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Use of an ATP Bioluminescent Assay To Evaluate Viability of *Pneumocystis carinii* from Rats

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A bioluminescent assay which employs the luciferin-luciferase ATP-dependent reaction was used to evaluate the viability of populations of *Pneumocystis carinii* derived from infected rat lungs. Contamination with host cells was reduced by a purification method which involved a combination of low- and high-speed centrifugations resulting in a 1,000-fold reduction of the rat cells while enriching for the trophic form of *P. carinii*. A linear correlation for the number of *P. carinii* nuclei versus the amount of ATP was observed. The ATP content of the organism populations could be maintained at inoculum levels for one week, although the number of organisms did not increase. Addition of respiratory chain inhibitors dramatically decreased the ATP content of the *P. carinii* after 24 h of incubation, with the exception of the antibiotic oligomycin B. Low concentrations of trimethoprim-sulfamethoxazole and pentamidine isethionate reduced the organism ATP content by over 50% after 24 h of exposure, while no effect was observed with 100-fold greater concentrations of ampicillin. The bioluminescent assay was found to be a more sensitive indicator of viability than a dual fluorescent staining technique. This assay does not require replication of *P. carinii* and should be a useful method for in vitro drug screening and viability assessment of *P. carinii* populations.

Pneumocystis carinii organisms are eukaryotic protistan pathogens that can be detected in a wide variety of mammalian lungs by staining techniques such as methenamine silver, Giemsa, or immunofluorescence (5). In immunocompromized hosts, especially those patients with AIDS, *P. carinii* organisms are able to cause a pneumonia which remains a major source of morbidity and mortality. Diagnosis, drug screening, and almost every aspect of *P. carinii* research have been hindered by the lack of a continuous in vitro cultivation system. Several laboratories have been successful in the limited propagation of rat-derived *P. carinii* on cell monolayers (3, 4, 13, 27, 32, 38, 39; for reviews, see references 8 and 46) or in cell-free media (7, 9, 48), but no system can supply the investigator with a steady, renewable source of organisms or supply the diagnostician with a method to evaluate drug susceptibilities.

One of the problems associated with establishing a culture for *P. carinii* is assessment of the viability of the organisms used as inocula. Many procedures for the purification of organisms from infected rat lungs have been described (4, 12, 38, 42, 49), but few have focused on the vitality of the *P. carinii* postisolation (23, 41). As an initial step towards defining culture conditions for *P. carinii*, we have explored various methods for the evaluation of organism viability. In previous studies, fluorogenic stains, such as calcein acetoxymethyl ester-ethidium homodimer (Live/Dead kit; Molecular Probes, Eugene, Oreg.), and classical vital stains have been reported to be useful for this purpose (21, 22, 26, 41). The advantage of these staining techniques is that they permit a cell-by-cell evaluation of the viability of the total cell population present. However, manual counting (21, 22, 41) or flow cytometry (26) is required to assess the percent dead versus live cells, and thus the process can be quite time consuming. In addition, preliminary in vitro drug screening studies using fluorescent staining methods in our laboratory have shown that the response to standard anti-*P. carinii* drugs was inconsistent with previous in vitro evaluations and not predictive of in vivo efficacy. As an alternative method, we chose to explore the use of a bioluminescent assay to assess the ATP content of *P. carinii* preparations as an indicator of viability. The bioluminescent ATP assay is a responsive, rapid, and relatively inexpensive system with the potential for the large-scale screening of samples in a short time period.

The basis of the assay is the measurement of intracellular adenosine triphosphate. ATP is an universal energy unit in all living cells; when a cell dies, the intracellular ATP is rapidly degraded by ATPases and the ATP levels decline rapidly (15). In the presence of luciferase and the substrate luciferin, extracted from the firefly *Photinus pyralis*, ATP-dependent oxidation of the substrate luciferin in the presence of luciferase produces oxyluciferin, carbon dioxide, AMP, inorganic phosphate, and light (2, 30). The amount of light emitted is directly correlated with the amount of ATP present. The ATP bioluminescent assay has been used for a variety of applications including the screening of anticancer drugs in mammalian cell culture (1, 17, 24, 25, 45), assessment of the viability of fastidious microorganisms (40) and protozoan parasites in which *P. carinii* was evaluated (33), and the detection of microbes in clinical specimens (20, 28, 31, 44).

There is good evidence that *P. carinii* can respire aerobically and that the main source of ATP for this organism is the mitochondria. Ultrastructural analyses have revealed the presence of intact, apparently functional mitochondria in all of the life cycle stages of *P. carinii* (51). Supporting the concept of an active metabolic state of *P. carinii* mitochondria are our recent studies using fluorescent probes (6). *P. carinii* was observed to transport and sequester mitochondrion-specific probes reliant upon the enzymatic function of viable mitochondria. Addition of the respiratory inhibitor potassium cyanide prevented the sequestration. Biochemical analyses of isolated organisms kept in buffered salt solutions showed the incorporation of radiolabeled pyruvate and evolution of labeled CO_2 at a rate much

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higher than that of glucose, indicating that aerobic respiration was operational (35). Cytochemical and starch gel electrophoretic techniques identified *P. carinii*-specific enzymes of the tricarboxylic acid cycle: succinic dehydrogenase (29), malate dehydrogenase (36), and glutamate dehydrogenase (36). Cytochrome activities were predicted by inhibition studies with cyanide (37) and hydroxynaphthoquinones (19). Direct analysis of oxygen consumption by isolated *P. carinii* showed a low rate of uptake (37); however, this may have been due in part to unfavorable maintenance (in vitro) conditions in which the organism was tested. The efficacy of atovaquone, an inhibitor of the respiratory chain at the cytochrome bc_1 complex, for the treatment of *P. carinii* pneumonia also implies a crucial role for the mitochondria in the respiration of this organism (19). Collectively, these studies suggest that *P. carinii* maintains functional mitochondria and that it is through the process of oxidative phosphorylation that the major portion of the ATP pool is synthesized.

The ATP bioluminescent assay presented here is a rapid and reproducible technique for assessing the metabolic state of *P. carinii*. It measures the ATP pools in organism populations, the biosynthesis of which appears to originate largely in the mitochondria. This assay does not require replication of organisms and provides a useful method for screening of candidate compounds against *P. carinii*.

MATERIALS AND METHODS

Source and preparation of *P. carinii*. It is now well documented that *P. carinii* prepared from different mammalian hosts displays both antigenic and genetic diversity suggestive of species differences (47, 50). Electrophoretic karyotypic and gene sequence differences among organisms prepared from several rat colonies indicate that there are at least two species and probably several strains of *P. carinii* that can cause infection in rats (10, 14). The two provisional species of rat-derived *P. carinii* have been termed prototype and variant. Differences in electrophoretic karyotype patterns have been used to further differentiate prototype organisms into forms 1 through 4 (10). In the present study, we used a specific form of rat-derived *P. carinii*, prototype form 1, throughout the experiments as a way to begin to standardize such assays and promote direct comparisons by investigators.

Male Sprague-Dawley rats were obtained from the Sasco, Inc., O'Fallon colony located in Omaha, Nebr. These rats harbored *P. carinii* prototype form 1, as previously documented (10) and subsequently verified prior to these studies (data not shown). The rats were received at weights of 140 to 160 g, allowed to acclimate in standard housing facilities at the Cincinnati Veterans Affairs Medical Center animal facilities for 1 week, and then immunosuppressed for 8 to 12 weeks by administration of weekly injections of 4 mg of methylprednisolone acetate (Depo-Medrol; The Upjohn Co., Kalamazoo, Mich.) (10). The studies used *P. carinii* from a single rat or pooled organisms from several rats. Organisms were prepared according to a modification of previous procedures (11, 13). Infected lungs were removed en bloc, placed in RPMI 1640 medium (Gibco BRL, Life Technologies, Inc., Grand Island, N.Y.) supplemented with 20% fetal bovine serum (FBS) (lot 73HO721; Sigma Chemical Co., St. Louis, Mo.) and 200 μg of streptomycin, 200 U of penicillin, and 0.25 μg of amphotericin B (AB-AM; Gibco BRL) per ml, and held at 4°C. The lobes were extricated from the trachea, esophagus, and other extraneous tissue and homogenized in fresh supplemented RPMI 1640 with a Stomacher Lab Blender 80 (Tekmar, Cincinnati, Ohio) for 5 min at the highest setting at room temperature. The homogenate was poured through sterile gauze to remove large tissue fragments and treated with aqueous 0.85% ammonium chloride to lyse erythrocytes and some nucleated host cells. After centrifugation at 1,000 × *g* to pellet the organisms and remaining lung cells, three washes with the supplemented RPMI 1640 were performed and the pellet was collected by centrifugation at 1,000 × *g*. A period of gravity sedimentation that served to reduce host cell debris was performed by reconstitution of the pellet in at least 15 ml of RPMI 1640 supplemented with 30% FBS with AB-AM, and incubation for 30 min at 37°C followed. Tinctorial staining of the sediment and liquid phase showed that most *P. carinii* organisms had settled, while host debris remained in the medium. The sediment containing the majority of organisms and some host cell contaminants was then subjected to three low-speed centrifugations (400 × *g*) in RPMI 1640 supplemented with 20% FBS with AB-AM to further eliminate host cell debris. The supernatants were combined and centrifuged at 2,400 × *g* to collect the organisms. Most of the organisms collected from the supernatants were trophic forms, while the cysts tended to settle out with the host debris (see Results). The number of organisms was enumerated by Diff-Quik staining and counting of *P. carinii* nuclei, as we have previously described (11). Host cells were stained and counted in a similar manner.

Media and conditions. After testing a variety of media, supplements, and temperature and culture conditions using the ATP assay presented herein, we found the medium which permitted the optimal maintenance of *P. carinii* viability to consist of the tissue culture medium RPMI 1640 (Gibco BRL) supplemented with 20% FBS (Sigma Chemical Co.), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Gibco BRL), 1% (vol/vol) minimal essential medium (MEM) vitamin mixture (Gibco BRL), 1% (vol/vol) MEM nonessential amino acid mixture (Gibco BRL), 1% (vol/vol) acid pyruvate (Gibco BRL), 0.01 mg of water-soluble cholesterol (40 mg of cholesterol per g; balance, 2-hydroxypropyl-β-cyclodextrin) (Sigma Chemical Co.) per ml, 0.001 mg of thymidine (Sigma Chemical Co.) per ml, 0.01 mg of L-glutamine (Gibco BRL) per ml, 0.00346 μg of sodium selenite (Sigma Chemical Co.) per ml, 0.01 mg of cobalt chloride (Sigma Chemical Co.) per ml, and AB-AM and adjusted to a pH of 8.0 with 0.1 M NaOH. This was termed the maintenance medium and had an osmolarity of 380 mosmol/kg. *P. carinii* organisms were inoculated at a density of 10^8 to 10^9 nuclei per ml into 10 ml of the maintenance medium in glass tubes fitted with black phenolic caps. The tubes were incubated at 37°C in an upright position with the caps loosely placed on the tubes. The atmosphere was humidified and contained 10% carbon dioxide. Three-fifths of the total medium was replaced with fresh medium after centrifugation at 1,000 × *g* on a daily basis to maintain the pH and the ATP at or above inoculum levels. Hydrogen ion concentration was monitored by use of a Micromodel pH meter (Horiba Instruments, Inc., Irvine, Calif.).

ATP assay. The extraction and preparation of reagents followed the vendor's instructions with few modifications. An ATP assay kit obtained from BioOrbit (Turku, Finland) was used for initial studies, and later, reagents were purchased separately and prepared in our laboratory. Bovine serum albumin (BSA), magnesium acetate, magnesium sulfate, Tris-acetate, EDTA, and inorganic pyrophosphate were purchased from Sigma Chemical Co., firefly luciferase and D-luciferin were purchased from BioOrbit, trichloroacetic acid was purchased from Fisher Scientific, Inc., Cincinnati, Ohio, and ATP standard was purchased from Sigma Chemical Co. At the reported time points, 0.1 ml of well-mixed inoculated maintenance medium was removed from each of triplicate tubes and

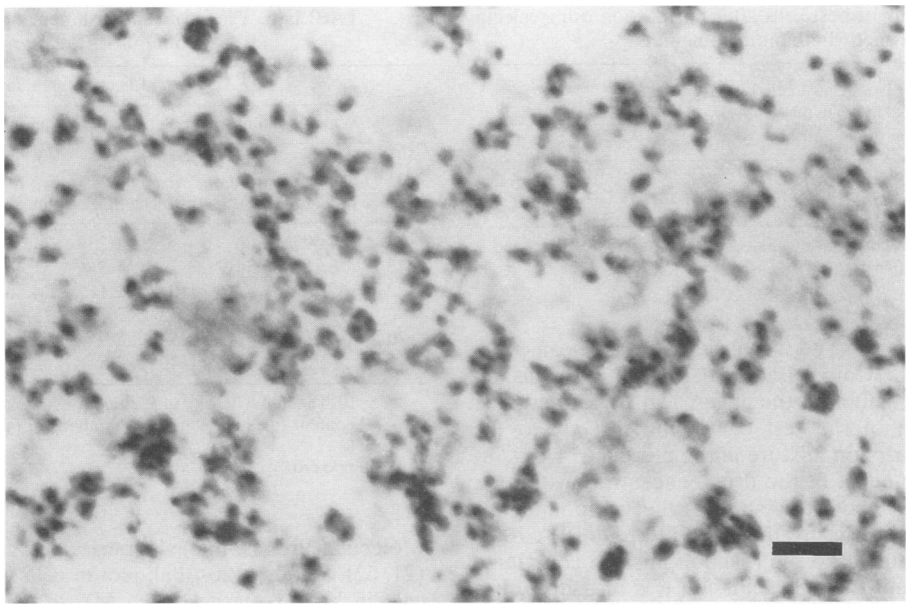

FIG. 1. Photomicrograph of a representative *P. carinii* preparation. Organisms were purified from infected rat lung by a series of low- and high-speed centrifugations, with an initial lysis step. Note the absence of intact host cells and the preponderance of trophic forms in this field. Bar = 10 μm.

added directly to 0.3 ml of 3.5% aqueous trichloracetic acid–2 mM EDTA solution (pH < 2.0), mixed well by vortexing, and incubated at room temperature for 10 min to extract the organism ATP.

All samples were measured in an AutoLumat LB 953 luminometer (Wallac, Inc., Gaithersburg, Md.). To initiate measurement of the ATP content of samples, the background light emission (B) was first measured by placing 50 μl of the ATP monitoring reagent (luciferase-luciferin, 50 mg of BSA, 0.5 mmol of magnesium acetate, 0.1 μmol of inorganic pyrophosphate) and 950 μl of Tris-acetate buffer (0.1 M Tris-acetate, 2 mM EDTA, pH 7.75) into a polystyrene tube (Fisher Scientific). Light emission was measured as relative light units (RLU). A 10-μl aliquot of the sample extract was then added to the same cuvette, and light emission was measured (S). For internal calibration (I), 10 μl of a 10^{-8} M standard ATP solution in the 0.1 M Tris-acetate buffer was added to the same tube and measured. The ATP concentration in each sample was calculated according to the following formula:

$$\text{ATP (M)} = \frac{\text{S(RLU)} - \text{B(RLU)}}{\text{I(RLU)}} \times \text{amount of ATP standard added (M)} \quad (1)$$

Evaluation of the ATP assay with mitochondrial inhibitors and anti-*P. carinii* compounds. Since the bioluminescent assay used in these studies quantifies ATP, which is produced in mitochondria for the most part, we used a series of mitochondrial inhibitors to evaluate the effects on the organism ATP content. Potassium cyanide, antimycin A, sodium azide, 2,4-dinitrophenol, and oligomycin B were obtained from Sigma Chemical Co. and added to the media in concentrations ranging from 0.01 to 10.00 μg/ml. A stock solution of oligomycin B was prepared in 25% ethanol, with a final concentration of ethanol never exceeding 0.2%. All other compounds were

TABLE 1. The reduction of host cells by an improved isolation procedure

No. of *P. carinii* nuclei/ml (mean ± SD)	No. of host cells (mean ± SD)		n^a
	Before purification	After purification	
$(1.0 \pm 0.22) \times 10^6$	$(2.3 \pm 0.25) \times 10^6$	$(0.4 \pm 0.12) \times 10^3$	3
$(2.0 \pm 0.16) \times 10^6$	$(2.7 \pm 0.24) \times 10^6$	$(0.8 \pm 0.31) \times 10^3$	4
$(1.0 \pm 0.15) \times 10^7$	$(1.3 \pm 0.18) \times 10^6$	$(0.7 \pm 0.25) \times 10^3$	4
$(2.0 \pm 0.13) \times 10^7$	$(1.4 \pm 0.16) \times 10^6$	$(1.2 \pm 0.31) \times 10^3$	3
$(1.0 \pm 0.14) \times 10^8$	$(2.5 \pm 0.34) \times 10^6$	$(1.3 \pm 0.28) \times 10^3$	5
$(2.0 \pm 0.17) \times 10^8$	$(2.9 \pm 0.47) \times 10^6$	$(2.3 \pm 0.22) \times 10^3$	4
$(4.0 \pm 0.25) \times 10^8$	$(2.8 \pm 0.34) \times 10^6$	$(3.6 \pm 0.19) \times 10^3$	3
$(6.0 \pm 0.33) \times 10^8$	$(3.1 \pm 0.18) \times 10^6$	$(3.5 \pm 0.25) \times 10^3$	4
$(8.0 \pm 0.36) \times 10^8$	$(3.6 \pm 0.34) \times 10^6$	$(4.5 \pm 0.33) \times 10^3$	3
$(1.0 \pm 0.12) \times 10^9$	$(6.8 \pm 0.37) \times 10^6$	$(6.4 \pm 0.35) \times 10^3$	4
$(2.0 \pm 0.18) \times 10^9$	$(7.1 \pm 0.47) \times 10^6$	$(7.3 \pm 0.42) \times 10^3$	3

[a] Number of separate rat preparations evaluated.

TABLE 2. The numbers of *P. carinii* cells versus ATP content

No. of *P. carinii* nuclei/ml (mean ± SD)	No. of host cell nuclei/ml (mean ± SD)	ATP content (10^{-9} M/10^8 nuclei; mean ± SD)	n^a
$(1.0 \pm 0.21) \times 10^6$	$(0.4 \pm 0.12) \times 10^3$	0.03 ± 0.01	6
$(2.0 \pm 0.16) \times 10^6$	$(0.8 \pm 0.31) \times 10^3$	0.04 ± 0.01	5
$(1.0 \pm 0.16) \times 10^7$	$(0.7 \pm 0.25) \times 10^3$	0.05 ± 0.02	5
$(2.0 \pm 0.14) \times 10^7$	$(1.2 \pm 0.31) \times 10^3$	0.50 ± 0.12	4
$(1.0 \pm 0.17) \times 10^8$	$(1.3 \pm 0.28) \times 10^3$	0.84 ± 0.23	6
$(2.0 \pm 0.20) \times 10^8$	$(2.3 \pm 0.22) \times 10^3$	5.93 ± 0.67	5
$(4.0 \pm 0.25) \times 10^8$	$(3.6 \pm 0.19) \times 10^3$	11.83 ± 1.56	9
$(6.0 \pm 0.36) \times 10^8$	$(3.5 \pm 0.25) \times 10^3$	17.46 ± 2.03	7
$(8.0 \pm 0.37) \times 10^8$	$(4.5 \pm 0.33) \times 10^3$	25.62 ± 3.62	5
$(1.0 \pm 0.13) \times 10^9$	$(6.4 \pm 0.35) \times 10^3$	33.56 ± 3.89	7
$(1.5 \pm 0.16) \times 10^9$	$(6.6 \pm 0.45) \times 10^3$	41.22 ± 4.56	8
$(2.0 \pm 0.18) \times 10^9$	$(1.2 \pm 0.23) \times 10^4$	54.35 ± 4.79	6
0[b]	$(2.3 \pm 0.18) \times 10^3$	0.0032 ± 0.0013	6
$(6.0 \pm 0.43) \times 10^8$[c]	$(3.3 \pm 0.23) \times 10^3$	—[d]	4
$(8.0 \pm 0.40) \times 10^8$[c]	$(4.0 \pm 0.34) \times 10^3$	—	3

[a] Number of separate rat preparations evaluated.
[b] Uninfected rat lungs.
[c] *P. carinii* cells were autoclaved.
[d] —, below level of detection.

TABLE 3. The viability of host cells after purification using calcein acetoxymethyl-ethidium homodimer

No. of *P. carinii* nuclei/ml (mean ± SD)	No. of host cell nuclei/ml (mean ± SD)	% Live cells (mean ± SD) of:		n^a
		P. carinii	Host	
$(1.04 \pm 0.23) \times 10^9$	$(3.6 \pm 0.56) \times 10^3$	99.4 ± 0.5	34.2 ± 3.6	5
$(2.46 \pm 0.36) \times 10^8$	$(1.5 \pm 0.21) \times 10^3$	99.2 ± 0.8	32.5 ± 2.4	6

[a] Number of separate preparations evaluated. A total of 500 host cells and *P. carinii* cells were counted per evaluation.

dissolved in distilled water and filtered through 0.22-μm-pore-size filters (Millipore Corp., Bedford, Mass.). Controls included vehicle (i.e., ethanol for the oligomycin B), untreated *P. carinii*, and addition of the test compound directly to the luciferase-luciferin reaction mixture in the presence of 10^{-8} M ATP to evaluate the effect of the drug concentrations on the ATP assay itself. For the latter studies, the compound was assayed at the highest concentration used (10 μg/ml). A 10-μl sample was added directly to the reaction mixture containing the ATP standard, and the light emission was measured. If a quenching of the reaction was observed, lower concentrations were evaluated. Only sodium azide was found to quench this reaction at all concentrations, and it was deleted from the study.

In some cases, it was desirable to evaluate the effects of selected inhibitors on the yeast *Saccharomyces cerevisiae* (strain S288C *MATα mal gal2*). For these studies, the yeast was cultured in YEPD (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% glucose) (Difco Laboratories, Inc., Detroit, Mich.) for 36 to 48 h at 32°C, adjusted to the same density as *P. carinii* (i.e., 10^8/ml), and inoculated into the concentration series of the compounds. The extraction and measurement of ATP followed the procedures described above.

The standard anti-*P. carinii* compounds, pentamidine isethionate (Pentam 300; Fujisawa Pharmaceutical Co., Deerfield, Ill.) and trimethoprim-sulfamethoxazole (TMP-SMX) (Sigma Chemical Co.), were evaluated for effect on ATP content of *P. carinii*. A 1:20 ratio of TMP-SMX was distributed in concentrations ranging from 0.01 through 10 μg/ml, and pentamidine concentrations followed the same range. Stock solutions of each of the treatments were made at 1.0 mg/ml in distilled water. Ampicillin at the same doses was included in the study to test the specificity of the assay.

Dual fluorescent staining. Staining of cells with the calcein acetoxymethyl-ethidium homodimer technique (Live/Dead kit; Molecular Probes, Inc.) was performed as previously described (21, 22). Briefly, a 20-μl aliquot of cells was mixed with 5 μl of calcein acetoxymethyl (80 μM) and 5 μl of ethidium homodimer (150 μM) and incubated for 30 min at 37°C. A 5-μl drop was then examined for live (green fluorescence) or dead (red fluorescence) cells by the counting of at least 100 cells.

TABLE 4. Decrease in pH value and ATP content of *P. carinii* in unsupplemented medium[a]

Time (h)	pH (mean ± SD) of[b]:		ATP content (10^{-9} M/10^8 nuclei; mean ± SD)
	P. carinii in medium	Medium alone	
24	7.13 ± 0.23	7.85 ± 0.32	30.11 ± 3.42
48	6.85 ± 0.33	7.74 ± 0.22	24.16 ± 3.20
72	6.74 ± 0.21	7.79 ± 0.27	17.52 ± 1.73
96	6.64 ± 0.44	7.80 ± 0.31	10.33 ± 1.82
120	6.61 ± 0.23	7.78 ± 0.35	7.21 ± 1.41
144	6.14 ± 0.11	7.88 ± 0.25	4.56 ± 1.72

[a] $n = 10$.
[b] The original pH of the medium was 8.0, and the osmolality was 280 mOsmol/kg. The mean density of *P. carinii* organisms used as inocula was 1.32×10^9 nuclei per ml, with an ATP content (mean ± SD) of $(36.48 \pm 3.24) \times 10^{-9}$ M/10^8 nuclei.

TABLE 5. Effect of replenishment of medium on ATP content of *P. carinii*[a]

Time (h)	Effect on medium			
	When medium was replenished		When medium was not replenished	
	ATP content (10^{-9} M/10^8nuclei; mean ± SD)	% Difference from t_0[b] (mean ± SD)	ATP content (10^{-9} M/10^8 nuclei; mean ± SD)	% Difference from t_0 (mean ± SD)
24	37.42 ± 3.56	−9.3 ± 1.2	34.56 ± 3.42	−16.2 ± 2.4
48	38.18 ± 2.34	−7.4 ± 1.3	31.45 ± 3.12	−24.0 ± 4.5
72	45.45 ± 1.77	10.0 ± 2.1	24.56 ± 4.34	−40.5 ± 4.7
96	51.38 ± 2.65	24.6 ± 3.7	15.34 ± 2.45	−62.8 ± 5.7
120	50.98 ± 2.77	23.6 ± 3.9	7.83 ± 2.65	−81.0 ± 5.4
144	51.22 ± 3.45	24.2 ± 3.5	2.45 ± 1.55	−94.0 ± 5.8

[a] $n = 10$. The initial density of *P. carinii* organisms was 1.66×10^9 nuclei, with an ATP content (mean ± standard deviation) of $(40.14 \pm 2.68) \times 10^{-9}$ M/10^8 nuclei.
[b] t_0, time zero.

RESULTS

Isolation of organisms. The purification procedure for obtaining *P. carinii* organisms suitable for ATP measurement differed from our previous isolation procedures by use of gravity sedimentation and low-speed centrifugation and by the elimination of the use of 10-μm filtration steps to reduce host cells. A photomicrograph of a representative preparation is shown in Fig. 1. Trophic forms were the predominant form in these preparations, although some cysts could be detected. Microscopic examination of at least three different preparations stained with Diff-Quik produced an average distribution of about 96% trophic forms and 4% cysts. For these analyses, all single-nucleus forms were counted as trophic forms and multinucleated forms were counted as cysts.

A comparison of the numbers of host cells in organism preparations purified by our previously published method (11, 13) and by the method described above is shown in Table 1. The numbers of *P. carinii* organisms were held constant. Without the purification procedure, host cell numbers ranged from 1 to 7 million per milliliter of preparation, whereas the purified organisms contained at least 1,000-fold fewer host cells. The ATP contents of the purified preparations are shown

TABLE 6. ATP content and number of *P. carinii* nuclei over time[a]

Time (h)	No. of *P. carinii* nuclei (10^9; mean ± SD)	ATP content (10^{-9} M/10^8 nuclei; mean ± SD)	% Difference from t_0[b]
24	1.50 ± 0.16	37.42 ± 3.56	−9.3
48	1.60 ± 0.20	38.18 ± 2.34	−7.5
72	1.70 ± 0.14	45.45 ± 1.77	9.6
96	1.94 ± 0.26	51.38 ± 2.65	24.55
120	1.92 ± 0.17	50.98 ± 2.77	23.58
144	1.93 ± 0.22	51.22 ± 3.45	24.16

[a] $n = 12$. The medium was replenished every 24 h. The initial mean density of *P. carinii* organisms was 1.56×10^9 nuclei per ml, with a mean ATP content of 41.25×10^{-9} M/10^8 nuclei.
[b] t_0, time zero.

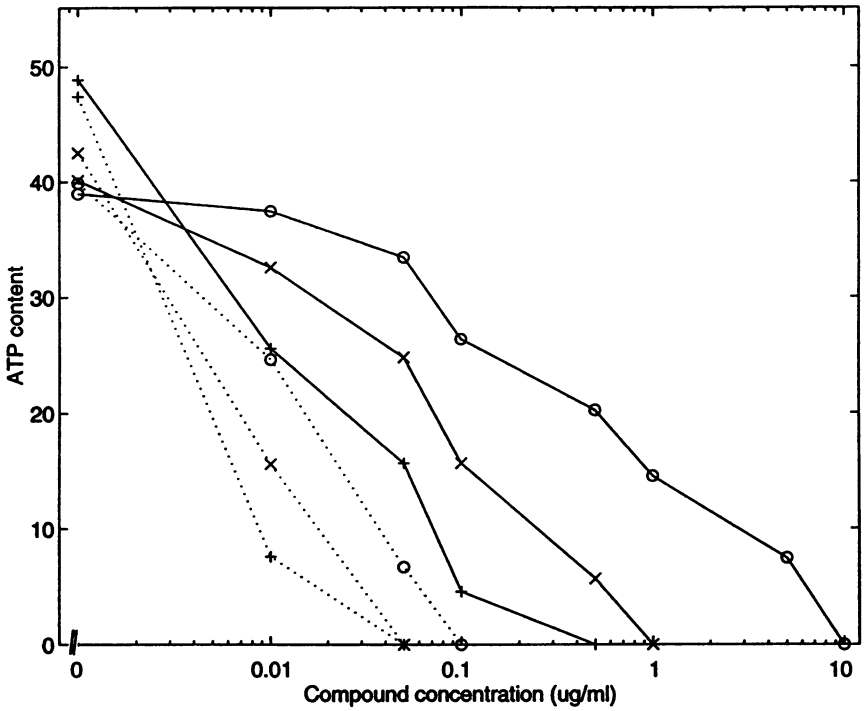

FIG. 2. Effects of potassium cyanide and 2,4-dinitrophenol on the ATP content of *P. carinii*. ATP content (10^{-9} M/10^8 *P. carinii* nuclei) is plotted versus inhibitor concentration on a logarithmic axis. Dotted lines (·····), potassium cyanide-treated *P. carinii*; solid lines (———), 2,4-dinitrophenol-treated *P. carinii*. Time points postinoculation: ○, 24 h; ×, 48 h; +, 72 h. Untreated *P. carinii* controls were 39.25 × 10^{-9}, 39.90 × 10^{-9}, 42.50 × 10^{-9}, and 47.40 × 10^{-9} M ATP per 10^8 *P. carinii* nuclei at the time of inoculation and at three subsequent time points in the potassium cyanide study and 41.12 × 10^{-9}, 38.97 × 10^{-9}, 40.12 × 10^{-9} and 48.89 × 10^{-9} M ATP per 10^8 *P. carinii* nuclei, respectively, in the 2,4-dinitrophenol study.

in Table 2. The ATP content and numbers of *P. carinii* nuclei exhibited a linear relationship (r = 0.9876) over 3 orders of magnitude. The most consistent responses were observed to be between 10^7 and 10^9 organism nuclei. On the basis of the ATP content of normal rat lung cells and the minimal ATP levels in preparations with low numbers of organisms, it appeared that host cell ATP did not influence greatly the total ATP content of the preparations. To further address the influence of host cells, selected organism preparations were stained with a dual fluorescent staining technique to determine the viability of the host cells and of *P. carinii* after isolation. The results in Table 3 show that of the host cells remaining in the preparations, only one-third were viable. Hence, the contribution by host cells appears to be quite minimal. In contrast, the viability of the *P. carinii* in the preparations was quite high, proving that the purification process was not deleterious to the organisms. The ATP content of organism preparations ranged from 27.32 × 10^{-9} to 43.56 × 10^{-9} M ATP per 10^8 *P. carinii* nuclei, with an average of 39.64 × 10^{-9} M ATP per 10^8 *P. carinii* nuclei. The average ATP content per organism (per nucleus) would be approximately 39 fmol, although the ATP content of the different developmental forms may be quite variable.

Medium and conditions. The ATP content of organisms inoculated into unsupplemented medium gradually declined, to a loss of over 50% after 72 h of incubation at 37°C (Table 4). Measurement of the pH of the medium throughout the culture period showed a rapid acidification after 24 h and at subsequent daily intervals. To prevent acidification in the event that it influenced the ATP content of the organisms present and to offer a renewable source of nutritional supplements, 60% of the culture medium was replenished on a daily basis. This was done by collection of the organisms at the bottom of the glass tube by centrifugation with subsequent removal of 6 ml of the uppermost supernatant. In this way, the original pH at the time of inoculation was maintained throughout the assay period. Such treatment was shown to retain the original level of ATP with a slight but consistent increase after 72 h of incubation in the supplemented maintenance medium (Table 5). Organism populations that were not replenished lost ATP content over time (Table 5). Although the ATP content could be kept near inoculum levels or slightly higher, there was no concomitant rise in the numbers of organism nuclei, which remained at or near inoculum density (Table 6). This slight increase in ATP may be explained by the death of a subpopulation of *P. carinii* with an equivalent rise in another or by activation of life cycle stages to a higher metabolic state.

Response to mitochondrial inhibitors. The response of the *P. carinii* population to specific mitochondrial inhibitors was tested. Inoculation of the organisms into maintenance medium with concentrations of 0.01 to 10.0 μg/ml of the respiratory chain inhibitor potassium cyanide and the uncoupler 2,4-dinitrophenol dramatically decreased the ATP content of the organism population after 24 h of exposure (Fig. 2). By the 24-h time point, all but the lowest concentrations of potassium cyanide reduced the ATP content of organism populations to undetectable levels (Fig. 2). After 72 h, only the organisms exposed to the lowest concentration of potassium cyanide, 0.01 μg/ml, had a detectable ATP content, although the ATP level had decreased by >80% compared with untreated control organisms. The 2,4-dinitrophenol also was able to dramatically decrease the ATP content of the exposed *P. carinii* populations in a dose-dependent manner, with negligible amounts of ATP

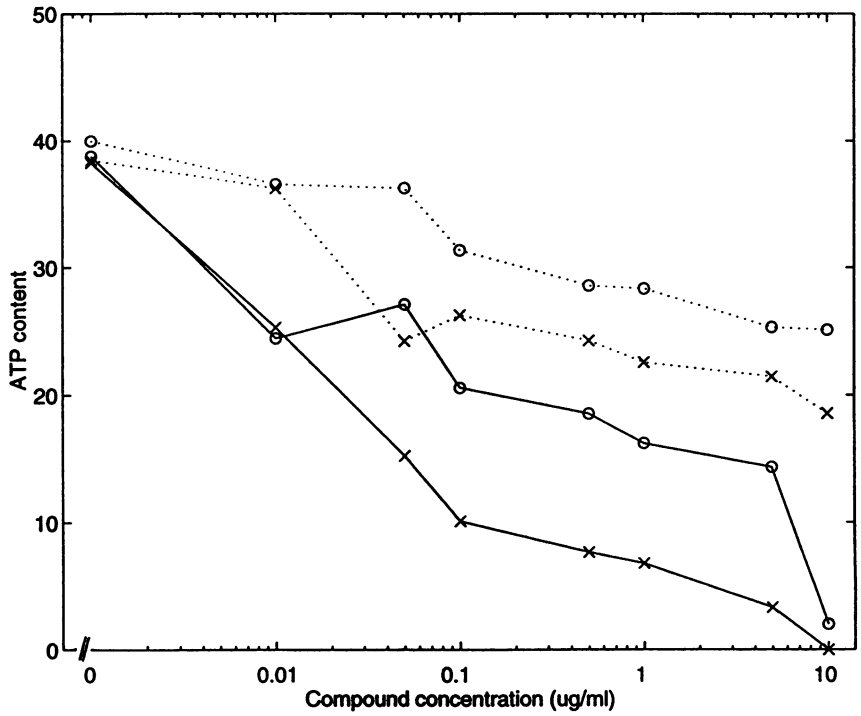

FIG. 3. Effects of antimycin A and oligomycin B on the ATP content of *P. carinii*. ATP content (10^{-9} M/10^8 *P. carinii* nuclei) is plotted versus inhibitor concentration on a logarithmic axis. Dotted lines (·····), oligomycin B-treated *P. carinii*; solid lines (———), antimycin A-treated *P. carinii*. Time points postinoculation: ○, 24 h; ×, 48 h. Untreated *P. carinii* controls were 42.25×10^{-9}, 39.96×10^{-9}, and 38.45×10^{-9} M ATP per 10^8 *P. carinii* nuclei at the time of inoculation and two subsequent time points in the oligomycin B study and 40.03×10^{-9}, 38.75×10^{-9}, and 38.22×10^{-9} M ATP per 10^8 *P. carinii* nuclei, respectively, in the antimycin A study.

remaining after 24, 48, or 72 h of incubation with concentrations greater than 1.0 μg/ml (Fig. 2).

The treatment of *P. carinii* with another respiratory chain inhibitor, antimycin A, reduced the ATP pool by 50% or more with concentrations of 0.10 to 10 μg/ml (Fig. 3). At 10 μg of the compound per ml, a decrease of 95% was observed. Continued incubation for an additional 24 h resulted in a 50% reduction of the ATP content of the organism populations treated with antimycin A at concentrations of 0.05 μg/ml or more. Exposure of antimycin A to *S. cerevisiae* was performed as a control for efficacy of the inhibitor, to permit direct comparison with another member of the fungi, and to evaluate whether the compound was administered in physiologic concentrations. As shown in Fig. 4, antimycin A had a profound effect on the ATP content of the yeast cultured in YEPD for 24 h at 32°C at a density (10^8) identical to that of *P. carinii*. In contrast to the *P. carinii* populations, the *S. cerevisiae* yeast organisms were more susceptible to the inhibitor at lower concentrations after 24 h with only slight augmentation of this effect after 48 h of incubation.

The antibiotic oligomycin B has been reported to bind with the F_0 subunit of ATP synthase and inhibit the biosynthesis of ATP (16, 34, 43). Both mammalian cells and wild-type yeast cells have been shown to be susceptible to the action of this compound at the concentrations that we evaluated (18). As shown in Fig. 3, *P. carinii* did not appear to respond to this antibiotic, with only a slight decrease in ATP after 24 h of incubation. A decrease in ATP content of less than 50% was observed at all concentrations and time points with the exception of a 48-h incubation in which the highest concentration reached was 10.0 μg/ml and in which about a decrease of about 50% was realized. In contrast, the ATP content of the *S. cerevisiae* populations was reduced by 90 and 75% after exposure to concentrations of 10 μg/ml for 24 or 48 h (Fig. 4).

The cytochrome oxidase inhibitor sodium azide could not be used to assay the effect on the population ATP levels because it directly interfered with the luciferase-luciferin reaction when controls with compound and ATP standard were performed.

To evaluate the potential of the bioluminescent assay for in vitro drug screening, we exposed the organism populations to two standard, anti-*P. carinii* compounds, TMP-SMX and pentamidine, and one control compound, ampicillin, which has no known in vitro or in vivo effect on *P. carinii*. As shown in Fig. 5, a 1:20 ratio of TMP-SMX, at total concentrations of 0.1 through 10.0 μg/ml, effectively reduced the ATP content of *P. carinii* by 24 h at all of the concentrations. After 48 h of incubation, the amount of ATP present in the populations was at or below the level of detection. Likewise, the same concentrations of pentamidine effectively reduced the ATP content after exposure to all three of the concentrations (Fig. 5). In contrast, incubation with ampicillin at any concentration did not cause any decrease in the ATP content during the entire 72-h assay period (Fig. 5).

Comparison of the ATP bioluminescent assay with a dual fluorescent staining method. To validate the ATP assay as a measure of *P. carinii* viability, we conducted comparative studies with another viability indicator, a dual fluorescent staining procedure (21, 22). This technique utilizes a nonfluorescent compound, calcein acetoxymethyl, and a nuclear counter stain, ethidium bromide. The nonfluorescent compound is cleaved by nonspecific esterases in a viable cell, converting the molecule into one that emits green fluorescence. The DNA intercalator acts as a positive probe for nonviable or morbid cells that have lost membrane integrity. A

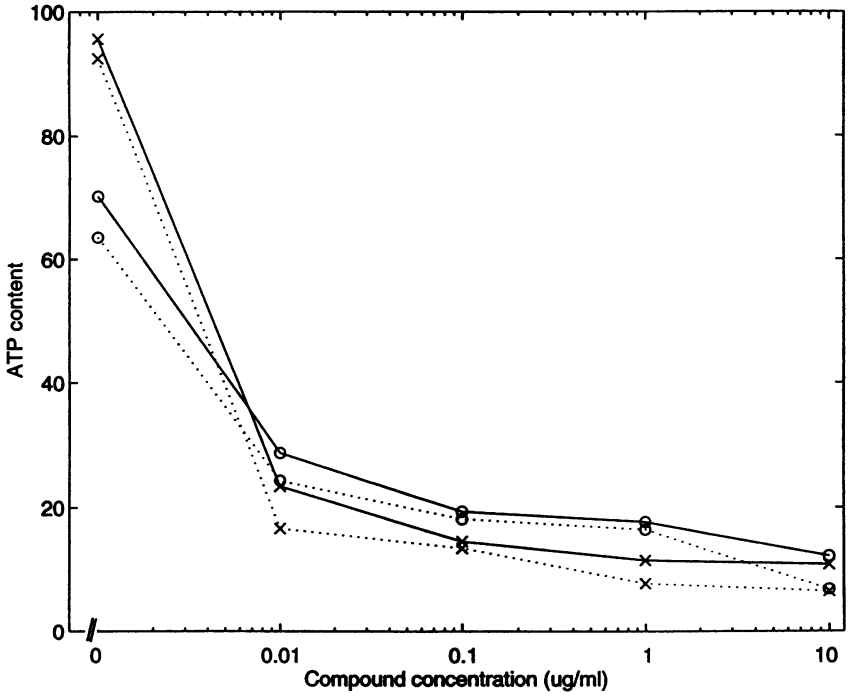

FIG. 4. Effects of antimycin A and oligomycin B on the ATP content of *S. cerevisiae*. ATP content (10^{-9}M/10^8 yeasts) is plotted versus inhibitor concentration on a logarithmic axis. Dotted lines (·····), oligomycin B-treated *S. cerevisiae*; solid lines (———), antimycin A-treated *S. cerevisiae*. Time points postinoculation: ○, 24 h; ×, 48 h. Untreated *S. cerevisiae* controls were 47.23×10^{-9}, 63.56×10^{-9}, and 92.56×10^{-9} M ATP per 10^8 yeast cells at the time of inoculation and two subsequent time points in the oligomycin B study and 51.23×10^{-9}, 70.21×10^{-9}, and 95.68×10^{-9} M ATP per 10^8 yeast cells, respectively, in the antimycin A study.

comparison of the ATP bioluminescent assay and the dual fluorescent staining technique was performed on the same *P. carinii* preparation incubated at suboptimal conditions (in RPMI 1640 supplemented only with 20% FBS, 37°C) for a period of 9 days (Table 7). It is evident from Table 7 that the ATP assay detected a more dramatic decline in viability during the first 72 h of incubation, as determined by the reduction in ATP, than did the staining method. After this time point, the values were quite similar, with more than 50% of the population determined to be nonviable by either method.

DISCUSSION

The primary question in the establishment of any in vitro assay using organisms derived from a host is the degree of contamination with cells that could influence the outcome of the assay. In the case of the bioluminescent assay, an evaluation of the amount of host cell contaminants is of paramount importance since ATP is not specific for any one cell type. Several lines of evidence showed that host cells did not significantly contribute to the ATP being measured. (i) Additional steps in the organism isolation procedure reduced the numbers of host cells by a factor of 1,000 as evaluated by tinctorial staining. (ii) A dual fluorescent staining technique showed that only about one-third of the remaining host cells were viable. (iii) Direct assessment of host cells using the procedures outlined for organism isolation revealed that very low levels of ATP remained in these cells. (iv) The relative insensitivity of the antibiotic oligomycin B provides indirect evidence that the ATP being measured is largely from the organisms, since mammalian cells are quite sensitive to this compound. (v) The responsiveness of standard anti-*P. carinii* drugs, TMP-SMX and pentamidine, in this ATP system provided further evidence that it is the ATP levels of the organism population that are being measured. Furthermore, comparison of the ATP bioluminescent assay with another viability indicator showed some correlation, although it was evident that the bioluminescent assay was a more sensitive technique than the dual fluorescent staining technique.

It is apparent from the addition of the respiratory chain inhibitors that the assay is assessing the mitochondrial function of the *P. carinii* populations. Those inhibitors that targeted the cytochrome oxidases in the electron transport chain, antimycin A and potassium cyanide, and 2,4-dinitrophenol, which disrupts the proton gradient of the inner mitochondrial membrane, manifested a dramatic decrease of ATP in the population after 24 h, the first time period evaluated. It is of interest to note that oligomycin B did not lower the ATP pools in a significant manner. This antibiotic is thought to inhibit the final step in the biosynthesis of ATP by blocking the translocation of H^+ across the mitochondrial membrane at F_0 (16). In contrast, wild-type *S. cerevisiae* cells proved quite sensitive to these antibiotics at concentrations of 0.25 μg/ml or more when a growth assay was used to evaluate the effects (18). Conversely, mutants made from sensitive parental yeast strains were resistant to >10 μg of oligomycin per ml in the same set of experiments. In the present experiment, we compared directly the effects of oligomycin B on yeast organisms using the bioluminescent assay. A decrease in ATP of more than 70% was observed at oligomycin B concentrations found to inhibit the growth of the wild-type yeast. *P. carinii* populations were more similar to mitochondrial yeast mutants in resistance to the inhibitors, as concentrations of 10 μg/ml decreased the ATP levels by only 27%. This observation may be important in future considerations of drug design targeted at the F_0 subunit.

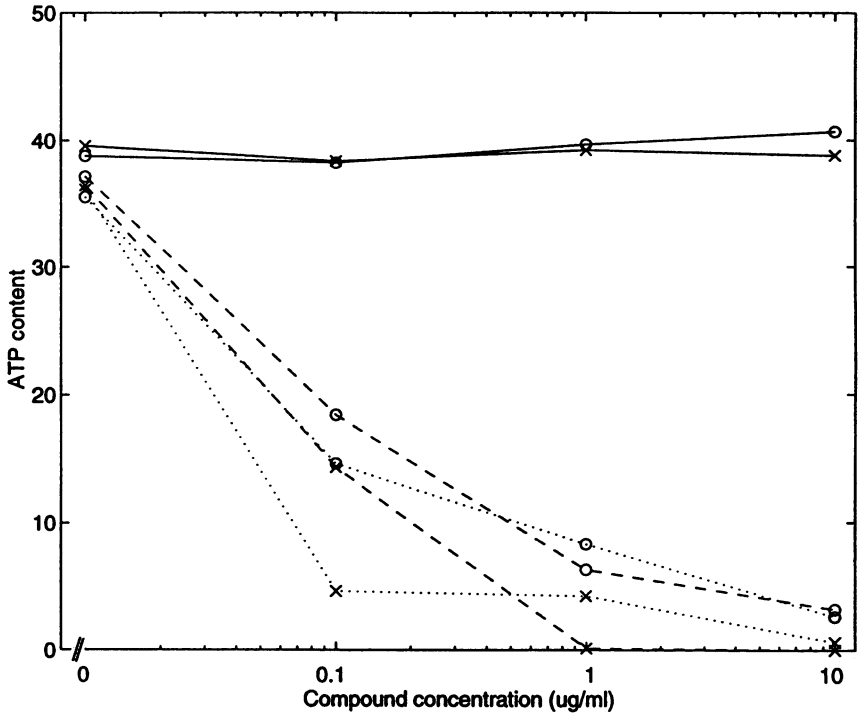

FIG. 5. Effects of TMP-SMX, pentamidine isethionate, and ampicillin on the ATP content of *P. carinii*. ATP content (10^{-9} M/10^8 *P. carinii* nuclei) is plotted versus inhibitor concentration on a logarithmic axis. Dotted lines (·····), pentamidine-treated *P. carinii*; dashed lines (– – –), TMP-SMX-treated *P. carinii*; solid lines (———), ampicillin-treated *P. carinii*. Time points postinoculation: ○, 24 h; ×, 48 h. Untreated *P. carinii* controls were 38.12×10^{-9}, 35.52×10^{-9}, and 36.12×10^{-9} M ATP per 10^8 *P. carinii* nuclei at the time of inoculation and two subsequent time points in the pentamidine study; 39.48×10^{-9}, 37.15×10^{-9}, and 36.43×10^{-9} M ATP per 10^8 *P. carinii* nuclei, respectively, in the TMP-SMX study; and 41.25×10^{-9}, 38.77×10^{-9}, and 39.56×10^{-9} M ATP per 10^8 *P. carinii* nuclei, respectively, in the ampicillin study.

Further experiments to probe the specificity of the block by use of related compounds such as ossamycin or venturicidin are planned.

That the assay was specifically evaluating the ATP levels of the *P. carinii* populations was shown by the reduction in levels by the known anti-*P. carinii* drugs TMP-SMX and pentamidine and by the stasis in ATP levels when ampicillin was added to the medium. The ATP levels were decreased at similar concentrations of the TMP-SMX and pentamidine, previously shown to inhibit the growth of *P. carinii* in tissue culture (12) and cell-free culture (9). Although we did not observe any significant increase in the number of organisms in our system, the ATP levels did remain constant over the time period of the assay. This system holds promise as an in vitro screening technique that does not require the establishment of a culture system. Identification of an in vitro method that eliminates the tedious counting of organism nuclei or developmental stages represents a significant step forward.

TABLE 7. Comparison of calcein acetoxymethyl-ethidium homodimer viability assay with ATP bioluminescent assay

Time (h)	Loss of viability as measured by[a]:		
	ATP assay		Calcein AM-ethidium homodimer (% nonviable)[b]
	ATP content (10^{-9} M/10^8 nuclei; mean ± SD)	% Decrease from t_0[c]	
24	29.56 ± 1.34	9.0	2.0
48	22.16 ± 0.98	31.8	14.3
72	17.06 ± 2.12	47.5	59.0
96	12.14 ± 1.76	62.7	57.5
120	8.45 ± 1.22	74.0	70.0
144	6.52 ± 1.11	80.0	83.6

[a] Values are means of three samples withdrawn from each of three tubes in a single experiment. The initial inoculum (mean ± standard deviation) was (8.3 ± 0.67) × 10^8 nuclei per ml, with an ATP content (mean ± standard deviation) of (32.22 ± 1.56) × 10^{-9} M/10^8 nuclei.

[b] AM, acetoxymethyl.

[c] t_0, time zero.

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