977). Phagocytosis in leprosy. 1. The levels of diaphorase, β-glucuronidase, acid phosphatase and lipase in circulating leukocytes. *Lepr. Rev.* **48,** 17.

Goren, M. B. (1977). Phagocyte lysosomes: interactions with infectious agents, phagosomes, and experimental perturbations in function. *Ann. Rev. Microbiol.* **31,** 507.

Hanks, J. H., Chatterjee, B. R. and Lechat, M. F. (1964). A guide to the counting of mycobacteria in clinical and experimental materials. Int. J. Lepr. **32,** 156.

Job, C. K., Kirchheimer, W. F. and Sanchez, R. M. (1978). Liver lesions in experimental lepromatoid leprosy of the armadillo—a histopathological study. *Int. J. Lepr.* **46,** 1.

Kanai, K. (1967). Detection of host-originated acid phosphatase on the surface of *in vivo*-grown tubercle bacilli. *Jap. J. Med. Sci. Biol.* **20,** 73.

Kirchheimer, W. F. and Storrs, E. E. (1971). Attempts to establish the armadillo *(Dasypus novemcinctus* Linn.) as a model for the study of leprosy. I. Report of lepromatoid leprosy in an experimentally infected armadillo. *Int. J. Lepr.* **39,** 692.

Kirchheimer, W. F., Storrs, E. E. and Binford, C. H. (1972). Idem. II. Histopathologic and bacteriologic post-mortem findings in lepromatoid leprosy in the armadillo. *Int. J. Lepr.* **40,** 229.

Levy, G. A. and Marsh, C. A. (1959). Preparation and properties of β-glucuronidase. *Advan. Carbohydr. Chem.* **14,** 381.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265.

Matsuo, E. and Skinsnes, O. K. (1974). Acid mucopolysaccharide metabolism in leprosy. 2. Subcellular localization of hyaluronic acid and β-glucuronidase in leprous infiltrates suggestive of a host-*Mycobacterium leprae* metabolic relationship. *Int. J. Lepr.* **42,** 399.

Matsuo, E. and Skinsnes, O. K. (1974). Acid mucopolysaccharide metabolism in leprosy. 2. mycobacterial gtowth enhancement, and growth suppression by saccharic acid and vitamin C as inhibitors of β-glucuronidase. *Int. J. Lepr.* **43,** 1.

Prabhakaran, K., Harris, E. B. and Kirchheirner, W. F. (1973). Particulate nature of *o*-diphenoloxidase in *Mycobacterium leprae* and assay of the enzyme by the radioisotope technique. *Microbios* **8,** 151.

Prabhakaran, K., Harris, E. B. and Kirchheimer, W. F. (1976). Hypopigmentation of skin lesions in leprosy and occurrence of *o*-diphenoloixdase in *Mycobacterium leprae. Pigment Cell,* Vol. 3, pp. 152–164. V. Riley, Ed. S. Karger, Basel.

Talalay, P., Fishman, W. H. and Huggins, C. (1946). Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. *J. Biol. Chem.* **166,** 757.