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Adenosine Triphosphate Content of *Mycobacterium leprae* Isolated from Armadillo Tissue by Percoll Buoyant Density Centrifugation[1]

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In 1977, Dhople and Hanks ([5]) reported the continuous *in vitro* growth and multiplication of *Mycobacterium lepraemurium,* the rat leprosy bacillus. Their ability to monitor the organism's growth potential and biomass using ultrasensitive adenosine triphosphate (ATP) measurements at the time of bacterial recovery from infected mouse tissue and at subsequent periods under a variety of *in vitro* conditions proved vital to their success. It was shown that *M. lepraemurium* contained approximately 2.4 pg of ATP/10^6 cells ([4]) but rapidly lost ATP due to cell leakiness attributed to improper cell membrane function ([6]). Homogenization of infected tissue in albumin and yeast supplement B retarded ATP leakage, thereby enabling the bacteria to synthesize functional cell membranes *in vitro* ([6]). This improved *M. lepraemurium's* ability to survive *in vitro* and eventually to grow and multiply.

The application of ATP measurement to host-derived *M. lepraemurium* revealed a number of important findings which required technical innovation. Recovery of *M. lepraemurium* was made possible by merely diluting heavily infected tissue (10^{11}/g) homogenates which reduced tissue ATP and tissue debris to inconsequential concentrations but retained sufficient bacteria for extraction and ATP measurement ([3]). It was also shown that previous methods for extracting bacterial ATP were not adequate for mycobacterial cells, and led to the development of a heat/chloroform procedure ([3]) which recovered all intracellular mycobacterial ATP. A close correlation between the number of *M. lepraemurium* cells and the amount of ATP per milliliter of culture indicated that ATP could sensitively detect cell growth and multiplication ([4]). Conversely, a significant decrease in ATP per unit number of cells (ATP level) signaled

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unsuitable conditions for *in vitro* survival. The work of Dhople and Hanks established the feasibility and desirability of using ATP measurement to assess the ability of host-derived *M. lepraemurium* to survive *in vitro.* The method replaced error-prone cell counts ([1]) as the primary method of detecting bacterial multiplication, provided an accurate and sensitive measure of the physiological and metabolic quality of *M. lepraemurium,* and accelerated the pace of the research whose objective was cultivation of the microorganism *in vitro.*

Since the armadillo was first shown by Kirchheimer and Storrs ([13]) and Storrs ([23]) to be susceptible to systemic infection with *M. leprae,* useful quantities of this microorganism have become available for physiological, metabolic and immunological investigation. The procedure of Draper ([9]) has been adopted by the World Health Organization for the recovery and purification of *M. leprae* for immunological investigation and by Wheeler, *et al.* ([24]), with minor modification, for metabolic study. Dhople, *et al.* ([7, 8]) have developed a method for separating *M. leprae* cells from human tissue so that ATP measurements can be used to help evaluate the effectiveness of chemotherapy in lepromatous leprosy patients. Both recovery procedures have proven to be ideal for their respective purposes, but each requires considerable time and manipulation which risks unpredictable physiological and metabolic alteration to the cells.

Dhople, *et al.* have also found ([7, 8]) that *M. leprae* from human, armadillo, or mouse foot pad tissues possess approximately 1.3–1.4 pg of ATP/10^6 cells, which is in the same order of magnitude of *in vivo*-grown *M. lepraemurium.* They also detected a significant amount of host-derived ATP bound to the surface of *M. leprae* which could be removed by detergent and enzyme treatment ([8]). David, *et al.,* however, reported 400 pg of ATP/10^6 cells from armadillo tissue ([2]). This disparity is of considerable importance since the data of Dhople, *et al.* would suggest *M. leprae* is defective in its ability to generate and/or maintain biosynthetic energy when compared to cultivable bacteria ([10, 17–19]); whereas the findings of David, *et al.* would indicate the opposite.

The purpose of this research was to first develop a more rapid and mild method of recovering *M. leprae* from armadillo tissue to minimize structural and/or functional damage to the organisms. The purified *M. leprae* were used to help resolve the reported disparity in their ATP content, to determine if significant host ATP is bound to the cells and whether the cells are leaky *in vitro,* and to elucidate if the ATP level accurately reflects *M. leprae's in vitro* survivability.

MATERIALS AND METHODS

Bacteria. *M. leprae*-infected armadillo liver tissues containing 10^9–10^{10} bacteria per g were received on dry ice from the National Hansen's Disease Center, Carville, Louisiana, U.S.A. (#R-199 and R-277) and the Florida Institute of Technology, Melbourne, Florida, U.S.A. (#A-21 and A-102). Upon receipt, the tissues were thawed at 25°C (freeze-thaw cycle #1), subdivided into 2–4 g pieces, and refrozen at −76°C. Prior to purification, the tissues were thawed at 25°C (freeze-thaw cycle #2). In two cases (A-21 and A-102), a portion of the tissue was used to recover bacteria after the first thawing. The Armed Forces Institute of Pathology (AFIP) provided freshly harvested liver tissue (#729) on wet ice. One piece was processed after subdivision, but before freezing. Frozen, uninfected armadillo liver tissue was received from AFIP and handled in the same fashion as infected tissue.

In vivo-adapted *M. lepraemurium* (Hawaiian strain) was provided by Dr. A. Dhople, Florida Institute of Technology, and was grown on Löwenstein-Jensen slants at 35°C. *M. avium* (Pasteur Institute strain CIPT 130210005) was provided by Dr. F. Portaels, Instituut voor Tropische Geneeskunde Prins Leipold, Antwerp, Belgium, and was grown on Löwenstein-Jensen slants. *M. intracellulare* (ATCC 13950) and *M. smegmatis* (strain 607) were grown in Dubos broth containing 0.4% glycerol at 35°C. Laboratory strains of *Escherichia coli* and *Staphylococcus aureus* were cultured in Trypticase Soy Broth at 35°C.

Clofazimine solution. Clofazimine (Ciba-Geigy, Basel, Switzerland) was dissolved in dimethyl sulfoxide (DMSO) to yield a 5 mM (2.36 mg/ml) stock solution which was stored at 4°C. The stock was diluted 1:500 in 0.1 M potassium phosphate buffer, pH

7.0, immediately before use to give a 10 μM working solution in 0.2% DMSO.

2,4-Dinitrophenol solution. An aqueous solution of 2,4-dinitrophenol (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was prepared immediately before use and diluted in 0.1 M potassium phosphate buffer, pH 7.0, to give 0.05, 0.5 and 5 mM concentrations.

Single cell suspension. Single cell suspensions of all the mycobacterial species (except *M. leprae*) and *S. aureus* were prepared by passage through a 5 μm membrane filter. This was necessary to remove clumps of cells whose total numbers were variable.

Bacterial counts. Bacterial suspensions were counted with a Neubauer-Hausser counting chamber under bright field illumination of 400× magnification. The average was calculated for counts agreeing within ±10%. Mycobacterial counts performed in this fashion proved to be approximately 30% higher (unpublished results) than Ziehl-Neelsen-stained mycobacteria counted in standardized pinhead smears ([11]). The difference is most likely accounted for by the loss of bacteria during staining, washing, decolorization, and counter-staining during the Ziehl-Neelsen procedure.

Bacterial viability. The viability of *E. coli* and *S. aureus* was determined by plating on Trypticase Soy Agar with incubation at 35°C. Mycobacterial viability was determined using a previously described ([15]) fluorescein diacetate and ethidium bromide staining method which has been shown to give an accurate measure of viability. A method for accurately measuring the number of viable *M. leprae* is not available.

ATP measurement. A bioluminescent assay developed by Kvach, Funk and Hanks (unpublished results) was used to measure ATP. Briefly, 400 mg of luciferase-containing firefly extract (FLE-50; Sigma) was dissolved in 25 ml of distilled water and transferred to a flask containing 150 ml of Tris acetate buffer pH 7.7. One liter of buffer contained 3.6 g Tris acetate, 1.6 g magnesium acetate·4 H_2O, 0.3 g EDTA (free acid), 5 g bovine serum albumin, and sufficient crystalline Trizma base to adjust the pH to 6.7. Reduced, synthetic luciferin (Sigma) was dissolved in 25 ml of Tris acetate buffer to give 1 mg/ml and also transferred to the flask. After gentle mixing, the luciferin-luciferase system was incubated for 4 hr at 25°C in the dark to permit ATP contaminating the FLE-50 to "burn off" to a negligible concentration. Five and ten ml volumes were dispensed to amber bottles to protect light-sensitive luciferin and stored at −20°C. The assay system reproducibly detected 5 pg ATP/0.1 ml and gave a linear response up to 10,000 pg ATP/0.1 ml. The cost of the system is 10–13 times less expensive than commercially available preparations, and was the primary reason for its development and use.

All ATP assays were performed in triplicate with an Aminco Chem-Glo Photometer (SLM; Urbana, Illinois, U.S.A.) by injecting 0.2 ml of luciferin-luciferase into 0.1 ml of the test solution. A standard curve using 0, 5, 10 and 20 pg of ATP/0.1 ml was prepared immediately prior to measuring ATP in bacterial extracts or supernatant fluids.

Bacterial recovery and extraction of ATP. Bacterial ATP was extracted by a modification of the heat-chloroform method of Dhople and Hanks ([3]). Unless otherwise described, bacterial suspensions (10^7 bacteria/ml) were centrifuged at 2500 × *g* for 20 min at 4°C in 13 × 100 mm screw-cap test tubes, the supernatant fluids carefully removed, and the pellets resuspended in 0.1 ml of 10 mM EDTA (free acid; Sigma) previously adjusted to pH 7.7 with crystalline Trizma base (Sigma). Following the addition of 40 μl of chloroform, the test tubes were tightly sealed, heated for 9 min at 96–98°C in a boiling water bath, and dried under a vacuum for 1 min in the water bath. Immediately after drying, the extracted ATP was solubilized in 0.4 ml of distilled water and stored on ice. The ATP extracted and stored in this fashion remained stable for 2 hr due to the presence of EDTA and enabled all ATP assays to be performed at one time. Triplicate ATP assays were performed from triplicate suspensions using 0.1 ml of resolubilized extract. The mean and standard deviations were calculated for values agreeing within ±10% (i.e., if one of the triplicate determinations fell out of this range, it was deleted from the average).

Buoyant density purification of *M. lep-*

rae. Preweighed liver tissue (approximately 4 g) was thawed at 25°C, minced with scissors to the consistency of a paste, and transferred to a 100 ml stainless steel Sorvall Omni-Mix cup containing sufficient precooled 0.1 M potassium phosphate buffer, pH 7.2, to yield a 20% tissue suspension. Following homogenization for 30 sec at 35,000 rpm on ice, the suspension was cooled for 2 min and homogenized an additional 15 sec. After another 2 min cooling, the suspension was transferred to a Dounce homogenizer (Kontes, Vineland, New Jersey, U.S.A.), and a single mammalian cell suspension prepared with four strokes of pestle "A". The mammalian cells were lysed with six strokes of pestle "B" to release the intracellular bacteria. The volume of suspension was measured, transferred to a 40 ml polycarbonate Oakridge tube, and mixed with sufficient 100% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) to give 33% Percoll. Centrifugation at 27,000 × *g* for 20 min at 4°C produced a self-generated density gradient with four major bands containing bacteria and/or tissue debris. Ten ml of the bottom-most band (buoyant density = 1.062–1.143 g/ml) was removed, transferred to a clean 40 ml Oakridge tube, and brought to 20 ml with phosphate buffer.

Eighteen ml of 100% Percoll was added to the suspension to give 48% Percoll, and the mixture was recentrifuged as above. Two major bands resulted with the bacteria in the bottom band. Approximately 12 ml of the bacteria-containing, bottom band was transferred to a clean Oakridge tube, brought to 40 ml with ice-cold phosphate buffer, and centrifuged at 10,000 × *g* for 10 min at 4°C to pellet the bacteria. The supernatant fluid was removed, a 2 ml volume sterilized through a 0.22 μm membrane filter, and 0.1 ml assayed in triplicate for ATP. The pellet was resuspended in 40 ml of buffer and recentrifuged at 10,000 × *g*. The supernatant fluid was removed and assayed for ATP. No soluble ATP could be detected in 10 such supernatant fluids. The pellet was resuspended in 10 ml of phosphate buffer and the bacteria counted after diluting the suspension 1:100. The final suspension contained 0.8–1.4 × 10^9 bacteria/ml. The purified suspensions were either used immediately or subdivided into 1.0 ml aliquots and stored at −76°C.

ATP decay of freeze-thawed *M. leprae*. ATP decay was studied under defined conditions to determine if the ATP level (pg ATP/10^6 bacteria) is a reliable indicator of *M. leprae's* ability to survive *in vitro*. Bacteria frozen and thawed one time were recovered from liver tissue (A-21) by the Percoll procedure and suspended (10^9/ml) in phosphate buffer. An aliquot was diluted 1:100 in buffer, and 4.5 ml volumes transferred to test tubes. Three tubes were immediately assayed for ATP, and the remaining three tubes were assayed after 7 days' incubation at 25°C.

The same suspension (10^9/ml) was repeatedly frozen (−76°C) and thawed (25°C). After cycles 1, 3 and 5, the ATP level of diluted suspensions (10^7/ml) was determined immediately and after 7 days' incubation at 25°C.

Elevation of ATP levels. Elevation of ATP by purified *M. leprae* occurred rapidly after inoculation of pH 7, 0.1 M potassium phosphate buffer and required the procedure for handling the cells to be modified. Thirty ml of buffer in 50 ml Erlenmeyer screw-cap flasks was pre-warmed or cooled prior to inoculation to give 10^7 *M. leprae*/ml. Volumes (0.1 ml) of cell suspension were periodically transferred to 13 × 100 mm test tubes which contained 0.1 ml of Tris-EDTA buffer and 40 μl of chloroform. The tubes were immediately placed in a boiling water bath to stop the ATP increase, and the bacteria were extracted and measured for ATP as previously described. This method avoided the need to centrifuge the suspensions and decant supernatant fluids before ATP extraction, and permitted early time points to be taken.

RESULTS

Percoll buoyant density purification. It was first necessary to establish that neither soluble nor particle-contained (i.e., mitochondria and micells), host-derived ATP was carried over into the final bacterial suspension and thereby give erroneously high ATP values. Uninfected armadillo liver tissue was processed the same way as infected tissue, and the soluble and particle-associ-

TABLE 1. *Removal of soluble and particle-contained ATP from uninfected liver tissue during buoyant density purification.*

Step	Dilution per step	pg of ATP[a] per ml	
		Soluble	Particulate[b]
20% tissue suspension	1:5	ND[c]	ND
Percoll #1	1:1.5	ND	ND
Percoll #2	1:3.8	ND	19,200
Wash #1	1:3.3	300	1,480
Wash #2	1:21	0	16
Final resuspension	1:6	0	8
Inoculation of buffer	1:86	0	0.6

[a] ATP values are for uninfected armadillo liver tissue treated in the same fashion as infected tissue. Buoyant density marker beads permitted the location of volumes in the gradients corresponding to the bacterial bands.

[b] ATP was extracted with heat and chloroform.

[c] Not done due to quenching of bioluminescence during ATP assay.

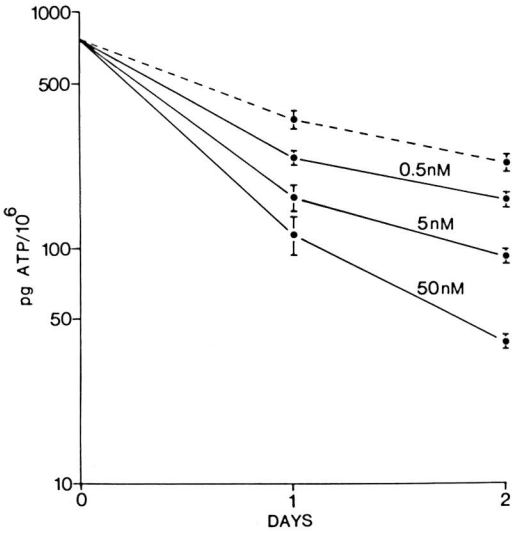

FIG. 1. The effect of clofazimine on the ATP content of *M. leprae* suspended at 10^7/ml in potassium phosphate buffer (pH 7.0) and incubated at 25°C. The control was the same as above but without clofazimine. Each data point is the mean of triplicate cultures assayed for ATP in triplicate. Bars represent ± one standard deviation.

ated ATP measured. The volumes in the Percoll gradient corresponding to those achieved with infected tissue homogenates were located using buoyant density marker beads (Pharmacia). The data in Table 1 show no soluble ATP present following the second buffer wash, and an insignificant concentration (less than 0.008% of bacterial ATP) after inoculation in buffer to give 10^7/ml. Additionally, no soluble ATP was detected in filter-sterilized supernatant fluids from twice-washed *M. leprae* purified by buoyant density centrifugation in each of 12 purification trials using infected tissue. Purified *M. leprae* suspensions (10^9/ml) were exposed to either distilled water for 30 min or one freeze-thaw cycle (−76°/25°C) in 0.1 M phosphate buffer with the intent of disrupting particles containing ATP. *M. leprae* exposed to distilled water or freeze-thawing retained 100% and 95% of their ATP, respectively. Both light and transmission electron microscopy further showed the purified suspensions to be free of tissue debris. These results indicated that soluble and membrane-bound ATP did not contribute significantly to the ATP pool of *M. leprae.*

Testing whether significant ATP was bound to the surface of *M. leprae* was approached by exposing suspensions (10^7/ml) to various concentrations of the antileprosy drug clofazimine. A demonstration of antimicrobial activity, as evidenced by a decrease in the ATP content of the *M. leprae* cells, would indicate interference with cellular function and the inability to replenish ATP. Figure 1 shows nanomolar concentrations of clofazimine accelerated the rate of ATP decay in direct proportion to drug concentration. Only 18% of the ATP content of the phosphate buffer control was present after 48 hr incubation at 25°C in the presence of 50 nM clofazimine.

The possibility that clofazimine directly expedited the hydrolysis of intracellular and/or surface-bound ATP or quenched bioluminescence during the assay was tested by mixing clofazimine with ATP to yield a 1.25 μM clofazimine solution containing 200 pg ATP/ml. The ATP was fully recoverable (data not shown), indicating the reduction of cellular ATP was due to drug action on the bacteria.

ATP content of *M. leprae.* Data presented in Table 2 show the ATP content of *M. leprae* cells purified from five armadillo livers in 12 trials. Eight of the 12 purifica-

TABLE 2. *ATP content of* M. leprae *isolated from armadillo livers by buoyant density centrifugation.*

Armadillo	Bacteria per g of tissue	Purification trial	Freeze-thaw cycles[a]	pg ATP/10^6 bacteria[b]
R-277	10^9	1	2	417 ± 7
		2	2	808 ± 8
		3	2	335 ± 22
729	10^9	1	0	597 ± 9
		2	1	236 ± 26
		3	2	345 ± 30
A-102	10^9	1	1	949 ± 33
A-21	10^{10}	1	1	618 ± 16
		2	2	462 ± 19
		3	2	832 ± 29
R-199	10^{10}	1	2	727 ± 74
		2	2	696 ± 89

[a] In tissue.

[b] For each purification trial, triplicate suspensions (10^7/ml) were prepared in phosphate buffer and assayed in triplicate. Each value is the mean ± one standard deviation for the triplicate suspensions.

TABLE 3. *ATP content of cultivable bacteria in stationary phase growth.*

Organism	pg ATP per 10^6 total bacteria[a]	Percent viability[b]	pg ATP per 10^6 viable bacteria
M. avium	91 ± 25	81	112 ± 31
M. intracellulare	103 ± 19	45	229 ± 43
M. lepraemurium	174 ± 58	70	248 ± 82
M. smegmatis	338 ± 110	69	490 ± 75
E. coli	993 ± 171	48	2069 ± 356
S. aureus	71 ± 11	16	441 ± 66

[a] Total counts were determined with a Neubauer counting chamber. ATP was measured from triplicate suspensions in triplicate. Each value is the mean ± one standard deviation for the triplicate suspensions.

[b] The viability of *M. avium, M. intracellulare, M. lepraemurium* and *M. smegmatis* was measured by staining with fluorescein diacetate and ethidium bromide. The viability of *E. coli* and *S. aureus* was determined by plating on Trypticase Soy Agar.

tions used tissue freeze-thawed (−76°/25°C) twice; whereas the remaining trials used either unfrozen or one-time frozen tissue. A range in the ATP content was observed for *M. leprae* recovered in separate purification trials from the same liver, and for bacteria recovered from different livers. No correlation was noted between the ATP level and the number of freeze-thaw cycles in tissue.

As a control on the methods of ATP extraction and measurement, a number of cultivable bacteria in stationary phase growth were assayed for their ATP content (Table 3). The values fall within the range reported by other investigators ([10, 16, 17, 19]) and indicate our methods were satisfactory. The mean ATP level of *M. leprae* (585 pg/10^6) is in the same order of magnitude as the cultivable bacteria.

Additionally, the data in Table 3 reflect the problem in reporting ATP levels based on total cell counts rather than on the number of viable cells. This is most pronounced with single cell suspensions of *S. aureus,* which had 71 pg of ATP per 10^6 total cells but 441 pg of ATP per 10^6 viable cells.

ATP level and freeze-thawing. A *M. leprae* suspension (10^9/ml) was repeatedly frozen and thawed to determine the effect upon the ATP level. This was intended to determine if the ATP level is a true indication

TABLE 4. *Effect of freeze-thawing on ATP level and ATP decay of* M. leprae.

Freeze-thaw cycles	Bacteria in[a]	Initial ATP level[b,c]	Percent of control	After 7 days in buffer[c,d]	
				ATP level	% original
1	Tissue	618 ± 16	100	250 ± 29	40
1	Buffer	588 ± 50	95	92 ± 6	16
3	Buffer	437 ± 4	63	30 ± 6	7
5	Buffer	369 ± 63	56	9 ± 0.3	2

[a] Bacteria were recovered from liver A-21 freeze-thawed one time. The purified bacterial suspension (10^9/ml) in 0.1 M potassium phosphate buffer was repeatedly freeze-thawed (−76°/25°C).

[b] Determined immediately after dilution to 10^7 bacteria/ml.

[c] ATP levels expressed as pg ATP/10^6 bacteria ± one standard deviation.

[d] The ATP level of *M. leprae* cells suspended (10^7/ml) in phosphate buffer was determined after seven days' incubation at 25°C. Percent of original ATP was calculated from the initial ATP level after each freeze-thaw cycle.

of *M. leprae's in vitro* survivability. Table 4 shows that *M. leprae* cells frozen and thawed once in tissue had an ATP level of 618 pg/10^6 cells immediately following purification and served as the control. The ATP level progressively decreased to 56% of the control after five freeze-thaw cycles in buffer.

Dilution of bacteria to 10^7/ml in buffer immediately after each freeze-thaw cycle, followed by incubation for seven days at 25°C, showed that the initial ATP level can be misleading. Even though no significant difference in ATP was observed between the control bacteria and those freeze-thawed once in buffer, an appreciable difference existed in the ATP content after seven days' incubation. Much greater differences in ATP levels after three and five freeze-thaw cycles more accurately reflected *M. leprae's in vitro* survivability.

ATP leakage. *M. leprae* were suspended in phosphate buffer (10^7/ml) to determine if the cells leaked ATP as evidenced by a reduction in ATP level. Rather than leakage, rapid, temperature-dependent ATP synthesis was observed (Fig. 2).

Effect of 2,4-dinitrophenol. *M. leprae* suspensions were exposed to various concentrations of 2,4-dinitrophenol (DNP) to determine if oxidative phosphorylation was primarily responsible for the increase in ATP. Table 5 shows the control cells in buffer alone nearly double their ATP level after 30 min with a more gradual increase thereafter. Bacteria exposed to 5 mM DNP experienced a rapid reduction in ATP level with time. This indicated ATP utilization, without the ability to synthesize it due to uncoupling of oxidative phosphorylation. Compared to the control, 0.5 mM DNP had a minimal effect after 2 hr; whereas 0.05 mM DNP stimulated an increase in ATP levels.

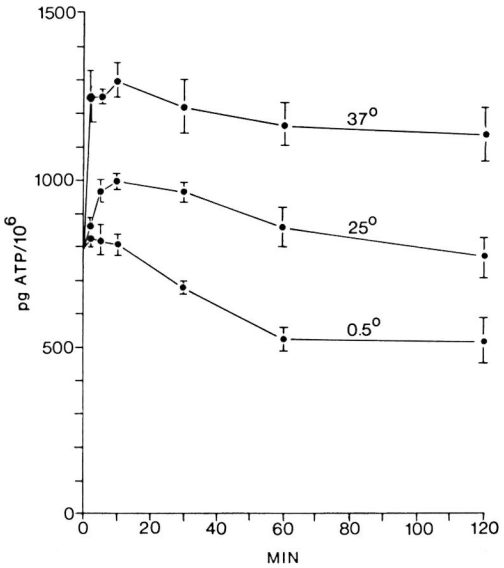

FIG. 2. The effect of temperature on the ATP synthesis of *M. leprae* suspended at 10^7/ml in potassium phosphate buffer (pH 7.0). Each data point is the mean of triplicate cultures assayed for ATP in triplicate. Bars represent ± one standard deviation.

DISCUSSION

The availability of *M. leprae* from heavily infected armadillo tissues has made it possible to study the physiology and metabolism of the organism. The Percoll buoyant density centrifugation method described in this paper was developed for recovering *M.*

TABLE 5. *The effect of 2,4-dinitrophenol (DNP) on the ATP content of* M. leprae.

DNP concentration (mM)	pg ATP/10^6 bacteria[a] (% of control) at min			
	0	30	60	120
0 (control)	588 ± 5 (100%)	1166 ± 55 (100%)	1198 ± 65 (100%)	1640 ± 76 (100%)
0.05	665 ± 45 (113%)	1522 ± 73 (131%)	1509 ± 8 (126%)	1887 ± 90 (115%)
0.5	670 ± 20 (114%)	1088 ± 66 (93%)	1281 ± 5 (107%)	1352 ± 13 (82%)
5.0	621 ± 36 (106%)	309 ± 11 (27%)	225 ± 8 (19%)	240 ± 21 (15%)

[a] *M. leprae* suspensions (10^7/ml) were incubated in 0.1 M potassium phosphate buffer pH 7 with and without DNP at 37°C. Triplicate tubes were assayed in triplicate and expressed as ATP level ± one standard deviation.

leprae in less time and with less manipulation than other methods ([8, 9]) to help minimize structural and/or functional damage to the cells.

Although relatively little is known about the effects of purification on *M. leprae,* it has been found by Buchanan (personal communication) that during and after tissue homogenation, tissue proteases remove antigenic determinants from the bacterial surface. Likewise, sodium hydroxide treatment is routinely used to remove surface-bound tissue components ([8, 9, 24]) and for decontamination ([8, 14]). Matsuo's mouse foot pad infectivity studies ([16]) have shown moderate-to-significant viability loss after treating *M. leprae* with 0.25 N NaOH for variable periods; whereas Shepard, *et al.* reported ([22]) that 0.1 N NaOH reduced the immunogenicity of the organism. Dhople, *et al.,* however, have demonstrated ([8]) that 1 N NaOH has no effect upon either the organism's ATP content or mouse foot pad infectivity.

Our method of recovering *M. leprae* does not require NaOH treatment and avoids intentional enzyme treatment used by others ([8, 9]). The bacteria are purified within 2–2½ hr under controlled conditions, with a 20–25% recovery rate, and are free of host ATP and tissue contamination. More efficient recovery is desirable but has not yet been achieved. As currently used, a sufficient number of organisms is obtained for at least six experiments.

Although it is possible some host ÀTP is bound to *M. leprae's* surface, the results indicate the amount is not significant, otherwise 50 nM clofazimine would not have reduced the ATP level to 18% of the control after 48 hr. Similarly, the rapid increase in ATP which was DNP-sensitive would be unlikely if the preponderance of the measured ATP was host-derived and also indicates *M. leprae* is capable of ATP synthesis *in vitro.* We currently do not know if the synthesis is *de novo* and/or due to the phosphorylation of adenosine diphosphate (ADP). The DNP sensitivity would tend to confirm previous studies ([12]) which showed *M. leprae* to have a functional cytochrome system.

Finding *M. leprae* capable of increasing its ATP level within minutes after thawing under nutrient-deficient conditions suggests that the organism can utilize unidentified, endogenous substrates for energy generation. The rapid increase may also help to explain the variation in ATP level of bacteria recovered from the same armadillo liver. Although an effort has been made to standardize the purification method, even short periods at room temperature would allow for ATP synthesis. Nevertheless, the lowest ATP level measured was 236 pg/10^6 cells, which is in the same order of magnitude as reported by David, *et al.* ([2]) and approximately 170 times higher than the level found by Dhople, *et al.* ([7, 8]). This places *M. leprae's* ATP content within the range of cultivable bacteria ([10, 17–19]), and would suggest that its reticence to grow *in vitro* is not due to the inability to generate sufficient biosynthetic energy.

Leakage of ATP by *M. leprae* at 25°C and 37°C is not indicated by our results. This confirms Dhople, *et al.* who also found ([8]) no evidence that the organism leaked ATP like *M. lepraemurium* ([6]). The decrease in ATP at 0.5°C may be due to ATP utilization without the ability to replenish it to the original level at the low temperature and/or alteration of cell membrane fluidity, resulting in leakage.

Our results show that initial ATP levels may inaccurately reflect *M. leprae's in vitro* survivability (Table 4). Incubation for seven days under defined conditions served to detect and to magnify the damage incurred during freeze-thawing and more accurately assessed the degree of damage. Repeated freeze-thawing in buffer eventually caused a significant reduction in the ATP level. This was not unexpected, since freeze-thawing is known to damage or kill cells.

The control bacteria recovered from tissue freeze-thawed one time (Table 4) maintained 40% of their ATP after seven days under nutrient-deficient conditions. We have subsequently confirmed this finding which raises important questions regarding the organism's ability to survive outside the animal host. Other workers have shown species of mycobacteria to survive up to six months in distilled water ([20]). Endogenous substrate utilization was postulated as responsible for the long-term survival, although the exact mechanism is unknown

([20]). It has been shown by Reeve, *et al.* ([21]) that *E. coli* and *Salmonella typhimurium* auxotrophs deficient in peptidases do not survive as well during carbon starvation as the wild-type strains. Perhaps peptidases also may have a roll in the *in vitro* survival of *M. leprae.*

Demonstrating the antimicrobial activity of clofazimine *in vitro* for the first time shows the potential usefulness of monitoring *M. leprae's* ATP decay rate over time and under defined conditions. With further study, the system may be used to detect clofazimine resistance in leprosy patients. It likewise might be used as an *in vitro* screening method for new antileprosy drugs.

It is important to know that *in vivo*-grown *M. leprae* have an ATP content similar to cultivable bacteria and that they are capable of rapidly synthesizing ATP *in vitro* and of maintaining their ATP level for a considerable period while deprived of nutrients. Likewise, it is important to recognize that initial ATP levels of similar magnitude do not always reflect the organism's ability to maintain itself *in vitro* and is a shortcoming of using the ATP level as an index of the physiological and metabolic quality of the bacteria. This can be circumvented by monitoring ATP over time while incorporating an internal control, such as potassium phosphate buffer, to which variables can be compared for their effect upon *M. leprae.*

SUMMARY

A buoyant density centrifugation procedure using Percoll was developed for the isolation and purification of *Mycobacterium leprae* from experimentally infected armadillo liver tissue. The method separates the bacteria from host adenosine triphosphate (ATP) and tissue debris and recovers 20–25% of the bacteria within 2–2½ hours under controlled conditions. The mean ATP content (585 pg/10^6) of the purified bacteria was similar to cultivable bacteria.

The organisms did not leak intracellular ATP when exposed to phosphate buffer. Temperature-dependent ATP synthesis was observed within minutes and could be inhibited by 2,4-dinitrophenol. Freeze-thawing *M. leprae* as purified suspensions in buffer damaged the organisms, resulting in decreased ATP levels and an accelerated loss of ATP upon incubation under defined conditions. *In vitro* treatment with the antileprosy drug clofazimine increased the rate of ATP decay directly proportional to drug concentration.

RESUMEN

Se desarrolló un procedimiento para aislar y purificar al *Mycobacterium leprae* a partir del tejido hepático de armadillos infectados experimentalmente. El procedimiento incluye la separación del bacilo por flotación sobre Percoll a una cierta densidad. El método permite separar a la bacteria del trifosfato de adenosina (ATP) y de los restos tisulares del huésped, y la recuperación del 20 al 25% de las bacterias en 2 a 2½ horas, bajo condiciones controladas. El contenido promedio de ATP (585 pg/10^6) de las bacterias purificadas fue similar al encontrado en bacterias cultivables.

Los organismos no dejaron escapar ATP intracelular cuando se trataron con regulador de fosfato. La síntesis de ATP dependiente de temperatura ocurrió en minutos y fue inhibida por el 2, 4-dinitrofenol. La descongelación de suspensiones purificadas de *M. leprae* dañó a los organismos dando como resultado niveles disminuidos de ATP y pérdida acelerada del mismo durante la incubación de los bacilos bajo condiciones definidas. El tratamiento *in vitro* con la droga clofazimina, aumentó la velocidad de decaimiento del ATP en forma directamente proporcional a la concentración de la droga.

RÉSUMÉ

Une méthode de centrifugation en densité de flottaison, à base de Percoll, a été développée pour isoler et purifier le *Mycobacterium leprae* à partir de tissu hépatique infecté d'armadillo. Cette méthode a consisté à séparer les bactéries des débris tissulaires et à les débarrasser de l'adénosine triphosphate (ATP) de l'animal. On peut ainsi récupérer 20 à 25% des bactéries, en 2 à 2½ heures, dans des conditions bien contrôlées. Le contenu moyen en ATP des bactéries purifiées (585 pg/10^6) était similaire à celui des bactéries cultivées.

Lorsqu'on les expose à un tampon phosphaté, les microorganismes ne libèrent pas d'ATP intracellulaire. Une synthèse de l'ATP, qui dépend de la température, a été mise en évidence dans un délai de quelques minutes; cette synthèse pouvait être inhibée par le 2,4-dinitrophénol. La congélation, suivie du dégel, de *M. leprae* en suspension purifiée dans le tampon, endommage les organismes, ce qui entraîne une diminution des taux d'ATP, ainsi qu'une perte accélérée de l'ATP lors de l'incubation dans des conditions bien définies. Le traitement *in vitro* par la clofazimine, un médicament antilépreux, augmente le taux de la dégradation de l'ATP, de manière proportionnelle à la concentration du produit.

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REFERENCES

1. COLLINS, F. M., MORRISON, N. E., DHOPLE, A. M. and WATSON, S. R. Microscopic counts carried out on *Mycobacterium leprae* and *M. tuberculosis* suspensions. A comparison of three staining procedures. Int. J. Lepr. **48** (1980) 402–407.
2. DAVID, H. L., RASTOGI, R., FREHEL, C. and GHEORGHIU, M. Reduction of potassium tellurite and ATP content in *Mycobacterium leprae.* Ann. Microbiol. (Paris) **133B** (1982) 129–139.
3. DHOPLE, A. M. and HANKS, J. H. Quantitative extraction of adenosine triphosphate from cultivable and host-grown microbes: calculation of adenosine triphosphate pools. Appl. Microbiol. **26** (1973) 399–403.
4. DHOPLE, A. M. and HANKS, J. H. Energetics (adenosine 5′-phosphate) of *Mycobacterium leprae* in diffusion chambers incubated *in vitro* and in mice. Infect. Immun. **8** (1973) 907–910.
5. DHOPLE, A. M. and HANKS, J. H. *In vitro* growth of *Mycobacterium lepraemurium,* an obligate intracellular microbe. Science **197** (1977) 370–381.
6. DHOPLE, A. M. and HANKS, J. H. Pedigreed stocks of *Mycobacterium lepraemurium* for cultivation and metabolic studies. Can. J. Microbiol. **26** (1980) 1247–1252.
7. DHOPLE, A. M. and HANKS, J. H. Adenosine triphosphate content in *Mycobacterium leprae.* A brief communication. Int. J. Lepr. **49** (1981) 57–59.
8. DHOPLE, A. M. and STORRS, E. E. Adenosine triphosphate content of *Mycobacterium leprae*: effect of purification procedures. Int. J. Lepr. **50** (1982) 83–89.
9. DRAPER, P. Annex 4, Report of the fifth meeting of the Scientific Working Group on the Immunology of Leprosy (IMMLEP). Geneva: World Health Organization, 1980, (5)80.3.
10. FORREST, W. W. Adenosine triphosphate pool during the growth cycle in *Steptococcus faecalis.* J. Bacteriol. **90** (1965) 1013–1016.
11. HANKS, J. H., CHATTERJEE, B. R. and LECHAT, M. F. A guide to the counting of mycobacteria in clinical and experimental materials. Int. J. Lepr. **32** (1964) 156–157.
12. ISHAQUE, M., KATO, L. and SKINSNES, O. K. Cytochrome-linked respiration in host grown *M. leprae* isolated from an armadillo (*Dasypus novemcinctus,* L.). Int. J. Lepr. **45** (1977) 114–119.
13. KIRCHHEIMER, W. F. and STORRS, E. E. Attempts to establish the armadillo (*Dasypus novemcinctus* Linn.) as a model for the study of leprosy. 1. Report of lepromatoid leprosy in an experimentally infected armadillo. Int. J. Lepr. **39**: (1971) 693–702.
14. KRASNOW, I. and WAYNE, L. G. Sputum digestion. I. The mortality rate of tubercle bacilli in various digestion systems. Techn. Bull. Regist. Med. Techn. **36** (1966) 34–37.
15. KVACH, J. T. and VERAS, J. R. A fluorescent staining procedure for determining the viability of mycobacterial cells. Int. J. Lepr. **50** (1982) 183–192.
16. MATSUO, Y. Effect of sodium hydroxide and trypsin on the viability of *Mycobacterium leprae.* Hiroshima J. Med. Sci. **23** (1974) 19–23.
17. MOLIN, O., NILSSON, L. and ANSEHN, G. Rapid detection of bacterial growth in blood cultures by bioluminescent assay of bacterial ATP. J. Clin. Microbiol. **3** (1983) 521–525.
18. NUZBACK, D. E., BARTLEY, E. E., DENNIS, S. M., NAGARAJA, T. G., GALITZER, S. J. and DAYTON, A. D. Relation of rumen ATP concentration to bacterial and protozoal numbers. Appl. Environ. Microbiol. **46** (1983) 533–538.
19. PICCIOLO, G. L., CHAPPELLE, E. W., KNUST, E. A., TUTTLE, S. A. and CURTIS, C. A. Problem areas in the use of the firefly luciferase assay for bacterial detection. In: *Analytical Applications of Bioluminescence and Chemiluminescence.* Chappelle, E. W. and Picciolo, G. L., eds. Washington, D. C.: National Aeronautics and Space Administration, 1975, pp. 1–26.
20. RATLEDGE, C. Nutrition, growth and metabolism. In: *The Biology of the Mycobacteria. Volume 1 Physiology, Identification and Classification.* Ratledge, C. and Stanford, J., eds. London: Academic Press, 1982, pp. 183–271.
21. REEVE, C. A., BOCKMAN, A. T. and MATIN, A. Role of protein degradation in the survival of carbon-starved *Escherichia coli* and *Salmonella typhimurium.* J. Bacteriol. **157** (1984) 758–763.
22. SHEPARD, C. C., DRAPER, P., REES, R. J. W. and LOWE, C. Effect of purification steps on the immunogenicity of *Mycobacterium leprae.* Br. J. Exp. Pathol. **61** (1980) 326–379.
23. STORRS, E. E. The nine-banded armadillo: a research model for leprosy and other biomedical research. Int. J. Lepr. **39** (1971) 703–714.
24. WHEELER, R. P. and GREGORY, D. Superoxide dismutase, peroxidatic activity and catalase in *Mycobacterium leprae* purified from armadillo liver. J. Gen. Microbiol. **121** (1971) 457–464.