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Use of the Firefly Bioluminescent Reaction for Rapid Detection and Counting of Bacteria[1]

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A rapid and routine procedure for the quantitative detection and counting of bacteria is frequently of vital importance. Classical techniques, which have the advantage of positive identification and preservation of the bacteria for future use, are usually slow and require rather complex media. The technique described here offers a high degree of sensitivity, rapidity, accuracy, and reproducibility. It exploits not only the high specificity of an enzymatic reaction, but also the advances in technological development of devices capable of measuring extremely low levels of light.

The universal presence of adenosine triphosphate (ATP) in all life forms (1) makes this compound an excellent indicator of the presence of bacteria. Of the various methods for quantitative assay of ATP (2, 3, 4, 5), the most sensitive is the ATP-dependent bioluminescent reaction occurring in the firefly (6). Although other investigators have used the firefly bioluminescent assay to measure ATP (7, 8), our method offers greater sensitivity through improvement in technique and in instrumentation. We have developed a simple, rapid, and unique method of extraction which permits determination of the ATP content of a variety of bacterial species.

[1] This investigation was initiated under Contract No. N178-8097, Bureau of Naval Weapons.

The emission of light in firefly bioluminescence results from a two-step reaction[2] (9, 10, 11, 12):

$$(1) \qquad E + ATP + LH_2 \overset{Mg^{++}}{\rightleftarrows} E - LH_2 - AMP + PP$$

$$(2) \qquad E - LH_2 - AMP + O_2 \rightarrow E - L - AMP + H_2O + h\nu$$

The total amount of light emitted during the reaction is a function of the concentrations of luciferase, luciferin, oxygen, and ATP. With all other

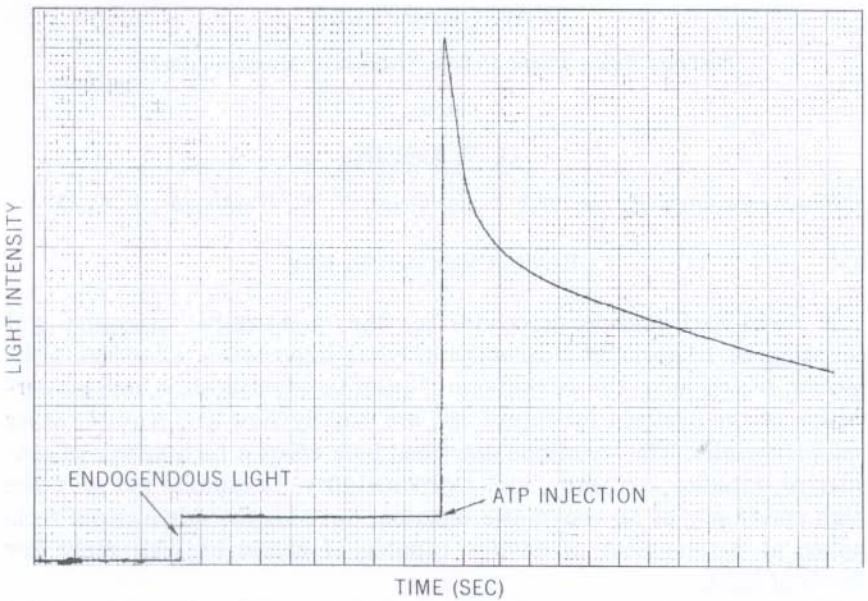

FIG. 1. A typical light-emission curve when ATP is injected into a solution containing luciferase, luciferin, Mg^{++}, and TRIS buffer.

necessary factors in excess, both the total emitted light and the peak light intensity are proportional to the quantity of ATP. The peak light intensity is reached almost instantaneously, whereas the measurement of total light emission requires a long time period (e.g., 30 minutes). Thus, for practical purposes, the peak light intensity was selected as the measure of the ATP concentration and is reported in arbitrary light units. Figure 1 is a typical light-emission curve.

[2] E = enzyme (luciferase), LH_2 = luciferin (reduced), PP = pyrophosphate, AMP = adenylic acid, L = dehydroluciferin.

MATERIALS AND METHODS

Materials

Luciferase

A crude luciferase preparation was obtained by the extraction of an acetone powder prepared from desiccated firefly tails.[3] The acetone powder prepared according to Green and McElroy (14) can be conveniently stored in large quantities, and our procedure succeeds in removing from it most of the interfering lipid material. The extraction was carried out by adding 1 gm of the acetone powder to 10 ml 0.05 M TRIS, pH 7.4, containing 0.001 M EDTA, and stirring it in the cold for 30 minutes. The suspension was centrifuged at 10,000 g for 15 minutes. The supernatant solution contained the luciferase activity.

Luciferin

Luciferin was synthesized in this laboratory according to a method described by White *et al.* (13).

Sephadex G-100

Sephadex G-100, medium grade (Pharmacia, Inc., New York, N. Y.), was allowed to swell in 0.05 M TRIS in the cold for approximately 24 hours before it was used to pack the chromatography column.

ATP

A stock solution of the disodium salt of ATP (Sigma Chemical Corp., St. Louis, Mo.) was made up in 0.05 M TRIS to 1 mg/ml and stored in 1 ml aliquots at —20°. Immediately before use, the ATP was thawed and diluted with distilled water to the desired concentration.

Bacterial Extracting Agent

The extracting agent was a solution of 0.1 M TRIS, 0.01 M potassium arsenate, 0.01 M EDTA, and 6% *n*-butanol.

Methods

Instrumentation

Figure 2 is a drawing of the light-measuring instrumentation, which consists of a reaction chamber and photomultiplier assembly, amplifier, power supply and recorder.

[3] Commercial sources of desiccated firefly tails are Sigma Chemical Company, St. Louis, Mo.; Worthington Biochemical Corporation, Freehold, N. J., and Carolina Biological Supply Company, Burlington, N. C. The species used in these studies was *Photinus pyralis*.

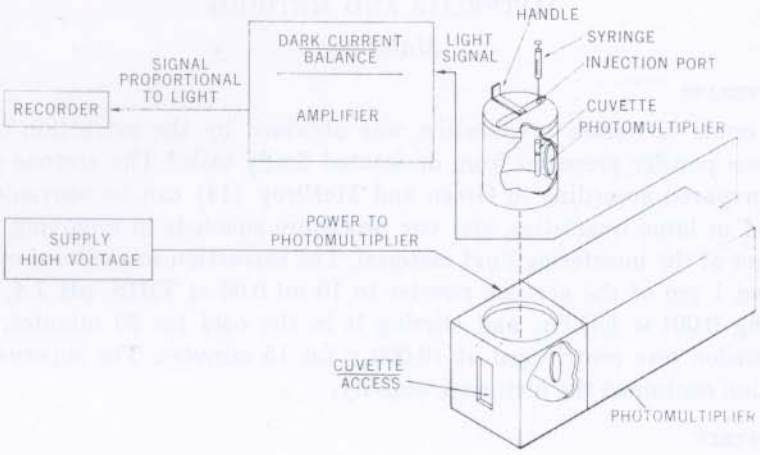

FIG. 2. A schematic drawing of the instrumentation used for the light measurements described in this paper.

The reaction chamber (Figs. 3, 4) which is coupled to an RCA 7265 photomultiplier, is a rotary drum designed so that access to and removal of the reaction cuvette is possible without exposing the photomultiplier to ambient light. The chamber can accommodate a 6 × 50 mm cuvette. Immediately above the cuvette holder, when rotated to the injection position, is a 0.040 inch injection port sealed by a replaceable, light-tight, rubber diaphragm to eliminate light leaks during injection. All surfaces of the reaction chamber assembly, exclusive of the mirror, are painted optic black.

A thin glass plate physically separates the photomultiplier and the drum assembly so that the photomultiplier chamber can be cooled to re-

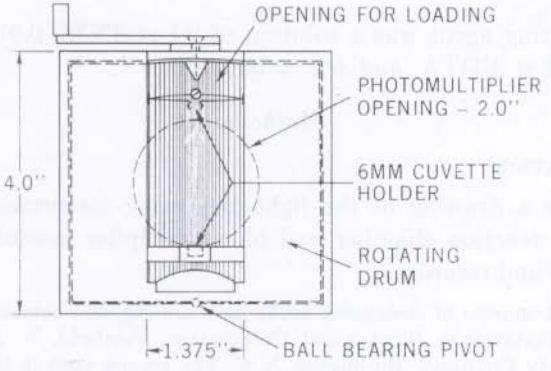

FIG. 3. Front view of rotating-drum reaction chamber.

duce the dark current. The photomultiplier, selected for low dark current, is spaced 0.0625 inches from the glass plate and 0.0250 inches from the center of the cuvette.

The voltage-divider network and focus controls (Fig. 5) are mounted concentrically within the photomultiplier housing, at the rear of the photomultiplier socket.

The remainder of the instrumentation is readily available commercial equipment. The amplifier routinely used was a General Radio 1230-A electrometer amplifier. For optimum stability, a well-regulated power

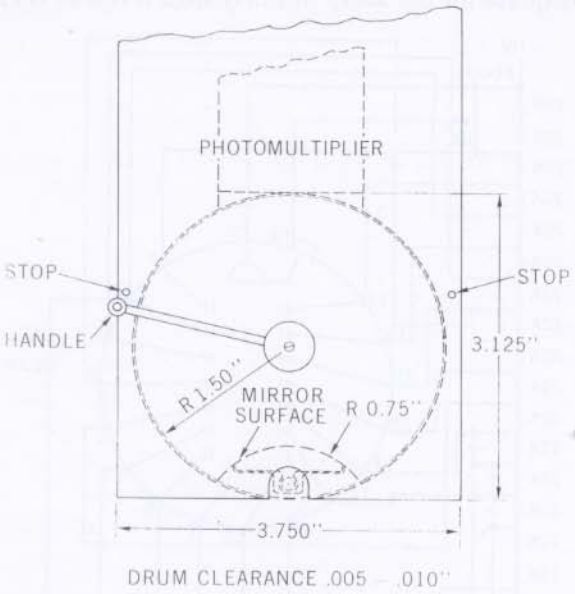

FIG. 4. Top view of rotating-drum reaction chamber.

supply was required; John Flukes 413-C was used. The best signal-to-noise ratio was obtained on applying —1200 V to the photomultiplier. The light emission was followed by means of a Moseley 2D-2 X-Y recorder, which has a response time for 0.5 seconds for full-scale deflection. The stability and sensitivity of the instrumentation were routinely monitored using a radioactive-liquid scintillator light standard.[4]

GENERAL ASSAY PROCEDURE

The assay procedure consists of using an extracting agent to extract the sample containing ATP. After suitable filtration or centrifugation to

[4] Kindly supplied by Prof. J. W. Hastings.

remove particulate matter, an aliquot of the soluble material is removed by needle and syringe. The contents of the syringe are then injected into the cuvette containing the enzyme mixture (luciferase, luciferin, Mg^{++}, and buffer) which has been positioned before the face of the photomultiplier by rotating the lightproof drum.

RESULTS AND DISCUSSION

Optimum Reaction Conditions

Preparation of Enzyme. The crude acetone powder extract from firefly lanterns is adequate for the assay of fairly high levels of ATP (i.e., above

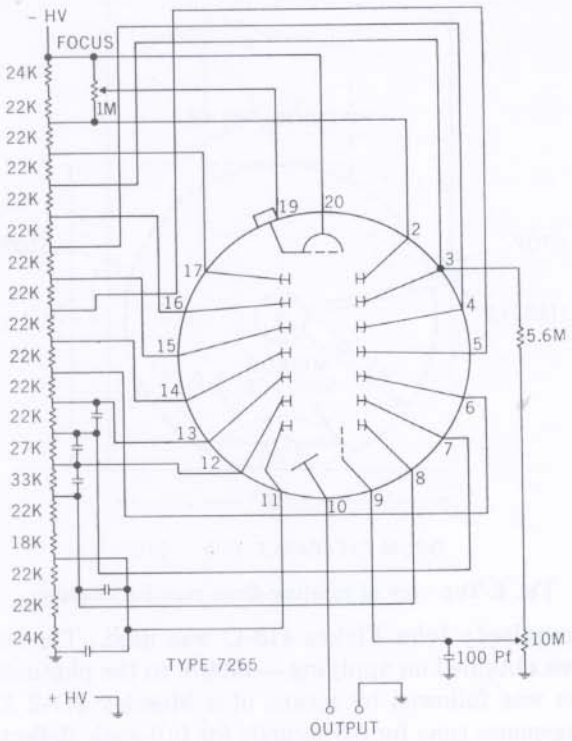

Fig. 5. Circuitry of instrumentation associated with photomultiplier.

10^{-3} μgm). However, the light in the crude system due to endogenous ATP does not permit the assay of lower concentrations of added ATP. Partial purification of the enzyme by passage of the crude extract through a Sephadex G-100 column will produce an enzyme which is very low in endogenous light. Luciferin and Mg^{++} must be added to the partially-purified enzyme, as they are removed during chromatography. The optimum con-

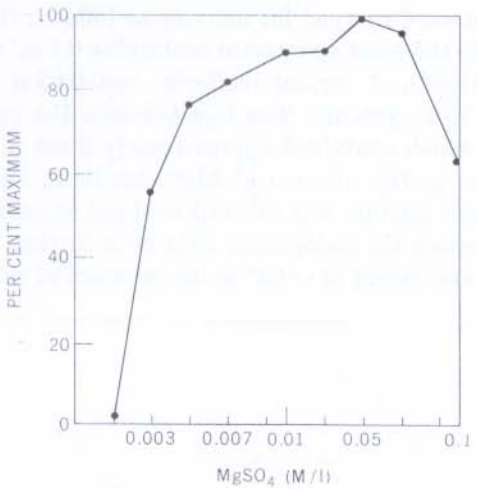

FIG. 6. Effect of Mg^{++} concentration on light response.

centration of Mg^{++} (Fig. 6) is in agreement with that reported by Green and McElroy (14), approximately 0.01 M. The luciferin concentration necessary for maximum light emission is shown in Fig. 7. The presence of impurities in our luciferin preparations may explain the fall-off in light emission at concentrations above 1 mg/ml. The fractions eluted from the

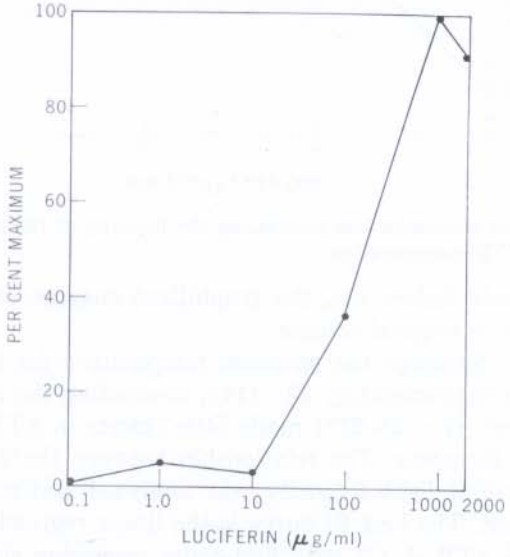

FIG. 7. Effect of luciferin concentration on light response.

Sephadex column were assayed for activity as follows: 0.2 ml of the fraction was placed in the reaction cuvette containing 0.1 ml of a solution containing 0.01 M $MgSO_4$, 1 mg/ml luciferin, and 0.05 M TRIS (pH 7.4). 0.1 ml of ATP (0.1 μgm/ml) was injected into the cuvette. The most active fractions which contained approximately 2 mg of protein/ml were pooled and added to the solution of Mg^{++}, luciferin, and TRIS in a 1:2 ratio. This reaction mixture was allowed to stand at ambient temperature for one hour to reduce the endogenous light even further. The solution was then lyophilized and stored at —60° in the presence of silica gel as a desic-

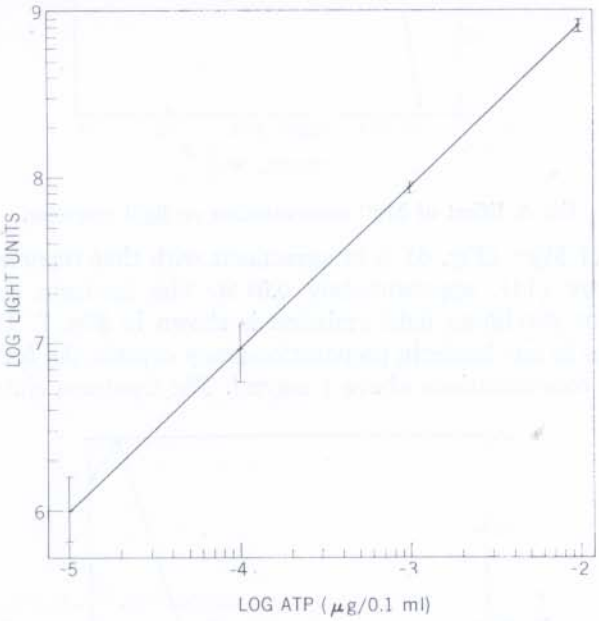

FIG. 8. Statistical regression line illustrating the linearity of the initial peak height as a function of ATP concentration.

cant. Immediately before use, the lyophilized enzyme was dissolved in distilled H_2O to its original volume.

Temperature. Although the optimum temperature for the bioluminescent reaction is approximately 23° (14), conducting the reaction at ambient temperature (i.e., 21–27°) made little change in ATP response.

Linearity of Response. The relationship between the ATP concentration and the initial light intensity was analyzed statistically by polynomial regression. The best fit curve is the linear regression (15) (Light units = 0.9375 ATP + C) with first-order regression significant at the 0.1% level. A typical concentration curve (Fig. 8) shows the regression

TABLE 1
ATP CONTENT IN BACTERIA

Organisms	Total cell/ml ($\times 10^8$)	μgm ATP/cell ($\times 10^{-10}$)
Aerobacter aerogenes	15	0.28
Bacillus cereus	3	1.1
Bacillus coagulans	8.8	1.7
Bacillus globigii	4.6	5.4
Brevibacterium helvolum	22	0.37
Erwina carotovora	6.4	0.44
Escherichia coli	16	1.0
Flavobacterium arborescens	6.1	1.5
Gaffkya tetragena	10	0.61
Klebsiella pneumoniae	9	5.0
Micrococcus lysodeikticus	14	1.3
Mycobacterium phlei	5.3	1.9
Mycobacterium smegmatis	6.5	8.9
Pseudomonas aeruginosa	33	1.0
Pseudomonas fluorescens	11	3.1
Proteus vulgaris	7.9	1.8
Sarcina lutea	32	0.37
Serratia marcescens	26	1.0
Staphylococcus aureus	23	0.64

line in light units $\pm\sigma$ vs. ATP concentration. Using partially purified enzyme, 10^{-6} μgm ATP can be detected. Use of more sensitive instrumentation (to be described elsewhere) permits measuring ATP levels down to 10^{-8} μgm.

ATP in Bacteria. The ATP in a number of bacteria (Table 1) was extracted by suspending 1 ml of washed cells in 5 ml of the extracting agent. Using *Escherichia coli* as a representative bacterium, maximum extraction occurs almost instantaneously (Table 2). The linearity of ATP response on dilution of a suspension of *B. globigii* is shown in Fig. 9. The variation in the activity of enzyme batches makes it necessary to run a standard

TABLE 2
MAXIMUM EXTRACTION TIME OF ATP FROM BACTERIA

Time (min.)	μgm ATP/cell ($\times 10^{-10}$)
0	1.3
1	1.0
10	1.5
30	1.9

ATP curve routinely; the ATP content of any sample assayed is then determined on the basis of the standard curve. All species were assayed in the stationary growth phase after being maintained under conditions optimum for each species. In routine studies with *E. coli,* the cell population calculated on the basis of ATP content agrees consistently with that determined by both plate counting and microscopic counting. Among

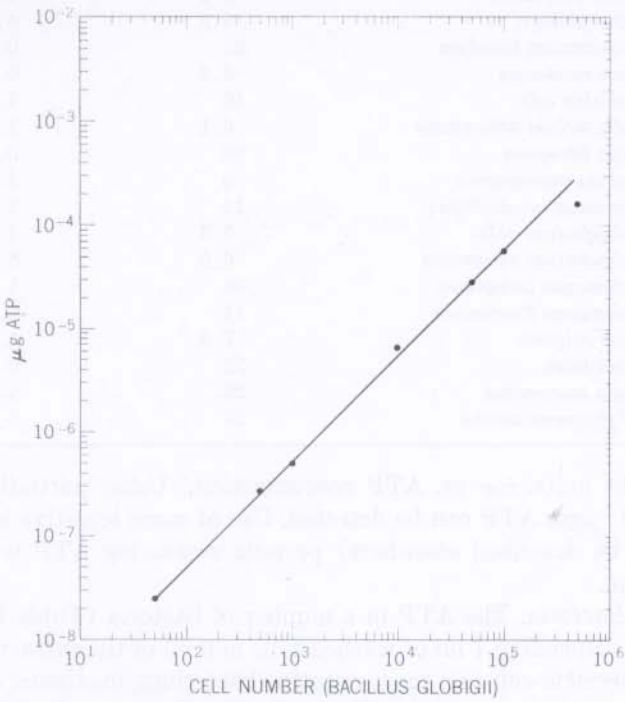

FIG. 9. A dilution curve of a suspension of *Bacillus globigii* indicating the linearity of response as a function of cell number.

the various species assayed, the ATP content varies by approximately one order of magnitude. Studies of three bacterial species (16) indicate that the ATP content of the organisms varied only slightly throughout the growth phases. The ATP contents for the species reported here are in accord with those reported by Levin *et al.* (17).

The results of our studies show that measuring bacterial populations on the basis of ATP content is a simple and rapid technique whose accuracy is comparable to that of slower and more classical means such as plate-counting and direct microscopic count.