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

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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 lepraemurium. 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. lepraemurium, 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 quantity 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 lepraemurium, the noncultivated agent of 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. lepraemurium. 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 as-
saying 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 pg 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 tough-
ness of cell walls, were diluted to an optical density
of 0.01, and 0.1 ml (approximately 10^7 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 H1S, was grown at maximal smoothness
for 20 h in modified Hanks basal medium
containing FeCl₂, 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
suspension 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 10^6 cells) used per
extraction. Further dilutions and plate counts on
Difco nutrient agar were made as described for E. coli.

The noncultivable M. lepraemurium cells were
harvested from pooled livers, spleens, and omentum
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 x g. The single-cell
bacterial suspensions contained on average 10^9
microscopically countable organisms per 0.1 ml (6).
Thus, dilution of the clarified homogenates to tissue
equivalent 0.2% provided approximately 10^6 bacilli
per 0.1-ml sample extracted.

Extraction procedures. The standard ATP, biolu-
minescent reagents, bacterial cells and extracted ATP
were held in crushed ice (0.5 C) except during manip-
ulations. Centrifugations were at 4 C. The recom-
ended 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. lepraemurium, 10 min) with shaking during
the last minute to release excess chloroform that
might "bump" during evaporation; (iv) during 1 fur-
ther min at 98 C, vacuum (750 mm of mercury) was
applied, which dried the samples within approxi-
ately 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 x g, 99% of the superna-
ant 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 precipi-
tates 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 precipi-
tates. Re-extractions were routine while developing
the reference methods and in the experiments with
n-butanol.

RESULTS

Calculation of ATP pools. If a given pro-
cedure 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 extrac-
tion 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 ef-
ects 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 correct-
ing 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 compar-
ing 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. lepraemurium to acids,
alikales, 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. lepraeum, 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. lepraeum. Since vortexing M. lepraemurium 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. lepraeum. 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. lepraemurium 10 min.

The reliability of the reference method was checked by “salting” M. lepraeum 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
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 impendence 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

| Item | Standard ATP (10 pg) | ATP extracted from |
|------|---------------------|-------------------|
|      | Units | E. coli | M. phlei |
| 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 | 46 92 169 92 121 77 |
| 4. PCA (5 min, room temp) | 96 155 84 118 75 |
| 0.10 N | 48 96 | 46 92 185 93 128 81 |
| 0.50 N | 47 94 | 167 91 142 90 |
| 5.00 N | 46 92 | 184 100 149 94 |
| 0.75 N | 47 94 182 99 144 91 |
| 1.00 N | 46 92 | 184 100 149 94 |
| 5. PCA (preheat 98 C, 10 min) | 96 155 84 118 75 |
| 0.10 N | 48 96 | 46 92 185 93 128 81 |
| 0.50 N | 47 94 | 167 91 142 90 |
| 5.00 N | 46 92 | 184 100 149 94 |
| 0.75 N | 47 94 182 99 144 91 |
| 1.00 N | 46 92 | 184 100 149 94 |

* E. coli and M. phlei = 2.5 x 10^4 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 obtainable 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 Mycobacterium leprae and 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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