ULTRASENSITIVE BIOLUMINESCENT DETERMINATIONS OF ADENOSINE TRIPHOSPHATE (ATP) FOR INVESTIGATING THE ENERGETICS OF HOST-GROWN MICROBES

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In all living things, biologically useful energy is captured in adenosine triphosphate (ATP). Our interest in ATP arose from the need for a biologically significant biochemical tool applicable to host-dependent microbes. The theoretical basis for regarding ATP as a key compound appears to be sound.

- The concentration of ATP pools within any species under defined different circumstances is controlled by the net balance between rates of generating energy and rates of biosynthesis.
- Minimal levels of ATP suffice for energy of maintenance; slightly higher levels stimulate minimal rates of growth.

The practical question was whether the desired sensitivity could be obtained for metabolic investigations of host-grown microbes, which have been handicapped by the great number of cells required and by the fact that "host-grown" species have not been stabilized or activated in vitro.

McElroy and associates were the first to demonstrate the role of ATP in illuminating the tails of fireflies and developed methods whereby the bioluminescence can assay ATP levels. The almost miraculous sensitivity of this principle has been refined by Chappelle and associates at the Goddard Space Flight Center. In view of the possibility of achieving a sensitivity applicable to routinely obtainable numbers of *Mycobacterium leprae* cells, we undertook to confirm the stability of the reagents and to define the optimal concentrations of each of the reactants.

Luciferase enzyme was prepared and purified by the method of Chappelle using Sephadex G-100. The lyophilized enzyme in the presence of luciferin and magnesium, when stored at 197 K (-76°C), is stable for over 30 months. When rehydrated and held at 273.6 to 277 K (0.5 to 4°C), 96 percent of the original activity is retained after 7 days and 93 percent is retained after 11

days. One unit of enzyme is defined as the amount of enzyme per 0.3-ml acceptor system containing 100-μg/ml luciferin and 0.005 M Mg^{++}, that elevates the peak of ATP response curves 50 units (1 cm (2.5 in.)) due to 10 pg ATP. We denote this system as R-system (routine) and use it for all routine purposes. Our H-system (high sensitivity) contains 3 units of enzyme in the presence of 300 μg/ml luciferin and 0.0075 M Mg^{++} and gives an elevation of 150 units with 10 pg of ATP. The character of the interactions between 10 pg of ATP and acceptor systems containing different concentrations of luciferase, luciferin, and magnesium were investigated systematically.

Table 1 shows the method and the results of titrating optimal balances between the three interacting components of ATP acceptor systems and demonstrates the effects of luciferin concentration. The data in the columns for 1 and 3 units of enzyme clearly define the optimal concentrations of reactants for the R- and H-systems.

Table 1
Interactions and Optimal Concentrations of
Luciferase, Magnesium, and Luciferin*

Enzyme units:	1	2	3	4
Mg (mM)	2.5 5.0 7.5 10.0	5.0 7.5 10.0	5.0 7.5 10.0	5.0 7.5 10.0
Luciferin (μg/ml)				
50	23 46 29			
(65)†	(33)			
100	25 (50) 37 27	68 75 66		
200	47 35	89 109 86	115 122 108	110 121 105
300	45 41	118 125 100	145 (150) 125	143 150 134
400	40 44 46 39	116 122 104	139 143 128	129 145 130

* All reactants = concentrations during bioluminescence.
All data = units of response/10 pg ATP.
Values circled = optima for the R- and H-systems.

† Sensitivity of the system of Chappelle and Levin (*Biochem. Med.*, **2**, 1968, p. 41-52) in the presence of 1 unit enzyme.
The R-system uses only 92 percent as much enzyme, but is 1.5 times more sensitive.

In figure 1, panel A illustrates interdependencies between enzyme and luciferin concentrations in presence of optimal Mg. The effects of increasing enzyme concentration in the two acceptor systems differed markedly. When the luciferin and Mg were balanced optimally for one enzyme unit, they were inadequate for higher concentrations of enzyme. With the concentrations

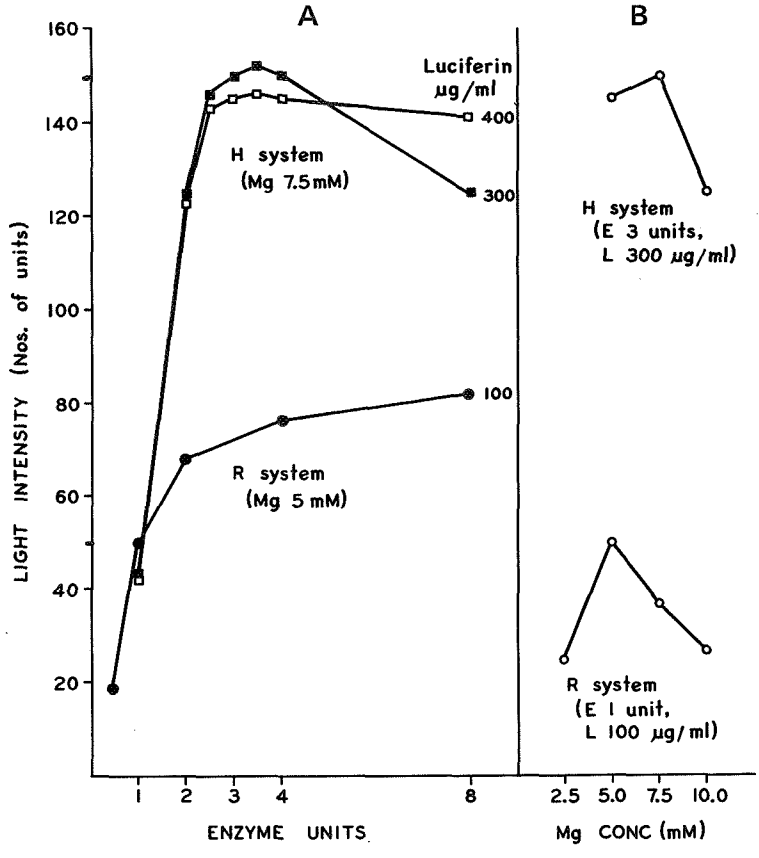

Figure 1. Panel A—the effect of enzyme concentration. Panel B—the effect of Mg concentration.

of luciferin (300 µg/ml) and Mg (0.0075 M) appropriate to the H-system, an increase from 1 to 2.5 units of enzyme caused a linear increase in sensitivity. Three and 3.5 enzyme units produced further, limited gains prior to the onset of inhibitions, which suggest that enzyme purity is a major limiter of sensitivity.

The data on panel B in figure 1 are restricted to optimal concentrations of enzyme and luciferin for the R- and H-systems and illustrate the differing optima with respect to Mg concentration. There were always losses of sensitivity when the designated optimal concentrations were altered.

The optimal ionic strength was confirmed to be 0.05 M for the four buffers tested. Figure 2 compares the luminescence in the presence of four buffers at pH 7.0, 7.5, and 8.0. The fact that a pH higher than 7.5 was always disadvantageous suggested that the optimal pH might be below 7.5. As

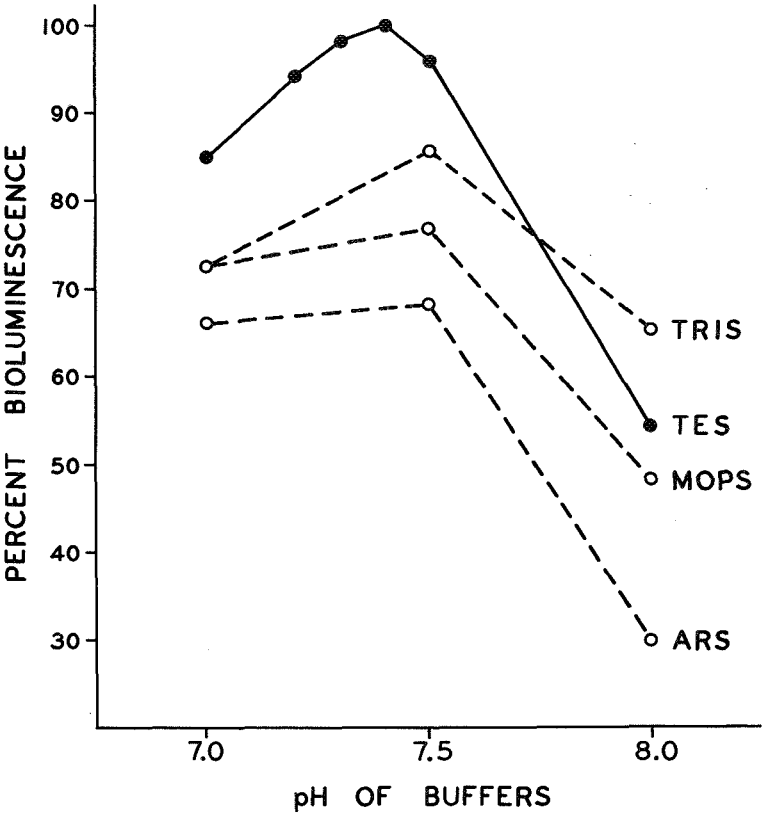

Figure 2. Comparison of luminescence in the presence of TRIS, TES, MOPS, and ARS buffers.

shown, pH 7.4 was optimal for the TES (N-tris [Hydroxymethyl] methyl-2-aminoethane sulfonic acid) buffer adopted. This pH provides the best margin of safety against errors in pH adjustments.

The features of the R- and H-systems as finally adopted are summarized in table 2. The two major points are that:

- The optima for buffer and pH are identical, and
- The optimal concentrations of enzyme, magnesium, and luciferin depend upon the sensitivity desired.

Before explaining the reference method for extracting ATP, I will present the procedures required to investigate the energetics of unwashed, host-grown microbes. In such cases, one must faithfully eliminate host ATP before releasing the bacterial ATP (see procedure 1). The first step in procedure 1 eliminates 99 percent of any soluble, nonbacterial ATP in the

Table 2
Optimal Concentrations of Bioluminescence Regulators

Acceptor:	*R-system	†H-system
Luciferase	1 unit	3 units
Luciferin	100 μg/ml	300 μg/ml
$MgSO_4$	0.005 M	0.0075 M
Buffer	TES, 0.05 M	TES, 0.05 M
pH	7.4	7.4

* R = Routine, for cultivable organisms.
† H = High sensitivity, for host-dependent organisms.
All reactants: concentrations during bioluminescence.

Procedure 1
Elimination of Host ATP and Extraction of Microbial ATP

Purpose of steps	Procedure	Dilution of soluble ATP
1. Remove soluble ATP	0.1 ml bacteria suspended in 10-mm or 13-mm glass tube +0.9 ml TES buffer. Centrifuge, cold, 20 min at 3,000 g; remove 99% supernatant with a Manostate Accropet. Resuspend sediment in 0.1 ml TES buffer.	100 X
2. Extract ATP	Add 0.03 ml chloroform (23% v/v), vortex 10 s. 331 K (98°C) water bath: (a) heat *n* min*; (b) apply 750-mm vacuum with shaking for 1 min to yield dry samples.	
3. Assay of ATP	Rehydrate in 0.4 ml TES buffer. Assay immediately by injecting 0.1 ml into 0.3 ml acceptor system.	4 X
Total dilution (safety) factor:		400 X

* *E. coli* = 1 min; *M. phlei* = 4 min; *M. lepraemurium* = 9 min.

sample. The second step extracts the bacterial ATP and produces a dried sample. After the third step, the bacterial ATP is at the volume required for triplicate assays. Meanwhile, the mammalian ATP has been diluted 400 times.

Each step in the foregoing methods was developed and validated by several criteria:

- By means of known amounts of ATP added to bacterial suspensions to represent soluble ATP;
- By using single-cell suspensions of *Mycobacterium phlei* and performing microscopic counts, plate counts, and ATP determinations; and
- By using both active and extracted *Mycobacterium lepraemurium* cells throughout the pertinent features of each promising method.

The classical extractors of ATP–PCA and n-butanol–were found incapable of extracting total microbial ATP, even from *E. coli*. These agents released only 70 to 85 percent of the ATP from a *saprophytic mycobacterium, M. phlei,* and some 50 to 70 percent of that in the pathogenic *M. lepraemurium.* Neutralization of PCA diluted the extracts and spoiled sensitivity. N-butanol quenched bioluminescence, even when diluted to 0.1 percent.

Table 3 summarizes the percentages of ATP pools released from representative microbes by heat and chloroform.

Previous investigations had uncovered only two agents–heat and chloroform–which promptly opened the *M. Lepraemurium* cells to dye penetration. Present work evolved a convenient reference method having minimal effects on reference standard ATP, giving maximal yields of dried ATP and no ATP upon reextraction of cell residues. The merits of chloroform are:

- It does not degrade reference standard ATP;
- It disrupts enormous clumps of mycobacteria instantaneously, thus exposing the surfaces of individual cells; it disrupts cell membranes; and
- Because of its low boiling point, it is readily removed by heat and cannot quench bioluminescence.

We have shown that heat at 331 K (98°C) degrades ATP so slowly that 98 percent of the reference standard ATP is assayable after 10 minutes. After sequential application of chloroform and heat for 10 minutes, 96 percent of the reference standard ATP was assayable.

The significant points in table 3 are:

- Items 1 and 2–neither heat nor chloroform alone suffice, even for *E. coli.* The other species were progressively more resistant, and

Table 3
Extraction of ATP by Heat and Chloroform
Basis of Reference Methods

Procedures	331 K (98°C)* (min)	% ATP pool released from: †		
		E. coli	*M. phlei*	*M. lepraemurium*
1. Heat only:	2	52	45	31
	5	78	69	54
	10	90	88	56
2. Chloroform only: ‡	-	94	93	62
3. Heat first, then	5	100	101	79
chloroform: §	10	-	-	100
4. Chloroform first,	2	100	96	70
then heat: ‖	5	-	100	86
	10	-	-	100

* Data = heating prior to vacuum drying.
† Calculated as shown previously (Dhople and Hanks, *Applied. Microb.*, **26**, 1973, p. 399-403).
‡ Chloroform = 0.03 ml added to 0.1 ml samples = 23% v/v; vortexed 10 s.
§ Reference procedure 3. Terminal heating = 1 min to warm the sample plus 1 min of vacuum drying.
‖ Reference procedure 4. Terminal heating = 1 min to vacuum dry.

chloroform was a more effective disruptor of membrane-wall complexes than heat.

- Item 4—Applying chloroform as the first step in the sequential use of the two agents was the most convenient method. The optimal heating period was 2 minutes for *E. coli*, 5 minutes for *M. phlei*, and 10 minutes for *M. lepraemurium*.

The overall gains in sensitivity for quantitation microbial ATP can be summarized as follows:

1. The R-system is 1.5 times more sensitive than that of Chappelle and Levin. The H-system is 3 times more sensitive than the R-system, a total gain of 4.5 times.
2. Because of a novel method of extracting total microbial ATP, the number of cells required per sample has been decreased to ±15 times.
3. Thus, 1.4 percent of the cell numbers originally required suffice for analyses of ATP.

Three years of working experience have enabled us to define the limits of sensitivity and the minimal concentrations of ATP that can be quantitated at present. These are shown in table 4.

Table 4
Quantitations of ATP

A. Limits of Sensitivity			B. Reliability
pg ATP/ assay	% Reference standard ATP assayed		
	†R-system Units %	†H-system Units %	1. The standard deviations of duplicate determinations of ATP are equivalent to those for triplicate plate and microscopic counts. 2. Average SDs, expressed as percentages of the observed values = ± 4%.
3	14 93	43 96	
1	3 60	14 93	
0.3	D*	3 67	
0.1		D	

* D = detected, not quantitated.
† R = routine; H = high sensitivity.

- Sensitivity. The efficiency of demonstrating ATP at less than 7 pg per assay falls off on a parabolic curve. This table sets the limits of the R-system at about 1 pg and that of the H-system at 0.3 pg. Correction factors compensate for the declining efficiency and yield data based on reference standard ATP.
- Reproducibility. The two goals were (a) to turn out duplicate assays having the same precision as triplicates and (b) to obtain a reproducibility of ± 2.5 percent. This was accomplished by means of mental arithmetic. When paired determinations agreed within 5 percent (that is, ± 2.5 percent of the mean), the results were accepted. When one value was more than 5 percent less than the higher value, a further (triplicate) assay was made. A review of accumulated data showed that triplicate assay had been required in only 10 percent of the total samples. Thus, 2.2 units of work yielded higher reproducibility than can be obtained from triplicates which are not monitored mentally while the work is in progress.

The estimate of ± 4 percent in table 4 was caused by the fact that the base data included determinations on bacterial suspensions prepared on different dates.

In conclusion, we have (1) redefined the optimal concentrations of the five reactants in the bioluminescent system, (2) devised a novel method of eliminating host ATP while extracting and drying total microbial ATP, and (3) now require only 1.4 percent of the number of cells originally needed.

The R-system for routine work is more efficient than the previous systems, while the H-system quantitates as little as 0.3 pg ATP. The usual triplicates can be replaced by 2.2 assays per sample, with a gain in reproducibility.