N-Acetyl-β-glucosaminidase, β-Glucuronidase and Acid Phosphatase in Mycobacterium leprae

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N-Acetyl-β-glucosaminidase, β-glucuronidase and acid phosphatase activities were detected in cell-free extracts of Mycobacterium leprae (from armadillo liver). Extracts of bacteria which had been treated with 7-diazonaphthalene-1,3-disulphonic acid to inactivate surface enzymes retained 30–45% of the activity of the glycosidases and 15% of the activity of the acid phosphatase. When intact bacteria were treated with 1 M-NaOH, the corresponding activity in the extracts was 4–9% for the glycosidases and 7% for the acid phosphatase. Inhibition studies with lactones and the use of concanavalin A–agarose showed differences between the glycosidases in extracts of M. leprae and those of armadillo liver. Inhibition studies with vanadate using extracts from NaOH-treated bacteria and extracts of armadillo liver showed differences between the acid phosphatases. Enzymes removed from the surface of M. leprae could have been adsorbed to the surface from host tissue (i.e. lysosomal enzymes) or they could have been extracellular enzymes or associated with the bacterial membrane.

INTRODUCTION

Very few enzymes of Mycobacterium leprae have been identified because of the problem of obtaining sufficient bacteria from tissues; growth in vitro remains an intractable problem, but much more M. leprae is now available from infected armadillos than from the only previous source, human biopsies. However, Skinsnes and co-workers demonstrated by histochemical methods the association of N-acetyl-β-glucosaminidase (EC 3.2.1.30; 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase) and β-glucuronidase (EC 3.2.1.31; β-D-glucuronide glucuronosohydrolase) with M. leprae in tissue from lepromatous leprosy patients (Matsuo & Skinsnes, 1974). They went on to show that hyaluronic acid had a 'growth priming effect' on M. leprae growing in mice, and that inhibitors of β-glucuronidase inhibited growth of M. leprae in mice (Matsuo et al., 1975). Although they showed that hyaluronic acid in sections of tissues infected with leprosy was host-derived (Matsuo & Skinsnes, 1974) they did not attempt to locate hyaluronidase (EC 3.2.1.36; hyaluronate 3-glycanohydrolase). Apart from the work by Skinsnes and co-workers, very little is known about glycosidases in mycobacteria.

Recently, the evidence for β-glucuronidase in M. leprae has been challenged (Prabhakaran et al., 1978) so we decided to study these glycosidases using unbroken M. leprae and extracts. Since acid hydrolases might be important in the metabolism and utilization of substrates we also studied acid phosphatase (EC 3.1.3.2; orthophosphoric-monoester phosphohydrolase: acid pH), which has been assayed in many mycobacteria, generally with the aim of classification.

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Except for hyaluronidase, all these hydrolases also occur in lysosomes from host tissue (De Duve, 1963). It has been shown that two lysosomal enzymes, acid phosphatase (Kanai, 1967a) and catalase (Wheeler & Gregory, 1980), were adsorbed to the surface of mycobacteria grown in vivo. It was therefore important, once activity of these hydrolases had been observed, to establish that each hydrolase was in fact from bacteria; enzyme activities from armadillo liver extracts (from which M. leprae was harvested) and from M. leprae (sometimes bacteria treated to inactivate superficially adsorbed enzymes from host tissue — were used) were compared, principally by using inhibitors.

**METHODS**

Organisms and bacterial extracts. *Mycobacterium leprae* and *Mycobacterium phlei* suspensions were obtained as described previously (Wheeler & Gregory, 1980). *Mycobacterium microti* OV254 was grown in modified Dubos medium and washed three times by centrifugation and resuspension in 1.5 mM-N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid (HEPES) plus 1 mM-MgCl₂, adjusted to pH 7.2 with KOH (HEPES/Mg buffer). Cell-free extracts were prepared by sonication of suspensions of 1-8 mg bacteria ml⁻¹ as described previously (Wheeler & Gregory, 1980) when sonicating *M. leprae*, but a 10 ml beaker was sometimes used to contain suspensions of *M. phlei* and *M. microti*. In all cases several sonication periods of less than 3 min were used and the apparatus was cooled on ice. At least 99.5% of the bacteria were broken. The sonicated material was centrifuged at 20 000 g for 10 min and the supernatant was re-centrifuged for 10 min to remove the remaining bacteria and debris. The final supernatant (extract), containing 0-25–2.0 mg protein ml⁻¹ was stored at −80 °C. For some experiments involving unbroken bacteria, *M. leprae* purified from γ-irradiated (90 Co; 2-5 mRad) liver was used instead of live *M. leprae*.

Armadillo liver extract. The method described previously was used to prepare an extract from armadillo liver (Wheeler & Gregory, 1980). Additionally, an infected Tris-treated armadillo liver extract was prepared from the supernatant of the first homogenate (0-2 mM-Tris; pH 8.7) from the preparation of pure *M. leprae* (Report, 1979) by the addition of HEPES and EDTA (to 20 mM and 10 μM, respectively) and adjustment to pH 7.8 with 1 mM citric acid followed by dialysis and lyophilization as described by Wheeler & Gregory (1980). Uninfected Tris-treated armadillo liver extract to which Tris (final concentration 0-2 mM) was added and adjusted to the pH recorded (pH 8.7) in the first stage of homogenization of infected liver. After 60 min (the duration of the first stage of homogenization), the pH was adjusted to pH 7.8 with 1 mM-citric acid and the material was used immediately or stored at −20 °C.

Fractionation of extracts. Some of the extracts of both armadillo liver and *M. leprae* were fractionated by centrifugation at 105 000 g for 1 h at 4 °C. The supernatant was collected and referred to as the soluble fraction. The pellet was resuspended in HEPES/Mg buffer and re-centrifuged at 105 000 g for 1 h; the resulting pellet was resuspended in HEPES/Mg buffer to the original volume before fractionation — this fraction was referred to as the membrane pellet.

Surface treatment of *M. leprae*. NaN₃ treatment was carried out by suspending *M. leprae* in 1 M-NaOH for 1 h or in 0-1 M-NaOH for 30 min using 2 mg dry wt bacteria ml⁻¹ at 25 °C. The suspensions were neutralized with 1 M-HEPES. Suspensions of *M. leprae* were also diazotized with 7-diazaenaphthalene-1,3-disulphonic acid (ANDS) prepared as described by Pardee & Watanabe (1968). ANDS (100 μl) was added to 750 μl bacterial suspension (5–10 mg dry wt ml⁻¹) and the reaction mixture was immediately adjusted to pH 7.0. After incubation at 25 °C for 1 h, 30 ml buffered Tween 80 (0.1% Tween 80 in 1.5 mM-2-(N-morpholino)ethanesulphonic acid (MES), pH 7.0) was added. Treated bacterial suspensions were centrifuged at 10 000 g for 10 min and the pellets were washed three times and suspended in HEPES/Mg buffer. The supernatant from the unbroken NaN₃-treated bacteria was desalted and concentrated to 2 ml using an Amicon CF-25 centrifuge ultrafiltration cone (700 g, 4 °C).

Enzyme assays. Acid phosphatase (Ohmori, 1937) was assayed with 5 mM-p-nitrophenyl phosphate in 0-1 M-citrate, pH 5.5 or pH 6.5. N-Acetyl-β-glucosaminidase (Findlay et al., 1958) was assayed with 5 mM-p-nitrophenyl-β-glucosaminide in 0-1 M-citrate, pH 4.4, and β-glucuronidase was assayed with 5 mM-p-nitrophenyl-β-glucuronide in 0.1 M-citrate, pH 4.4. All incubations were carried out in 150 μl for 5 h at 37 °C, then terminated by adding 300 μl 0.5 M-Na₂CO₃, or, for turbid solutions, 300 μl 0-7 M-NaOH. In the latter case, in incubations with nitrophenyl glycosides, the absorbance was read at 5 min intervals after the addition of NaOH and the absorbance at the time of adding NaOH (which slowly hydrolyses nitrophenyl glycosides) was calculated. All experiments with extracts included controls in which the extract and substrate were incubated separately for 5 h and combined just before the addition of alkali.
Hyaluronidase was assayed by the method of Linker (1974). Since this method relied on the detection of N-acetylglucosamine end-groups in oligosaccharides or free N-acetylglucosamine (Reissig et al., 1955), it was modified for use as an alternative method for assaying β-glucuronidase and N-acetyl-β-glucosaminidase. Hyaluronic acid (Koch-Light; 1-4 mg ml⁻¹) in 0-10 M-citrate, pH 4-4, was incubated with bovine testicular hyaluronidase (Sigma; 10⁻³ units) for 3 h, then cell-free extract (or distilled water as a control) was added to bring the concentrations of hyaluronic acid and citrate to 1 mg ml⁻¹ and 0-07 M, respectively. Incubation was carried out for 100 min, then the difference between the incubation including extract and the control was calculated.

Enzyme activities are expressed as units (U); one unit hydrolysed 1 µmol substrate min⁻¹. Specific activities are given as U (mg protein⁻¹), or U (mg protein equivalent⁻¹) for unbroken bacteria.

Enzyme inhibitors. Acetate, vanadate, molybdate and fluoride (sodium salts) were Analar grade from BDH; 2-acetamido-2-deoxyglcanolactone was from Koch-Light; saccharo-1:4-lactone was prepared by boiling 0-01 M-potassium hydrogen saccharate (pH 3-7) for 30 min. Concentrations of saccharo-1:4-lactone were calculated on the basis of a 30% molar conversion of saccharate to the lactone (Levy, 1952). Inhibitors were added to assays immediately before the addition of enzyme (4-8 × 10⁻⁵ U) and substrates.

Polyacrylamide gel electrophoresis. Whenever sufficient sample was available at high enough specific activity, it was concentrated to between 20 and 50 µl and applied, with 2 vol. glycerol and 5 µl of 0-1% (w/v) aqueous Bromophenol Blue, directly to cylindrical (70 x 5 mm diam.) small pore gels of 7.5% (w/v) acrylamide. The gel system was that described by Chang et al. (1979), modified so that citrate replaced acetate. After electrophoresis, gels were incubated at 25 °C for 15 min in 0-5 M-citrate at the pH corresponding to the buffer used for staining, then incubated for 2-18 h at 25 °C with a solution containing the appropriate naphthol AS-BI (6-glucosaminidase (Sigma) in 2 ml 2-methoxyethanol; for P-glucuronidase (Hayashi et al., 1979), modified for use as an alternative method for assaying P-glucuronidase and N-acetyl-P-glucosaminidase. The glycosidases in extracts of M. leprae were at such low specific activities that they were unsuitable for application to gels. Extracts were applied to a concanavalin A-agarose column (Glycosilex-A; Miles Research Products) from which certain glycosidases could be eluted with methyl α-mannoside (Skudlarek & Swank, 1979). The methods and buffers described by Skudlarek & Swank (1979) were used with the modification that 0-05% of the protein digest Polypep (Sigma) was added to all buffers, and eluates were desalted and concentrated using Amicon CF-25 cones.

RESULTS

Acid hydrolases associated with M. leprae

The specific activity of acid phosphatase associated with whole M. leprae was 21 × 10⁻⁴ U (mg protein equiv.)⁻¹; treatment with 1 M-NaOH completely inactivated the acid phosphatase activity that could be assayed using whole bacteria. When the supernatant from the NaOH-treatment of bacteria was assayed, no activity was detected, indicating complete inactivation of acid phosphatase exposed to 1 M-NaOH rather than release from the bacterial surface. Acid phosphatase was, however, detected when cell-free extracts were prepared from NaOH-treated M. leprae (Table 1); 7% of the acid phosphatase activity compared with the activity in a cell-free extract of untreated M. leprae was detected. After sonicating M. leprae, acid phosphatase activity in the cell-free extract (Table 1) was 25% of the activity assayed with the unbroken bacteria. Acid phosphatase activity after treatment with ANDS (see Methods), compared with untreated controls, was 15% in extracts and 10% with unbroken bacteria.

N-Acetyl-β-glucosaminidase and β-glucuronidase were detected in extracts of M. leprae (Table 2) and in suspensions of unbroken bacteria at similar levels: in unbroken bacteria activities were 1-84 × 10⁻⁴ U (mg protein equiv.)⁻¹ and 0-72 × 10⁻⁴ U (mg protein equiv.)⁻¹, respectively. However, hyaluronidase was not detected in extracts of M. leprae or with unbroken bacteria.

N-Acetyl-β-glucosaminidase and β-glucuronidase were affected similarly by each of the surface treatments of M. leprae. Treatment with 1 M-NaOH effectively abolished the
Table 1. *Acid phosphatase in cell-free extracts of* M. leprae *and armadillo liver*

Bacterial and armadillo liver extracts (Tris treatment of liver extracts is described in Methods) were incubated with and without inhibitors; inhibition experiments with extracts from treated *M. leprae* were limited because of the lack of extract. Results for specific activity show the mean value ± standard error where three or more separate cell-free extracts were made, polyacrylamide gels were run twice and inhibition experiments were carried out at least twice.

<table>
<thead>
<tr>
<th>Extract</th>
<th>10^4 × Specific activity [U (mg protein)^{-1}]</th>
<th>Relative mobility (7.5% gel)</th>
<th>Enzyme activity with inhibitor (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. leprae (untreated)</td>
<td>5.2 ± 1.4</td>
<td>0.11</td>
<td>65 50 25 90</td>
</tr>
<tr>
<td>M. leprae treated with 1 M-NaOH</td>
<td>0.36 ± 0.09</td>
<td>ND</td>
<td>85 50 ND ND</td>
</tr>
<tr>
<td>M. leprae treated with ANDS</td>
<td>0.78 ± 0.04</td>
<td>ND</td>
<td>70 50 ND ND</td>
</tr>
<tr>
<td>M. phlei</td>
<td>120 ± 39</td>
<td>0.45, 0.66</td>
<td>95 25 70 100</td>
</tr>
<tr>
<td>Armadillo liver</td>
<td>72</td>
<td>0.11*, 0.21,</td>
<td>50 60 30 85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.36, 0.41</td>
<td></td>
</tr>
<tr>
<td>Tris-treated armadillo liver</td>
<td>15.5</td>
<td>ND</td>
<td>ND ND ND ND</td>
</tr>
<tr>
<td>Infected Tris-treated armadillo liver</td>
<td>91</td>
<td>ND</td>
<td>ND ND ND ND</td>
</tr>
</tbody>
</table>

ND, Not determined.  * Major band.
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Table 2. Glycosidases in cell-free extracts of M. leprae and armadillo liver

\[ N\text{-Acetyl-}\beta\text{-glucosaminidase and } \beta\text{-glucuronidase were assayed in cell-free extracts of } M. \text{leprae (treated and untreated) and armadillo liver. When extracts were incubated with lactones or acetate the enzyme activity as a percentage of controls with no inhibitors was calculated. Results for specific activity show the mean value } \pm \text{ standard error where three (for } \beta\text{-glucuronidase) or four (for } N\text{-acetyl-}\beta\text{-glucosaminidase) extracts were made.} \]

\[ \text{N-Acetyl-}\beta\text{-glucosaminidase} \]

<table>
<thead>
<tr>
<th>Extract</th>
<th>Specific activity [U (mg protein)(^{-1})]</th>
<th>Enzyme activity with inhibitor (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10^4\times)</td>
<td>0.02 mM-2-acetamido-2-deoxyglucosonolactone</td>
</tr>
<tr>
<td>M. leprae (untreated)</td>
<td>2.31 ± 0.61</td>
<td>65</td>
</tr>
<tr>
<td>M. leprae treated with ANDS</td>
<td>0.75*</td>
<td>70</td>
</tr>
<tr>
<td>Armadillo liver</td>
<td>242</td>
<td>30</td>
</tr>
</tbody>
</table>

\[ \beta\text{-Glucuronidase} \]

<table>
<thead>
<tr>
<th>Extract</th>
<th>Specific activity [U (mg protein)(^{-1})]</th>
<th>Enzyme activity with inhibitor (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10^4\times)</td>
<td>0.3 mM-saccharo-1:4-lactone</td>
</tr>
<tr>
<td>M. leprae (untreated)</td>
<td>0.81 ± 0.18</td>
<td>85</td>
</tr>
<tr>
<td>M. leprae treated with ANDS</td>
<td>0.30</td>
<td>80</td>
</tr>
<tr>
<td>Armadillo liver</td>
<td>27</td>
<td>5</td>
</tr>
</tbody>
</table>

\[ \text{ND, Not determined.} \]
\[ * \text{Prepared from extracts with specific activity below the mean value.} \]

activities; cell-free extracts from NaOH-treated bacteria contained only 9\% of the \( N\text{-acetyl-}\beta\text{-glucosaminidase} \) and 4\% of the \( \beta\text{-glucuronidase} \) activity compared with extracts from untreated \( M. \text{leprae} \) harvested from the same tissue. After treatment with ANDS, 45\% of the \( N\text{-acetyl-}\beta\text{-glucosaminidase} \) and 33\% of the \( \beta\text{-glucuronidase} \) could still be detected in extracts (compared with extracts from untreated bacteria). Acid hydrolases were all located in the soluble fraction of extracts of \( M. \text{leprae}. \)

\[ \text{Acid hydrolases in extracts of armadillo liver} \]

Acid phosphatase (Table 1), \( N\text{-acetyl-}\beta\text{-glucosaminidase} \) and \( \beta\text{-glucuronidase} \) (Table 2) were present in extracts from armadillo liver at higher specific activities than in extracts from \( M. \text{leprae}. \) By comparing Tris-treated liver extracts it was shown that the acid phosphatase activity was increased sixfold when the liver was infected (Table 1). However, exposure to high pH (used in the homogenization step during isolation of \( M. \text{leprae} \) from host liver) inactivated 80\% of the acid phosphatase of liver (cf. armadillo liver and Tris-treated armadillo liver extracts; Table 1). Most \( N\text{-acetyl-}\beta\text{-glucosaminidase} \) and \( \beta\text{-glucuronidase} \) also appeared to be inactivated at high pH, since early attempts to run these glycosidases from liver on gels where the pH was pH 8.5 to 9 (Tris/glycine: Williams & Reisfeld, 1964) were unsuccessful.

\[ \text{Acid hydrolases in } M. \text{phlei and } M. \text{microti} \]

All the acid phosphatase activity that was detected in extracts of \( M. \text{phlei} \) could be detected using unbroken bacteria (Table 3). Conversely, very little of the acid phosphatase of \( M. \text{microti} \) (only just above background values) could be detected using unbroken bacteria (Table 3). ANDS inactivated 60\% of the acid phosphatase of \( M. \text{phlei} \) (Table 3). For \( M. \text{phlei} \) and \( M. \text{microti} \), the rate of hydrolysis of \( p\text{-nitrophenyl-}N\text{-acetyl-}\beta\text{-glucosaminide} \) was,
Table 3. Assay of hydrolases with unbroken and sonicated mycobacteria grown in vitro

Mycobacteria were grown as described in Methods. They were assayed before sonication, and after the preparation of cell-free extracts. Specific activities are expressed relative to protein equivalent when suspensions of bacteria were used.

<table>
<thead>
<tr>
<th>Bacterial suspension or extract</th>
<th>Acid phosphatase</th>
<th>N-Acetyl-β-glucosaminidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. phlei</td>
<td>$1.77 	imes 10^4$</td>
<td>$0.63$</td>
</tr>
<tr>
<td>Extract of M. phlei</td>
<td>$1.66 	imes 10^4$</td>
<td>$9.7$</td>
</tr>
<tr>
<td>Extract of ANDS-treated M. phlei</td>
<td>$6.6 	imes 10^4$</td>
<td>$9.0$</td>
</tr>
<tr>
<td>M. microti</td>
<td>$8.0 	imes 10^3$</td>
<td>$0.131$</td>
</tr>
<tr>
<td>Extract of M. microti</td>
<td>$9.2 	imes 10^3$</td>
<td>$3.20$</td>
</tr>
</tbody>
</table>

respectively, 15 and 24 times lower with unbroken bacteria than in cell-free extracts (Table 3) and (for M. phlei) treatment with ANDS did not inactivate N-acetyl-β-glucosaminidase (Table 3). β-Glucuronidase was present in extracts of M. phlei and M. microti at $7.36 \times 10^{-4} \text{U mg}^{-1}$ and $2.58 \times 10^{-4} \text{U mg}^{-1}$, respectively. Acid hydrolases in these bacteria were in the soluble fraction.

Effect of surface treatments of M. leprae on enzyme inhibition

Irradiated unbroken M. leprae (which retained some of the activity of hydrolases which could be assayed before irradiation) were treated, so that enzyme activity could still be assayed after treatment, with ANDS or 0.1 M-NaOH. Results of inhibition experiments were the same for the activities of both treated (with ANDS or NaOH) and untreated bacteria. Acid phosphatase activity compared with controls (with no inhibitor present) was 60–65% with 10 mM-$\text{VO}_3^-$ and 25–30% with 10 mM-$\text{F}^-$. N-Acetyl-β-glucosaminidase activity compared with controls was 55–65% with 0.02 mM-2-acetamido-2-deoxygluconolactone.

Properties of acid phosphatases

When acid phosphatase was assayed in extracts from untreated M. leprae, M. phlei and armadillo liver, some differences in the pattern of inhibition were observed between the M. phlei and liver enzyme but not convincingly between M. leprae and liver enzymes (Table 1). However, when extracts from NaOH-treated M. leprae were used, a reproducible pattern of inhibition of acid phosphatase was obtained which differed distinctly from that of armadillo liver acid phosphatase in that $\text{VO}_3^-$ was a far less potent inhibitor (Table 1). Acid phosphatase in extracts from ANDS-treated M. leprae, however, could not be distinguished from the enzyme in extracts from untreated M. leprae (Table 1). Application of extracts to polyacrylamide gels (Table 1) showed that the major isoenzyme from liver was present in the extract from untreated M. leprae.

Extracts were incubated at pH 6.5 as well as pH 5.5. The ratios of activity (at pH 6.5 to pH 5.5) were 0.34 in extracts from liver, 0.46 from untreated M. leprae, and 0.72 from NaOH-treated M. leprae.

Properties of glycosidases

Inhibition studies using lactones showed that the patterns of inhibition of glycosidases assayed in cell-free extracts of ANDS-treated and untreated bacteria were the same. The lactones were more potent inhibitors of the corresponding glycosidases from armadillo liver (Table 2). Acetate was also used as an inhibitor of liver N-acetyl-β-glucosaminidase but was found to stimulate the activity of the enzyme in extracts of M. leprae (Table 2).

An attempt was made to purify N-acetyl-β-glucosaminidase by affinity chromatography using a Glycosilex column (see Methods). At least a 30-fold purification was obtained (the
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presence of some Polypep, even after desalting, in the partly purified material made it impossible to detect low levels of protein of both *M. leprae* and liver enzyme; the yield of the enzyme was about 50%. *N*-Acetyl-β-glucosaminidase from liver (2 × 10^{-4} U) and from *M. leprae* (4 × 10^{-4} U) was run on 7-5% (w/v) polyacrylamide gels and had a mobility of 0-13 from both sources. Electrophoresis on 5% (w/v) gels did not resolve the enzyme from the two sources. However, 80% of the liver enzyme was recovered when the Glycosilex column was eluted with methyl α-mannoside compared with only 40% of the enzyme in *M. leprae* extracts, the remainder, in each case, being eluted by Tris/NaCl buffer. Similarly, β-glucuronidase from liver extract showed a greater affinity for the Glycosilex column (80% recovered by elution with methyl α-mannoside) than β-glucuronidase from an extract of *M. leprae* (all activity was eluted through the column by Tris/NaCl buffer). Neither extracts from armadillo liver nor *M. leprae* affected the carbohydrate-binding properties of concanavalin A (checked with concanavalin A from Sigma and washed Group O red blood cells). Hydrolysis of a substrate other than the model substrates (i.e. the nitrophenyl derivatives) by these glycosidases was demonstrated: using hyaluronic acid partly hydrolysed by hyaluronidase (see Methods) it was shown that an extract of *M. leprae* capable of hydrolysing 100 pmol p-nitrophenyl-β-glucosaminide min^{-1} and 30 pmol p-nitrophenyl-β-glucuronide min^{-1} exposed 50 pmol N-acetyl-β-glucosaminide min^{-1} from the hyaluronic acid oligosaccharides. When experiments with lactones were carried out using extracts from ANDS-treated *M. leprae*, the results were almost identical to the results obtained with extracts from untreated *M. leprae* (Table 2).

DISCUSSION

The acid hydrolases studied were all present in host liver, and their activities were elevated in liver infected with *M. leprae*, an observation consistent with the enhancement of activities of lysosomal hydrolases in infected macrophages (Karnovsky *et al.*, 1975). It was therefore essential to establish that acid hydrolases in extracts of *M. leprae* were authentic bacterial enzymes.

Enzymes adsorbed to the surface or located in the bacterial cell membrane were inactivated by incubating purified suspensions of *M. leprae* in NaOH, after which *M. leprae* (A. C. R. E. Lowe, unpublished results) and *M. tuberculosis* grown *in vivo* (Kanai, 1967b) remain viable, or in ANDS, which has been shown to partially (at least 60%) inhibit surface-located enzymes while having no effect upon cytoplasmic enzymes (Day & Ingram, 1971; Garber & Nachson, 1980).

It was necessary to ensure that observed differences in enzyme inhibition between enzyme activities from liver and *M. leprae* would not be interpreted as evidence for authentic *M. leprae* enzymes when there was an alternative possible explanation. It was established that neither NaOH nor ANDS treatment affected the pattern of enzyme inhibition, but attachment of enzyme to the surface of *M. leprae* did; using untreated bacteria, acid phosphatase in extracts and surface-attached acid phosphatase were inhibited 50% and 75%, respectively, by 10 mM-F^{-}. However, in this work all the acid hydrolases in extracts from both bacteria and armadillo liver were in the soluble fractions after ultracentrifugation, so results obtained with these extracts and inhibitors could not be influenced by the attachment of the enzyme to membranes.

Using inhibitors which had previously been used in differentiating mycobacterial acid phosphatases (Andrejew & Renard, 1968; Andrejew, 1968), and also VO_{4}^{2-} (Van Etten *et al.*, 1974) – not previously used for mycobacteria but the most useful in this work – as well as pH/activity measurements (Kanai, 1964; Andrejew & Renard, 1968), it was shown that acid phosphatase in extracts from *M. leprae* could only be differentiated from armadillo liver enzyme when NaOH-treated *M. leprae* were used. These results were interpreted as showing that NaOH removed the host enzyme which was otherwise present in extracts of *M. leprae*, and that the remaining enzyme was bacterial.
Incubation of N-acetyl-β-glucosaminidase and β-glucuronidase with inhibitors showed that these glycosidases in extracts from *M. leprae* were authentic bacterial enzymes. Acetate was used to inhibit a mammalian N-acetyl-β-glucosaminidase (Pugh *et al.*, 1957), and while it inhibited the armadillo enzyme it stimulated the enzyme from *M. leprae*. Aldonolactones corresponding to substrates inhibited mammalian glycosidases more strongly than bacterial glycosidases (Levvy, 1952; Findlay *et al.*, 1958) and we have shown this pattern for *M. leprae* and its host tissue. Prabhakaran *et al.* (1978) claimed that they could not distinguish β-glucuronidase from *M. leprae* and armadillo liver using saccharo-1:4-lactone, but this appears to be a result of using a single very high concentration (5 mM) of lactone in their experiments. Like Levvy (1952), we used up to 0.3 mM-lactone. Further evidence for the glycosidases in extracts from *M. leprae* being bacterial was provided by studies of their affinity for concanavalin A, but not by gel electrophoresis.

An indication of the location of the acid hydrolases could be obtained from our results, partly on the basis that cell membrane enzymes would be inactivated by surface-treatments, although care in interpretation was required as host enzymes adsorbed to the bacteria would also be inactivated. Previous work showed that bacterial cell membranes are not permeable to nitrophenyl phosphate (Brockman & Heppel, 1968) and, although nitrophenyl glycosides were transported across bacterial cell membranes (Kennedy, 1967), transport was limiting to hydrolysis to the extent that when the membrane barrier was removed by treating bacteria with toluene, nitrophenyl glycosides were hydrolysed over ten times more rapidly (Ulitzur, 1970; Lieberman *et al.*, 1977). Therefore, in our experiments, activity detected only in extracts would indicate intracellular hydrolases. Acid hydrolases of *M. phlei* and *M. microti* were intracellular, except for the acid phosphatases of *M. phlei* which were surface-located; experiments with mycobacteria to which contaminating enzymes could not have been adsorbed confirmed the usefulness of this approach. N-Acetyl-β-glucosaminidase and β-glucuronidase of *M. leprae* were shown to be cell membrane enzymes; comparison of these enzymes in extracts of *M. leprae* and armadillo liver indicated that ANDS partially inactivated bacterial cell membrane glycosidases. Acid phosphatase remaining after NaOH treatment of *M. leprae* was intracellular, although any cell membrane located enzyme would have been destroyed by NaOH treatment. The distribution of enzyme activity after ultracentrifugation of extracts was not used to indicate location; while o-diphenoloxidase was located as a particulate enzyme in extracts from sonicated *M. leprae* (Prabhakaran *et al.*, 1973), particulate enzymes were removed from the membranes of *M. phlei* by prolonged sonication (Brodie & Adelson, 1965).

Acid hydrolases were present at relatively low activities in *M. leprae*; acid phosphatase activity in extracts from surface-treated *M. leprae* was present at 0.3–0.6% of the levels detected from *M. avium* (Andrejew, 1968), *M. phlei* and *M. microti*. N-Acetyl-β-glucosaminidase activity in *M. leprae* was 24% of the activity in *M. phlei* and 70% of the activity in *M. microti*, and β-glucuronidase activity in *M. leprae* was 11% and 32% of the activities in *M. phlei* and *M. microti*, respectively. Such low enzyme activities may be partly a reflection of the low viability of suspensions of *M. leprae*, and may be one factor in the slow growth of *M. leprae*. It has been suggested that the glycosidases play a part in the utilization of hyaluronic acid as a substrate (Matsuo *et al.*, 1975) but hyaluronidase has not been detected (hyaluronic acid is not a substrate for N-acetyl-β-glucosaminidase and β-glucuronidase) in *M. leprae*.

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**REFERENCES**


Hydrolases in Mycobacterium leprae


