Measurement of ATP Generation and Decay in *Mycobacterium leprae* *in vitro*

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The intracellular ATP content of *Mycobacterium leprae* isolated from armadillo tissue was approximately 1.5×10^{-16} g per bacillus. During *in vitro* incubation of bacilli at 4 °C, 33 °C or 37 °C there was an exponential decrease in ATP content, the rate depending on the medium and the temperature. *M. leprae* incorporated phosphate into ATP and into other nucleotide materials during *in vitro* incubation.

INTRODUCTION

Mycobacterium leprae, the human leprosy bacillus, has not been grown *in vitro*. However, the availability of large numbers of organisms from experimentally infected nine-banded armadillos has enabled studies on the metabolism and biochemistry of *M. leprae* to be initiated (for a review see Wheeler, 1984*a*). Such studies have revealed, for example, that *M. leprae* is capable of *in vitro* incorporation of purines and pyrimidines (Khanolkar & Wheeler, 1983) and of catabolizing carbon sources such as glucose, glycerol and 6-phosphogluconate (Wheeler, 1983), and that it possesses key enzymes of the tricarboxylic acid cycle (Wheeler, 1984*b*).

One of the main objectives in studying the intermediary metabolism of *M. leprae* is to gain a better understanding of the biochemical relationship between the organisms and the host, and hence to arrive at a rational basis for devising culture media. However, the main problem with studying the *in vitro* cultivation of *M. leprae* is that multiplication or viability of the organism is difficult to demonstrate, requiring passage into mice and observation of the mice over many months. If methods for assessing the metabolic status of the organism could be devised, it should be possible to obtain much more rapid information on what happens to *M. leprae* during *in vitro* incubation.

In this study the possibility of measuring bacterial ATP as an indicator of metabolic status has been investigated. Using this approach successful cultivation of *M. lepraemurium* has been achieved in the last few years, and attempts have also been made to measure the concentration of ATP in *M. leprae* (Dhople & Hanks, 1981; Dhople & Storrs, 1982). In the absence of successful cultivation, the measurement of bacterial ATP could provide a means of assessing bacterial viability. We have measured the intracellular ATP content of *M. leprae* isolated from armadillo tissues and have followed the changes in ATP content during *in vitro* incubation in a variety of culture conditions, and we have demonstrated that, in spite of its inability to multiply, *M. leprae* is capable of synthesizing ATP *in vitro*.

METHODS

Organisms and media. *Mycobacterium leprae* was purified from the spleen or liver of experimentally infected nine-banded armadillos according to the most recent IMMLEP procedure (World Health Organization, 1980).

Abbreviations: BT, buffered-Tween; HBSS, (modified) Hanks' balanced salts solution; NT, nutrient-Tween medium; TG, thioglycollate medium; PEI-cellulose, polyethyleneimine-cellulose.

Briefly, this involves the release of bacteria from tissue by homogenizing in hypotonic-alkaline buffer (0.135 M-NaCl/0.2 M-Tris base/1 mM-MgSO₄/1 mM-benzamidine hydrochloride), differential centrifugation, DNAase treatment, separation of bacteria from tissue debris on a Percoll gradient (30%, w/v) and further purification using a two-phase system consisting of aqueous dextran and polyethylene glycol. Organisms were purified from fresh tissue or from tissue which had been frozen and stored at -80 °C. In some experiments irradiated *M. leprae* cells were used; these were purified from tissues which had been treated with 2.5 mrad ⁶⁰Co prior to storage at -80 °C. Purified *M. leprae* cells were suspended in Hanks' balanced salts solution containing 0.05% Tween-80 but without phenol red (HBSS) (Kvach & Veras, 1982), in nutrient-Tween medium (NT) (MacNaughton & Winder, 1977), or in thioglycollate medium (TG; Difco). Contamination of *M. leprae* suspensions with cultivable organisms was monitored by incubating samples in brain heart infusion broth, on blood agar and on Lowenstein-Jensen medium. In experiments where *M. leprae* was treated with NaOH, suspensions were incubated in 0.5 M-NaOH for 1 h at room temperature, neutralized with 1 M-HEPES buffer and washed three times with buffered-Tween (BT; 0.1% Tween 80 in 1 mM-MES, pH 6.8) (Wheeler *et al.*, 1982). Total counts of *M. leprae* were carried out according to published procedures (Hart & Rees, 1960).

Mycobacterium smegmatis NCTC 10265 was grown aerobically in either NT or a glucose medium (MacNaughton & Winder, 1977) modified by containing 0.136 g instead of 5 g KH₂PO₄ l⁻¹ (low-phosphate/glucose medium).

Extraction of ATP from mycobacteria. One volume of bacterial suspension, usually 20–30 µl, was mixed with two volumes of 100 mM-Tris/2 mM-EDTA buffer, pH 7.75, and the mixture placed in a boiling water bath for 5 min.

ATP Assay. ATP in bacterial extracts was measured by the firefly luciferin-luciferase assay system (Chappelle & Levin, 1968). Picozyme F (40 µl; equivalent to 4 × 10⁵ units of luciferin-luciferase, Packard Instrument Co.) was added to 10 µl bacterial extract. After a 10 s delay, luminescent counts were recorded for 30 s at 24 °C on a Packard PicoLite model 6100 Luminometer. The concentration of ATP in the extracts was calculated by comparing luminescent counts obtained using a standard ATP solution (Picochech, Packard).

Treatment of *M. leprae* with apyrase. *M. leprae* was incubated with 1 unit of apyrase (EC 3.6.1.5, Sigma) in 50 mM-MES buffer, pH 6.5 for 20 min at 37 °C. The organisms were then washed three times with BT.

Uptake of [U-¹⁴C]ATP by *M. leprae*. *M. leprae* cells were incubated in low-phosphate/glucose medium supplemented with 5 µCi [U-¹⁴C]ATP (specific radioactivity 515 mCi mmol⁻¹, 19.1 GBq mmol⁻¹; Amersham) ml⁻¹ for 24 h at 37 °C. Incorporation of [¹⁴C]ATP into bacteria was terminated by adding cold trichloroacetic acid (TCA) to 5% (w/v) concentration. Bacteria were collected on glass microfibre filter discs (Whatman GF/C) and washed with an excess volume (approximately 50 ml) of cold 5% TCA. Radioactivity incorporated into the bacteria was measured on a Beckman LS7000 counter.

Incorporation of [³²P]phosphate into nucleotides of *M. leprae*. Freshly purified *M. leprae* (2 × 10⁹ bacilli) were incubated in 5 ml of low-phosphate/glucose medium supplemented with 5 µCi (185 kBq) ³²P (as carrier-free orthophosphate) per ml medium for 24 h at 36 °C, with occasional shaking. The same number of heat-killed *M. leprae* cells (boiled for 15 min) was used in a parallel experiment as a measure of non-specific incorporation of ³²P into the nucleotide fraction of *M. leprae*. It was confirmed that the *M. leprae* suspension was free of contamination with cultivable mycobacteria as well as other bacteria.

Extraction of ³²P-labelled nucleotides from in vitro-incubated *M. leprae*. Cells of *M. leprae* were collected on type GS Millipore filter discs (pore size 0.22 µm) and the discs allowed to stand for 20 min, with occasional vortexing, in 2 ml ice-chilled 1 M-formic acid saturated with 1-butanol (10%, v/v, 1-butanol) (Olempska-Beer & Freeze, 1984). Acid-soluble materials in the supernate were obtained by centrifugation for 5 min at 5000 r.p.m. (3500 g) (Bactifuge, Heraeus Christ), and then about 500 nmol ATP (Sigma) was added to the supernate as an unlabelled carrier. Nucleotide materials were adsorbed onto acid-washed charcoal by addition of 30 mg charcoal per ml of sample. After 10 min on ice, the charcoal was collected by centrifugation for 5 min at 3500 g at room temperature. Nucleotide materials were then eluted from the charcoal with 4 ml of an ethanol/distilled and deionized H₂O/NH₄OH solution (50:45:5, by vol.) (Sargent, 1977) through a Millipore HVLP Duromembrane filter disc (pore size 0.45 µm) (Olempska-Beer & Freeze, 1984). The eluate was concentrated under vacuum at room temperature overnight and the residue resuspended in a minimum volume of distilled and deionized H₂O.

Two-dimensional chromatography of ³²P-labelled nucleotides extracted from *M. leprae*. Samples of the nucleotide extract were spotted onto polyethyleneimine (PEI)-cellulose F thin-layer plastic sheets (Merck) and chromatographed first with 3.3 M-ammonium formate plus 4.2% (w/v) boric acid (pH 7.0) and then with 0.75 M-KH₂PO₄ (pH 3.4). In between chromatography, the plates were soaked in methanol for 5 min, in distilled water for 15 min and then dried (Cashel *et al.*, 1969). After chromatography, the plates were subjected to autoradiography.

RESULTS

*Intracellular ATP content of *M. leprae**

The intracellular ATP content of *M. leprae* freshly purified from infected armadillo tissue ranged from 1.1 to 1.7 × 10⁻¹⁶ g ATP per bacterium, with a mean of 1.45 × 10⁻¹⁶ g

Table 1. *Effect of apyrase on quantification of ATP associated with M. leprae*

The results of a single representative experiment are shown. A second experiment gave essentially identical results.

Source of ATP	Apyrase treatment	Concn of ATP measured (M)	Percentage decrease due to apyrase treatment
Standard (10^{-7} M)	—	10^{-7}	—
Standard (10^{-6} M)	0.5 units	1.5×10^{-10}	99.99
<i>M. leprae</i> (5×10^6 cells)	—	5.3×10^{-7} *	—
<i>M. leprae</i> (5×10^6 cells)	1.0 units	5.7×10^{-7} *	0

* Mean of four determinations.

(SE 0.09×10^{-16} , $n = 6$). There was no difference in the values obtained for bacteria isolated from liver and those obtained for bacteria isolated from spleens. Freezing (1°C min^{-1} to -70°C followed by immersion in liquid nitrogen) and thawing of *M. leprae* resulted in a 30–40% decrease in ATP content when the suspending medium was HBSS, and in a 20% decrease when it was TG. The ATP content of *M. leprae* purified from irradiated tissue (1.57×10^{-17} g per bacterium; SE 0.31×10^{-17} , $n = 3$) was only about 10% of the value for bacteria from fresh tissue. The ATP content of the cultivable mycobacterium *M. smegmatis* during the exponential growth phase (2.17×10^{-15} g per viable bacterium; SE 0.36×10^{-16} , $n = 3$) was an order of magnitude greater than that in freshly purified *M. leprae*, though it should be noted that this calculation was based on *viable* counts of *M. smegmatis* as opposed to *total* microscope counts for *M. leprae*.

The question then arose as to whether the ATP detected in *M. leprae* was genuine intracellular ATP or whether it could have been due to contamination with host tissue. Treatment of the purified bacteria with NaOH, a procedure extensively used to eliminate host tissue contamination (Wheeler *et al.*, 1982), did not produce a significant decrease in ATP levels. In two experiments ATP contents of 1.3×10^{-16} g per bacterium and 1.7×10^{-16} g per bacterium before NaOH treatment became 2.1×10^{-16} and 2.7×10^{-16} g per bacterium, respectively, after NaOH treatment. In addition, treatment of the bacteria with apyrase, under suitable conditions, prior to extraction of ATP, did not result in a significant change in ATP concentration (Table 1). This confirmed that the ATP was genuine *M. leprae* intracellular ATP rather than host-tissue-derived or bacterial-surface-bound extracellular ATP.

Decay of intracellular ATP during in vitro storage or incubation of M. leprae

The change in the intracellular ATP content of *M. leprae* during storage in different media at 4°C is shown in Fig. 1. There was an exponential fall in ATP content over the two-week period of observation. ATP extracted from *M. leprae* and stored at 4°C was stable, and there was no change in the ATP content of irradiated *M. leprae* during such storage. There was a marked difference in decay rates in different media, with the fastest decay occurring in BT (half-life 1.7 d), and the slowest in TG medium (half-life 4.5 d). NT and HBSS gave similar decay rates (half-life 2.7 and 2.5 d, respectively).

The decay rate of *M. leprae* ATP during *in vitro* incubation was also dependent on the incubation temperature. At 37°C the half-life of ATP in *M. leprae* suspended in HBSS was 10 h, compared to 21 h at 33°C and 2.5 d at 4°C (Fig. 2a). Similar results were obtained when the suspending medium was TG (Fig. 2b), although ATP decayed more rapidly in TG than in HBSS at 37°C and 33°C .

Uptake of exogenous ATP by M. leprae in vitro

When viable *M. leprae* cells were incubated *in vitro* in low-phosphate/glucose medium containing [^{14}C]ATP, the level of ^{14}C incorporation was lower than that seen with heat-killed controls (Table 2). The higher value seen in the controls was probably due to the clumping of bacilli during heat treatment, resulting in less effective washing. The addition of excess

Fig. 1. Decay of intracellular ATP of *M. leprae* during storage of cells in different media at 4 °C. ATP concentration was measured in 10 μ l samples extracted from 10^6 *M. leprae* cells. Each point represents the mean of two determinations, with the range shown by the bars; where there are no bars, the two determinations were identical. ■, BT; ▲, HBSS; □, NT; ○, TG.

Fig. 2. Decay of intracellular ATP of *M. leprae* during incubation of cells at different temperatures (a) in HBSS and (b) in TG. ATP concentration was measured in 10 μ l samples extracted from 10^6 *M. leprae* cells. Each point represents the mean of two determinations, with the range shown by the bars; where there are no bars, the two determinations were identical. ●, 37 °C; ■, 33 °C; ▲, 4 °C.

unlabelled adenosine did not result in reduced levels of ^{14}C incorporation, suggesting that phosphatase activity was negligible. Using much higher numbers of *M. leprae*, phosphatase activity has been detected (P. R. Wheeler, personal communication). These results suggest that *M. leprae* is not able to take up ATP from its surrounding environment.

Incorporation of phosphate into M. leprae ATP in vitro

Both viable and heat-killed *M. leprae* were incubated for 24 h at 37 °C in low-phosphate/glucose medium containing [^{32}P]phosphate, and nucleotides were extracted and

Fig. 3. Two-dimensional chromatography on PEI-cellulose plates of ^{32}P -labelled nucleotides extracted from *M. leprae* after incubation with ^{32}P phosphate for 24 h at 37°C . (a) Fluorescence of unlabelled carrier ATP added during the extraction procedure; (b) autoradiogram of the ^{32}P -labelled nucleotides extracted from *M. leprae*.

Table 2. Uptake of $[U\text{-}^{14}\text{C}]\text{ATP}$ by *M. leprae*

Cells were incubated for 24 h at 37°C in low-phosphate/glucose medium supplemented with $5\text{ }\mu\text{Ci}$ $[^{14}\text{C}]\text{ATP ml}^{-1}$. Where indicated, unlabelled adenosine (25.7 mmol m^{-1} , equivalent to 10 times the concentration of $[^{14}\text{C}]\text{ATP}$) was also added. The results (means $\pm$ SE, $n = 3$) of a single, representative experiment are shown. The experiment was repeated twice and essentially identical results were obtained.

<i>M. leprae</i> (2.5×10^8 bacteria)	Unlabelled adenosine	Radioactivity in TCA-insoluble material (c.p.m.)
Purified from armadillo, heated at 100°C for 15 min	—	2575 ± 70
Purified from armadillo, untreated	—	1711 ± 58
Purified from armadillo, untreated	+	2188 ± 66

subjected to two-dimensional thin-layer chromatography. Autoradiography of the thin-layer plate after chromatography of extracts from viable cells (Fig. 3b) revealed a radiolabelled spot (arrowed) which exactly corresponded to the unlabelled ATP spot viewed under UV light (Fig. 3a). No such spot was observed when heat-killed *M. leprae* was used. A much more intense radiolabelled spot (A) with a minor spot (A_1) beneath it, was also observed in extracts from viable cells; these spots were apparent after a shorter (14 h) period of incubation, a time at which there was no detectable ^{32}P ATP. An additional spot (B) remained at the origin and a fourth spot (C) indicates the free ^{32}P phosphate front of the chromatogram. These results indicate that *M. leprae* is capable of taking up phosphate and incorporating it into ATP, and into other phosphate metabolic pathways.

DISCUSSION

There are two reasons why the measurement of intracellular ATP in *M. leprae* is important. First, it might provide a rapid means of measuring bacterial viability for studies involving cultivation, drug sensitivity measurement or drug screening. Secondly, studies of ATP generation and decay might be expected to give important information as to the factors affecting energy metabolism and hence lead to a better understanding of the nutritional requirements of

the organism. In this study we have shown that ATP from *M. leprae* can be readily measured, the sensitivity of the assay system permitting significant levels of ATP to be detected in as few as 10^5 – 10^6 bacteria. The ATP content of *M. leprae* reported here (1.45×10^{-16} g per bacterium) is approximately 100 times that reported by Dhople & Hanks (1981). The reason for this discrepancy is not clear, but is unlikely to involve contamination with host-tissue-derived ATP since treatment of the bacilli with either NaOH or apyrase prior to extraction did not result in a significant change in ATP concentration. A more likely explanation for the discrepancy is that technical differences in the storage and handling of the infected armadillo tissue (for example a 30–40% decrease in ATP content was found after freezing and thawing) and in the ATP extraction procedure were responsible. In any event, our results with *M. smegmatis* are in close agreement with ATP contents of other cultivable mycobacteria (Prioli *et al.*, 1985; V. Katoch, personal communication) as well as with those reported for other bacteria (Watanabe *et al.*, 1979). The fact that the ATP content of *M. leprae*, based on total bacillary counts, was approximately 10% of that in *M. smegmatis*, based on viable counts, is compatible with the view that the majority of *M. leprae* organisms in infected tissue are dead (McRae & Shepard, 1971; Sharp *et al.*, 1985).

There was an exponential decrease in ATP concentration during *in vitro* incubation of *M. leprae*, although sometimes this followed a transient increase during the first 4 or 5 h incubation. The rate of decrease was dependent on the temperature of incubation and the nature of the suspending medium, with incubation at 37 °C in nutrient-rich media (e.g. TG) producing the most rapid effect, suggesting that the decrease may be due to active utilization of ATP by *M. leprae*. Two important questions were posed by these results. First, what was the origin of the *M. leprae* ATP, i.e. does *M. leprae* generate its own ATP when growing in host tissue or is it capable of taking up and utilizing host ATP in the same way as host-energy-dependent organisms such as chlamydia (Hatch *et al.*, 1982) and rickettsia (Winkler, 1976)? Second, if *M. leprae* does generate its own ATP, does the mechanism by which it does so cease on *in vitro* incubation, or does ATP production continue but at a rate which does not match ATP utilization, hence resulting in a net decline in ATP concentration?

From the results shown in Table 2, it seems unlikely that *M. leprae* relies on its host for a supply of ATP, since it was unable to take up exogenous ATP when incubated *in vitro*. The fact that *M. leprae* is capable of generating its own ATP, and, in addition, that it continues to do so even during *in vitro* incubation is shown by the results illustrated in Fig. 3. Incorporation of ^{32}P into ATP was readily detectable after 24 h *in vitro* incubation. Whether this production is a transient phenomenon, or whether it continues for prolonged periods of *in vitro* incubation must be determined. Such studies on the energy metabolism of *in vitro*-incubated *M. leprae* should permit a more rational approach to the development of conditions favourable for cultivation. The results illustrated in Fig. 3 also indicate that ^{32}P is incorporated into other nucleotide materials. Attempts are being made to confirm the identity of these labelled metabolites with a view to gaining further insight into the ways in which the leprosy bacillus can generate and store energy.

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