## Phosphatases of *Mycobacterium* 607

Considerable data exist regarding phosphatases of animals and plants[1], but relatively less is known about bacterial phosphatases. Information about the enzymes of mycobacteria has been reviewed[2], and the presence of UDPG phosphatase has been mentioned. The occurrence of polyphosphatase has also been reported in *Mycobacterium smegmatis*[3]. This report records observations on some other phosphatases of *Mycobacterium* 607, which will be of interest to those working on the metabolism of bacteria, especially that of mycobacteria.

*Materials and methods.* *M.* 607 grown as described previously[4] was sonicated with three times its volume of water for 20 min in a 9 Kc Raytheon sonic oscillator. The cell-free extract, obtained after centrifugation for 20 min at 10,000 *g*, was brought to full saturation with ammonium sulphate and the separated protein recovered by centrifugation. This protein was dissolved in $0.005M$ Tris-HCl (pH 7.4) and dialysed overnight against the same buffer. The dialysed preparation was subsequently used as enzyme source. All operations were carried out at 0–4°C. The preparation so obtained contained no more than traces of phosphorus, as determined by the BARTLETT procedure[5] after preliminary acid hydrolysis for labile phosphates. Thus the possibility of interference or complications due to the presence of bacterial phosphates was ruled out, which remained not only in cell-free preparations obtained by direct cell grinding or sonication, but were also carried in the acetone dried preparations.

For the enzyme assay 100 μmoles Tris-HCl or Tris maleate buffer (pH 8.0), 5μ moles substrate, 7 μmoles $MgCl_2$, 5 μmoles reduced glutathione (GSH) and enzyme protein in a final volume of 1 ml was incubated at 37°C for 15 min. At the end of the incubation period, the enzyme was inactivated by the addition of 2 ml of chilled 5% TCA, and the solutions were chilled. After centrifuging down the protein, colour for orthophosphorus (Pi) was developed by the FISKE and SUBBAROW method[6] on suitable aliquots. A control was run simultaneously, which was given identical treatment except that enzyme addition was made subsequent to the addition of TCA, at the end of the incubation period. Protein was estimated by the method of LOWRY et al.[7].

*Results and discussion.* Table I shows that, under the assay conditions employed, orthophosphate was split enzymically from ATP, ADP, F-1,6-di-phosphate and pyrophosphate, and the rate of reaction was different with each substrate. All enzyme assays were performed under linear kinetic conditions. No orthophosphate liberation was seen with α-glycerophosphate, β-glycerophosphate, G-1-P, G-6-P and AMP using up to 3 mg enzyme protein.

The pH optima for ATP and pyrophosphate hydrolysis tested between pH 5.2–8.8 using Tris-maleate was found to lie at pH 7.8–8.0. Activity in Tris-HCl was the same as in Tris-maleate. The requirement of bivalent cation, $Mg^{2+}$, appears essential since, when tested with ATP as substrate, omission of $Mg^{2+}$ reduced the activity (95% of that in the presence of $7 \times 10^{-3}M$ $MgCl_2$); and inclusion of EDTA ($2 \times 10^{-3}M$) in the absence of $MgCl_2$ abolished the activity completely towards all the effective substrates. Fluoride inhibited the reaction rate by 20% at $4 \times 10^{-2}M$ concentration with ATP as substrate. For complete inhibition, relatively high concentration, $0.14M$, fluoride was required. Omission of GSH from the assay system had no effect on activity, but addition of para-hydroxy mercuribenzoate (PHMB) ($1 \times 10^{-4}M$) inhibited the activity to the extent of 75%. The PHMB inhibition was completely reversed by the addition of GSH ($5 \times 10^{-3}M$).

When an excess of enzyme protein was incubated with limiting substrate, the quantitative liberation of two equivalents of orthophosphate was seen for both ATP and pyrophosphate and only one for ADP. To determine whether the same enzyme was involved in the hydrolysis of ATP and ADP, the rate of reaction was followed by including both the substrates together in the assay system. The amounts of ATP and ADP employed were not limiting, since doubling their concentration was without effect. The amount of orthophosphorus liberated was found to be equal to the sum of orthophosphate liberated with ATP and ADP alone (Table I), suggesting that different enzymes are involved for the hydrolysis of these two substances. This was more clearly shown by the effect of heat treatment on enzyme preparation. With unheated

Table I. Activity of *Mycobacterium* 607 phosphatases with different substrates

| Substrate | μmole Pi liberated/15 min/mg protein |
|---|---|
| ATP | 1.04 |
| ADP | 0.48 |
| F-1,6-diphosphate | 0.31 |
| Pyrophosphate | 30.10 |
| ATP+ ADP | 1.65 |
| *After heat treatment* | |
| ATP | 0.13 |
| ADP | 0.47 |
| Pyrophosphate | 4.59 |

Table II. Effect of inhibitors on phosphatase activity

| Inhibitors | | μmole Pi liberated[a]/15 min/mg protein |
|---|---|---|
| NaF | $4 \times 10^{-2}M$ | 0.82 |
| NaF | $0.14M$ | 0.00 |
| PHMB | $1 \times 10^{-4}M$ | 0.26 |
| PHMB | $2 \times 10^{-4}M$ | 0.19 |
| PHMB | $1 \times 10^{-4}M$ + GSH | |
| | $5 \times 10^{-3}M$ | 1.10 |

[a] ATP was used as substrate.


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enzyme preparations, reaction rate with ADP was half of that with ATP, whereas, after 5 min heat treatment at 60°C, activity towards ATP dropped considerably (87%) while that with ADP remained unaffected. Thus there are at least two enzymes responsible for ATP and ADP hydrolysis. The presence of extremely active pyrophosphatase activity having similar pH optima and susceptibility to heat treatment as that of ATP hydrolysing enzyme activity precludes any speculation about the nature of ATP hydrolysis.

The occurrence of F-1,6-di-phosphatase activity in mycobacteria is of interest because of its known involvement in hexose monophosphate pathway[1,2] and in reversal of glycolysis[8]. Both operation of hexose monophosphate pathway[9] and glycolysis reversal – as judged by the formation of glycogen and other polysaccharides together with the presence of other glycolytic enzymes in this organism grown on glycerol as carbon source[2] – are known in mycobacteria. Further, for the interconversion of F-6-P to F-1,6-diphosphate, two different enzymes catalysing the unidirectional reactions are involved. The presence of phosphofructokinase in mycobacteria was reported by GOLDMAN[2], and the present demonstration of F-1,6-diphosphatase activity suggests that a similar diversion of metabolic route occurs here also as in animals[8,10,11].

*Zusammenfassung.* Die Phosphatase(*n*)-Aktivität der zellfreien Extrakte von *Mycobacterium* 607 wurde untersucht und Pyrophosphat, ATP, ADP und Fructose-1,6-diphosphat hydrolisiert. Eigene Eigenschaften der Phosphatasen wurden studiert. Auch wurde nachgewiesen, dass die Hydrolyse von ATP und ADP durch mindestens zwei verschiedene Enzyme hervorzurufen ist.

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## Electron Microscopic Observation of Bacteria Growing on the Skin Surface

The fixation of specimens of human skin containing bacteria, using in our laboratory conventional methods for mammalian tissues (osmium tetroxide, glutaraldehyde, potassium permanganate) failed to result in adequate bacterial fixation. Therefore we decided to try the RYTER-KELLENBERGER (R-K) fixation technique[1].

The areas of the skin to be biopsied were known to possess bacteria because positive cultures of various organisms were obtained either by swabbing or scraping the skin surface. A small punch 2 mm in diameter was used following local infiltration with 2% xylocaine. Immediately after removal, each specimen was immersed in

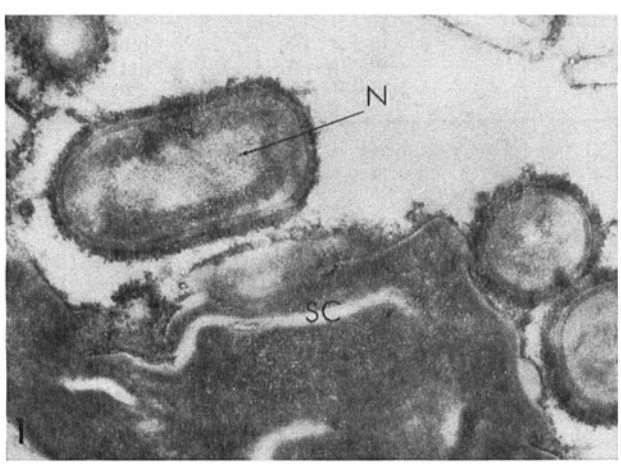


Fig. 1. Bacteria proliferating on the skin surface. The nucleoplasm of one of them (N) and the superficial stratum corneum (SC) are shown ( × 36,000).

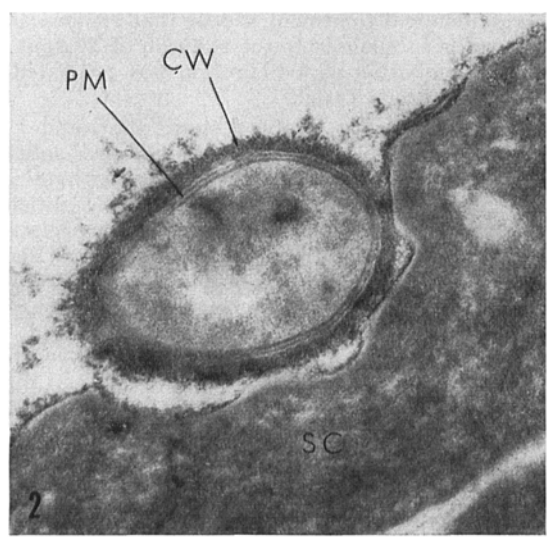


Fig. 2. Bacterium observed right above the stratum corneum (SC). The cell wall (CW) and a double-layered plasma membrane (PM) are seen ( × 52,000).

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