Quantitative Extraction of Adenosine Triphosphate from Cultivable and Host-Grown Microbes: Calculation of Adenosine Triphosphate Pools

ARVIND M. DHOPLE AND JOHN H. HANKS

Department of Pathobiology, Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland 21205

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Existing data on adenosine triphosphate (ATP) pools in microbes are deficient for two reasons: (i) incomplete extractions of ATP, and (ii) the failure to take into account that the adverse effects of extracting procedures on standard ATP exert analogous effects on the ATP released from bacterial cells. Methods for correcting observed yields and calculating ATP pools have been demonstrated. Three bacterial species were used in the studies on extraction of ATP: Escherichia coli, Mycobacterium phlei, and Mycobacterium leprae. Perchloric acid and n-butanol were disqualified because of their failure to extract total bacterial ATP even from E. coli and because of inconvenient procedures. The new extraction procedure had minimal effects on standard ATP, liberated 100% of the ATP pools from the three representative species of microbes, and caused no ionic imbalance or quenching of bioluminescence. This method involves vortexing of cell suspensions for 10 s with 23% chloroform (vol/vol), heating at 98 C for the required time (E. coli, 3 min; M. phlei, 5 min; M. lepra, 10 min) and then 1 min at 98 C with vacuum to dry the samples. Heat or chloroform alone may suffice for some microbes and release total ATP from plant and animal cells.

Existing data on adenosine triphosphate (ATP) pools in microbes are misleading because of incomplete extractions and failures to quantify the deleterious effects of extracting agents and procedures on standard ATP. All-purpose methods should meet the following requirements: minimal destruction of standard ATP; recovery of total ATP by a single extraction, as judged by maximal yields and inability to recover further ATP by a second extraction; and ability to release total ATP from "waxy-walled" microbes such as pathogenic mycobacteria. To attain the sensitivities required for investigating the energetics of severely limited supplies of host-grown microbes, the extracted ATP cannot be further diluted by the neutralization of acids or to decrease the quenching of bioluminescence by solvents. Optimal methods should produce dried samples of ATP which can be dissolved in the minimal volumes required for assays.

In order to examine minimal, moderate, and maximal resistance to the release of ATP, three bacterial species were used. They were: Escherichia coli to represent the eubacteriales; Mycobacterium phlei, the most heat-resistant saprophytic mycobacterium, to represent also the nocardiae, actinomycetes, and streptomycetes; and Mycobacterium leprae, the noncultivated agent of rat leprosy and the most heat-resistant and impenetrable of the pathogenic mycobacteria (5). The requirements outlined were met by focusing first on methods adequate for M. leprae. Experience in measuring the effects of extraction procedures on standard ATP and in evaluating the efficiencies of classical extractors such as PCA (perchloric acid) and n-butanol afforded insight into the calculation of ATP pools.

MATERIALS AND METHODS

Bioluminescence. ATP was determined by the firefly bioluminescent reaction, utilizing the reagents of Chappelle and Levin (3) in optimal concentrations, which increased the original sensitivity 1.5-fold (J. H. Hanks and A. M. Dhople, submitted for publication). The preparation and stabilization of reagents, the
concentrations that yield optimal sensitivity for assaying ATP, and the character of the instrument response have been summarized elsewhere (J. H. Hanks and A. M. Dhople, submitted for publication). The instrument used in measuring bioluminescence has been designed and fabricated by Chappelle and associates (3). The enzyme system was so adjusted that 10 ng of ATP per 0.1 ml sample, when injected into 0.3 ml of acceptor system (luciferase, 1 U; luciferin, 13 μg/ml; and Mg, 6.7 mM in 0.05 M N-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid (TES) buffer, pH 7.4, caused an instrument response of 50 U, i.e., 2.5 in (approximately 6.25 cm) elevation on the recorder.

Organisms. E. coli, strain B, was grown for 20 h in Difco nutrient broth containing 0.5% glucose. The post-log-phase cells, with presumed maximal roughness of cell walls, were diluted to an optical density of 0.01, and 0.1 ml (approximately 10⁵ cells) was used per extraction. A sample of this suspension was diluted and plated immediately on Difco nutrient agar so that the number of colonies permitted calculating ATP pools in terms of viable cells.

M. phlei, strain HMS, was grown at maximal smoothness for 20 h in modified Hanks basal medium containing FeCl₃, 6H₂O (0.005%); MgSO₄, 7H₂O (0.1%); ZnSO₄, 7H₂O (0.001%); dibasic potassium phosphate (0.5%); dibasic ammonium citrate (0.25%); sodium acetate (0.5%); glucose (0.2%); Tween 80 (0.3%) and bovine serum albumin fraction V (0.4%). The pH was adjusted to 7.0 with 1 N NaOH. The log-phase cells were stirred during filtration of the suspensions through membranes filters (8 μm pore size; Millipore Corp.) to obtain single-cell suspensions. The cells were then diluted to optical density of 0.02 and 0.1 ml (approximately 10⁷ cells) used per extraction. Further dilutions and plate counts on Difco nutrient agar were made as described for E. coli.

The noncultivable M. lepraeumurium cells were harvested from pooled livers, spleens, and omental fatty pads of CFW mice during the terminal illness of animals that had been challenged systemically (A. M. Dhople and J. H. Hanks, unpublished data). The organ homogenates (tissue = 20%) were freed of mammalian cells and debris and of clumped bacteria by centrifugation for 5 min at 200 × g. The single-cell bacterial suspensions contained on average 10¹⁸ microscopically countable organisms per 0.1 ml (6). Thus, dilution of the clarified homogenates to tissue equivalent 0.2% provided approximately 10¹⁰ bacilli per 0.1 ml sample extracted.

Extraction procedures. The standard ATP, bioluminescent reagents, bacterial cells and extracted ATP were held in crushed ice (0.5 C) except during manipulations. Centrifugations were at 4 C. The recommended extraction method was conducted as follows: (i) 0.1 ml of bacterial suspension was placed in a Pyrex tube (10 by 75 mm); (ii) 0.03 ml of chloroform (25% vol/vol) was added and vortexed 10 s to insure maximal contact of the agent with the bacterial cell walls and membranes; (iii) the tube was placed in a 98 C (boiling) bath for the time required to complete the liberation of the ATP (E. coli, 2 min; M. phlei, 5 min; M. lepraeumurium, 10 min) with shaking during the last minute to release excess chloroform that might "bump" during evaporation; (iv) during 1 further min at 98 C, vacuum (750 mm of mercury) was applied, which dried the samples within approximately 40 s. Since the stability of ATP was decreased by heating 10 min, all samples were rehydrated immediately with 0.4 ml TES buffer and assayed immediately.

To maintain convenient volume relationships in extractions with PCA and n-butanol, the 0.1 ml samples of bacterial cells in some instances were centrifuged 15 min at 3,000 × g. 99% of the supernatant fluid was removed, and the 0.01 ml of sedimented cells was treated. Cells extracted by the reference method were handled in the same manner. In view of the variety of methods and procedures not to be recommended, details can be found under each type of experiment.

The PCA used for extracting ATP and the KOH required for neutralization contained 0.001% phenol red to insure that the terminal pH of the small volumes was 7.4. The potassium perchlorate precipitates were removed by centrifugation and the clear supernatant fluids were assayed. Re-extractions of PCA-treated cells to test for further recoveries of ATP were not feasible because of the bulky white precipitates. Re-extractions were routine while developing the reference methods and in the experiments with n-butanol.

RESULTS

Calculation of ATP pools. If a given procedure permits detecting only 90% of the standard ATP, it can demonstrate only 90% of the ATP released from bacterial cells. It follows that valid estimations of bacterial ATP depend firstly upon determining the effects of extraction and handling procedures on standard ATP and secondly upon calculating the analogous effects on the bacterial ATP extracted.

Table 1 provides an example of the data and calculations required. Column A shows the effects of each procedure on standard ATP. The values for extracted ATP (column B) suggest that the release of bacterial ATP by the three methods differed significantly. Upon correcting for the effect of each procedure on standard ATP, the values in columns C and D reveal that the three methods had achieved an equivalent release of the ATP pool. The values in columns A, B, and C are given as units of instrument response, because such data suffice for comparing the effectiveness of extraction methods. For present purposes the ATP pools were defined as picograms of ATP per million viable cells.

Reference method. A previous investigation of factors that might alleviate the exceptional impenetrability of M. lepraeumurium to acids, alkalies, tetrazoles, and triphenylmethane dyes uncovered only two agents, heat and chloro-
form, which promptly opened the cells to dye penetration (5). The earliest attempts to release ATP from M. leprae, therefore, were comparisons of PCA and n-butanol versus heat and chloroform. The yields obtained by PCA and n-butanol were only a fraction of those obtained by applying heat and chloroform sequentially. The effects of heat and chloroform, therefore, were investigated systematically.

The background on optimal application of each agent is as follows. Heat at 98°C degrades ATP slowly; 99% of standard ATP is assayable after 5 min and 98% after 10 min. Heating beyond 10 min is destructive. Chloroform has the following merits: it does not degrade or sequester standard ATP; it disrupts enormous clumps of mycobacteria instantaneously, thus exposing the surfaces of individual cells. Because of its low boiling point, it is readily removed by heat or vacuum and cannot quench bioluminescence. The adopted concentration of chloroform (23% vol/vol) was chosen after vortexing the three types of cells in concentrations ranging from 17 to 50%, vol/vol, which degrades the acid-fastness of mycobacteria to a maximal degree. Chloroform at 17 to 23%, vol/vol, had maximal effects on E. coli and M. phlei; 50% produced a gain of only 3% with M. leprae. Since vortexing M. leprae with 23% chloroform for 3 s released only 68% of the ATP obtained by vortexing 10 s, 10-s vortexing was adopted for all species. When heat and chloroform were applied in either sequence, 23% chloroform sufficed for M. leprae. The foregoing guidelines defined the terms on which chloroform and heat could be applied in sequence and resulted in the development of two methods having minimal effects on standard ATP, giving maximal yields of extracted ATP and no ATP upon re-extractions of cell residues and dry samples.

Table 2 summarizes the percentages of ATP pools released from the representative microbes by heat and chloroform. Items 1 and 2 show that neither heat nor chloroform sufficed for E. coli and that the two mycobacterial species were progressively more resistant. Items 1 and 2 also demonstrate that chloroform was a more effective disruptor of membrane-wall complexes than was heat alone under the conditions defined.

Items 3 and 4 in the table show that applying either heat or chloroform as the first step was equally effective. The choice between methods was based on convenience. In the first method (item 3) the tubes were placed in the boiling bath twice, i.e., 10 min before chloroform treatment and later for 1 min to reheat plus 1 min to vacuum-dry. The reference method (item 4) is preferred because, once the cells have been vortexed with chloroform, the samples remain at 98°C until ready to be vacuum-dried. The minimal heating required for E. coli was 2 min, for M. phlei 5 min, and for M. leprae 10 min.

The reliability of the reference method was checked by "salting" M. leprae cells (the "toughest" species) with standard ATP before extraction. The total recoveries of ATP agreed with expected yields. Hence, no portion of the ATP liberated from bacterial cells remained adsorbed to the bacterial residues. These results were consistent with the failures to obtain further ATP by re-extracting cell residues.

PCA. The data in Table 3 characterize the

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**Table 1. Calculations of ATP pools in M. phlei after extracting by methods of apparently divergent effectiveness**

| Item | ATP pools | | | | |
|------|-----------|---|---|---|---|
|      | A. Standard ATP (10 pg)* | Extracted ATP | D. ATP pool† |
|      | Units | % | B. Units* | C. Units* | ATP (pg) | % |
| 1. Reference standard | 50 | 100 | 68 | 69.4 | 13.9 | 100 |
| 2. Reference method | 49 | 98 | 60 | 68.9 | 14.0 | 100.7 |
| 3. Experimental methods: | | | | | | |
| a | 43 | 86 | 62 | 68.9 | 13.8 | 99.3 |
| b | 45 | 90 | 62 | 68.9 | 13.8 | 99.3 |

* All values were calculated to 10^6 viable cells per assay.
* A. Units of instrument response to standard ATP and the percent response after each extraction procedure.
* B. Units of instrument response to the ATP extracted by each method.
* C. Units of instrument response corrected for the effect of extraction procedures on the standard ATP (column B = the percentage of standard ATP detected in column A).
* D. Conversion of units to picograms of ATP = column C × 0.2. (If 50 U of response = 10 pg ATP, 1 unit = 0.2 pg.)
shortcomings of PCA. At optimal concentrations (0.75 and 1.0 N) it hydrolyzed 6 and 8% of the standard ATP. After compensating for these effects, these optimal concentrations were roughly equivalent to heat for E. coli cells and inferior to heat for M. phlei cells. Higher concentrations impeded extraction. Item 5 (preheated cells) demonstrates that the impedance was due to interactions between PCA and undenatured membrane-wall complexes (see Discussion).

n-Butanol. The usefulness of this solvent is limited by low solubility in water, poor adsorption to bacterial cells, and the necessity of diluting to 0.001% to eliminate the quenching of bioluminescence. The high boiling point (118 C) complicated its complete distillation under the working conditions tolerated by ATP. After extracting bacterial cells with 5% n-butanol (vol/vol), vortexing 10 s, and allowing the solvent to act for 5 min at room temperature, extracts diluted five times caused a 14% quenching of bioluminescence. After calculations that compensated for quenching, single extractions liberated 88% of the ATP from E. coli cells and 69% from M. phlei. Single extractions were inadequate even when the cells were post-heated 10 min and vacuum-dried. The complicated double extractions released total ATP from E. coli, but not from M. phlei. As in the case of PCA, the total work on n-butanol uncovered no instance in which equivalent ratios of the ATP pools were liberated from the two test microbes.

DISCUSSION

Experience has disclosed three reasons why acids have earned a poor reputation as extractors of ATP. The first is that denaturing agents decrease the penetrability of microbial membrane-wall complexes. This observation explains why the extraction of ATP from E. coli by PCA (Table 3) declined at concentrations above 1 N and, in part, why Nazar et al. (7) reported that five successive extractions of E. coli with 1 N acetic acid liberated less than 50% of the triphosphate nucleotides. This general phenomenon is the basis of the notorious resistance of pathogenic mycobacteria to acids, alkalis, etc., of the observation that a primary effect of fixing agents is to impede their own penetration (4), and of the fact that 0.1 to 0.3 N NaOH is the only simple diluent that prevents the leaching of ATP from M. lepraemurium during the dilutions and washing required to separate these cells from host tissues. A second factor is the failure to make corrections for the hydrolysis of standard ATP by trichloroacetic acid (1) and PCA (2, 7). A third is the necessity for a carefully selected buffer (0.05 M TES) and

Table 2. Extraction of ATP by heat and chloroform: basis of reference method

| Procedures          | Time at 98 °C (min) | ATP pool released (%) from ATP | E. coli | M. phlei | M. lepraemurium |
|---------------------|---------------------|---------------------------------|---------|----------|-----------------|
| 1. Heat only        | 2                   | 52                             | 45      | 31       |                 |
|                     | 5                   | 78                             | 69      | 54       |                 |
|                     | 10                  | 90                             | 88      | 56       |                 |
| 2. Chloroform only*|                     |                                 |         |          |                 |
| 3. Heat first, then| 5                   | 100                            | 101     | 79       |                 |
| chloroform*         | 10                  | —                              | —       | 100      |                 |
| 4. Chloroform first,| 2                   | 100                            | 96      | 70       |                 |
| then heat*          | 5                   | —                              | —       | 100      |                 |
|                     | 10                  | —                              | —       | 100      |                 |

* Data refer to heating before vacuum-drying.
* Calculated as shown in Table 1.
* Chloroform: 0.03 ml added to 0.1-ml samples = 23% (vol/vol; vortexed 10 s).
* Terminal heating = 1 min to warm the sample plus 1 min of vacuum-drying.
* Reference method. Terminal heating = 1 min to vacuum-dry.

Table 3. PCA as an extractor of ATP

| Item               | Standard ATP (10 pg) | ATP extracted from |
|--------------------|----------------------|--------------------|
|                    | Units | % | E. coli | Units | % | M. phlei | Units | % |
| 1. Reference standard | 50   | 100 | 184 | 100 | 158 | 100 |
| 2. Reference method | 48   | 96  | 168 | 91  | 139 | 88  |
| 3. Heat 98 C, 10 min | 49   | 98  |      |      |      |      |
| 4. PCA (5 min, room temp) | | | | | | |
| 0.10 N             | 48   | 96  | 155 | 84  | 118 | 75  |
| 0.50 N             | 48   | 96  | 155 | 84  | 120 | 76  |
| 0.75 N             | 47   | 94  | 171 | 93  | 128 | 81  |
| 1.00 N             | 46   | 92  | 171 | 93  | 125 | 79  |
| 5.00 N             | 46   | 92  | 169 | 92  | 121 | 77  |
| 5. PCA (preheat 98 C, 10 min) | | | | | | |
| 0.10 N             | 47   | 94  | 167 | 91  | 142 | 90  |
| 0.50 N             | 47   | 94  | 182 | 99  | 144 | 91  |
| 0.75 N             | 46   | 92  | 185 | 100 | 149 | 94  |
| 1.00 N             | 46   | 92  | 184 | 100 | 149 | 94  |

* E. coli and M. phlei = 2.5 x 10⁴ viable units per assay. The units of response have been corrected for the effect of each procedure on standard ATP.
neutralizations titrated in the presence of a color indicator in order to optimize the detection of ATP.

The basic problem of extracting microbial ATP is reduced to simple terms by setting aside the mycobacteria as a special case and the necessity of dried samples as pertinent only to limited supplies of host-grown microbes. Even after making corrections that cancelled the adverse effects of PCA and n-butanol on standard ATP and bioluminescence, heating the representative eubacteriales for 10 min extracted ATP as efficiently as the optimal results obtained with PCA or n-butanol. Heat conserved 98% of the standard ATP and eliminated cumbersome procedures. Chloroform vortexed 10 s, then removed by vacuum-drying, was more efficient than heat. Heating for 2 min before vacuum-drying released total ATP and recovered 96% of the original ATP. This approach requires merely an extension of heating time to release total ATP from M. lepraemurium the most heat resistant and impenetrable mycobacterial pathogen. Expandable effectiveness is desirable since marginal methods applied to a given species may fail to liberate constant ratios of the ATP when cells differ by age and lipid content.

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