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DEVELOPMENT OF THE FIREFLY BIOLUMINESCENT ASSAY FOR THE RAPID, QUANTITATIVE DETECTION OF MICROBIAL CONTAMINATION OF WATER

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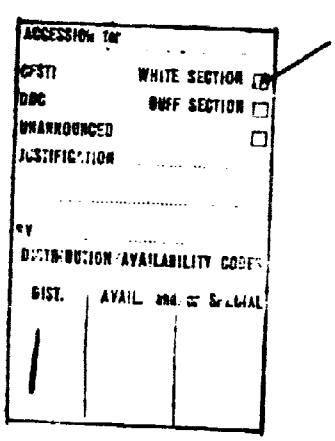

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FOREWORD

The findings published in this report were obtained during the course of applied research conducted under Contract No. AF 33(615)-3996 and in support of Project No. 7164, "Biomedical Criteria for Aerospace Flight," and Task No. 716410, "Aerospace Sanitation and Personnel Hygiene" for the Aerospace Medical Research Laboratories, Wright-Patterson Air Force Base, Ohio, 45433, by Hazleton Laboratories, Incorporated, P. O. Box 30, Falls Church, Virginia, 22046. The Air Force program monitor for this contract was Arnold R. Slonim, PhD, Biotechnology Branch, Life Support Division, Biomedical Laboratory. The research reported herein was conducted during the period from 1 May 1966 through 31 March 1967.

This technical report has been reviewed and is approved.

WAYNE H. McCANDLESS
Technical Director
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ABSTRACT

Research has been conducted and reported herein toward development of a sensitive, nonspecific microbial assay technique suitable for determining the biological quality of spacecraft water supplies. The firefly bioluminescent reaction is used to detect adenosinetriphosphate (ATP) extracted from microorganisms present in the test sample. Since ATP is ubiquitous in all organisms, this provides a means for detection of microorganisms. An instrument using a photomultiplier to detect the light output from the bioluminescent reaction has been designed, constructed, and tested. This instrument, a modification of one previously developed in this laboratory, was constructed primarily for the purpose of conducting the biological research required for this program. Extraction of ATP from cells was accomplished with dimethylsulfoxide (DMSO). Tests were conducted to find the optimum concentration of DMSO and the simplest extraction procedures. Cultures of a wide variety of microorganisms were grown, including various metabolically different bacteria as well as representative algae, fungi, and protozoa. The ATP content of measured numbers of these cells was determined. These tests have shown the ability to detect as few as several hundred cells of any of the test species (in many cases, less than 100 cells) in less than 30 seconds. Detection of less than 10 cells per ml of sample is feasible for any of the species tested with filtering or other concentration procedures. Excellent correlation between ATP quantity and light response over more than five orders of magnitude was demonstrated. Correlation between light response and numbers of cells among the bacterial species demonstrated a surprisingly constant ATP content per cell regardless of species or physiological state on the growth curve. Within an accuracy of approximately one order of magnitude, the light response amplitude measures the numbers of bacteria present. The assay technique may have applicability to terrestrial water supplies, particularly under hazardous or difficult conditions, as well as to spacecraft waters.

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LIST OF ABBREVIATIONS AND SYMBOLS

AMP	adenylic acid
ATCC	American Type Culture Collection
ATP	adenosinetriphosphate
C	Celsius
CRT	cathode ray tube
DMSO	dimethylsulfoxide
E	enzyme, luciferase
FTG	fluid thioglycollate broth
FTGA	fluid thioglycollate agar
L	dehydroluciferin
LH_2	luciferin
M	molar
mg	milligram
ml	milliliter
mp	melting point
mv	millivolt
NG	nitrate glucose broth
OD	optical density
P	Palmer chamber
pH	a measure of the relative acidity or alkalinity; the logarithm to the base 10 of the hydrogen ion concentration
PH	Petroff-Hausser chamber
PP	pyrophosphate
RC	resistance capacitance

R_x	reagent mixture
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
STA	synthetic thiobacillus agar
TB	Tetrahymena broth
TSB	tryptic soy broth
UV	ultraviolet
v/v	volume per volume
$[\alpha]_D^{22^\circ}$	optical activity; D-dextro-rotation; 22° - 22 degrees angular measure
γ	one microgram (gamma)
ε	absorption coefficient
λ	one microliter (lambda)
μ	one micron
$m\mu$	one millimicron

SECTION I

INTRODUCTION

This research was accomplished with the objective of developing a simple, sensitive and rapid technique for detecting microorganisms in spacecraft water supplies to enable astronauts to assess the biological quality of their drinking water.

In the small, closed environment of a spacecraft, the crew must be protected against microbial contamination of the environment. As flights lengthen in duration, the crew will be correspondingly more distant from medical treatment should disease occur. In addition, longer flights increase the hazard in that flight logistics will require the reclamation and reuse of wastes to supply drinking water. Even in short-term storage of drinking water, undesirable bacterial contamination may develop. To protect the crew and insure the performance of the mission, the astronauts must know the microbiological quality of their drinking water in order to take appropriate action when contamination develops.

Currently used standard methods (ref. 1) for the examination of drinking water are inadequate for use aboard the spacecraft for several reasons. First, the elapsed times, 24 to 96 hours, for completion of the tests are too long to permit corrective action to be taken prior to consumption of the water. Either the water would be consumed before the results of the tests were available or, if the water were held until the test results were read, its quality at the time of consumption would again be unknown. Furthermore, the criterion used by the U. S. Public Health Service for the determination of the bacteriological quality of drinking water (ref. 2) relates to the coliform group of organisms only. These organisms, of fecal origin, are an index of sewage contamination of water. While this index is satisfactory for municipal water supplies where the principal source of contamination is sewage, restricting the surveillance to coliform organisms aboard spacecraft may allow important pathogens to go undetected. The sources of contamination aboard the spacecraft may not always be directly of sewage origin and thus conveniently tagged with coliform organisms. Finally, the standard methods would require the continuous use of a 35° C incubator aboard the spacecraft with its concomitant requirements for temperature control of $\pm 0.5^{\circ}$ C and the need for power.

Unlike the rambling distribution system of a municipal water supply, a spacecraf water supply can be so carefully controlled that sterility could be maintained. This makes possible and desirable the

use of the total bacteria count as a sanitary quality index. The total bacteria test offers much greater protection than testing for the coliform group of organisms in that nonsewage associated pathogens will not escape detection. Except for this advantage, however, the same objections cited above for spacecraft use of the coliform detection method apply to the standard method (ref. 1) for the determination of total bacteria.

Based on a method conceived and developed at Hazleton Laboratories, Inc., research has been conducted to develop a very rapid and sensitive method for monitoring the presence of microorganisms in water reclamation units in order to enable anyone with a technical background, such as an astronaut, to assess the potability of drinking water. The system is based upon the presence of adenosinetriphosphate (ATP) in all living cells and upon the ability of the firefly bioluminescent reaction to detect minute quantities of ATP with great rapidity. It is a biologically nonspecific enzymatic method for detecting microorganisms.

Insofar as is known, ATP is ubiquitous in all living cells (ref. 3). Only under rare and contrived conditions can it be synthesized abiogenically (ref. 4). ATP released by dying microorganisms is rapidly acted upon by other organisms. Thus the extraction and detection of ATP from a suspect sample of water is excellent evidence for the presence of living organisms in the original sample. Through the use of physical or chemical means, ATP can be extracted from microorganisms. The extracted ATP can then be quantitatively assayed by means of the firefly bioluminescent reaction (ref. 5).

The firefly tail contains an enzyme, luciferase; a specific substrate, luciferin; and magnesium. When the firefly introduces ATP into these reactants in the presence of oxygen, light is produced;

$$E + ATP + LH_2 \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} E \cdot LH_2 - AMP + PP$$

$$E \cdot LH_2 - AMP + O_2 \longrightarrow E \cdot L - AMP + Light$$

where: LH_2 = luciferin, E = enzyme (luciferase),

PP = pyrophosphate, L = dehydroluciferin,

and AMP = adenylic acid.

The light output reaches its peak intensity in less than one second and, by means of a calibration curve made with standard ATP, the peak amplitude can be used as a quantitative assay for ATP in the unknown sample.

Readout is accomplished through the use of a photomultiplier tube coupled to an oscilloscope. Figure 1 is a typical oscilloscope readout during such an ATP assay.

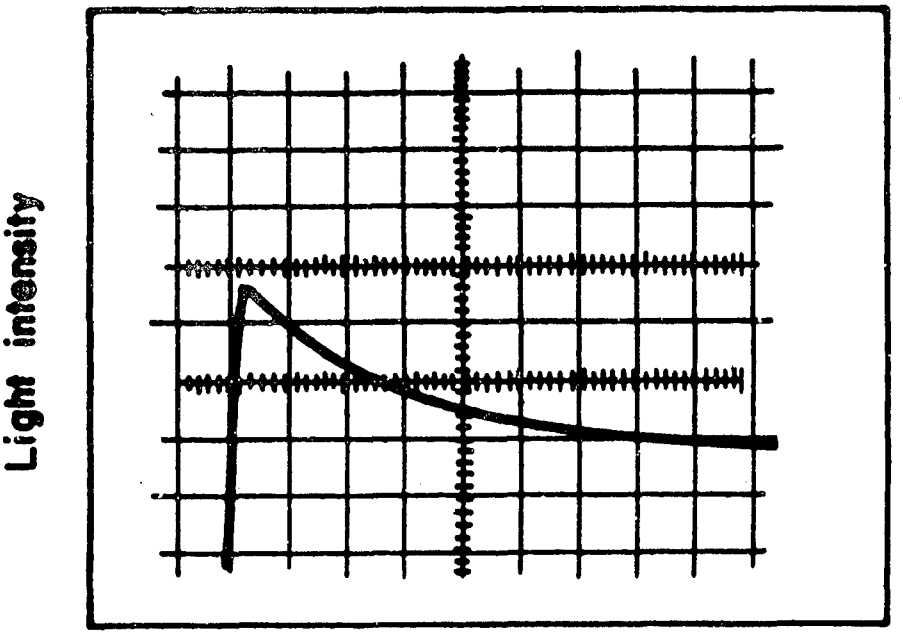


Figure 1 – Typical response of microbial ATP in firefly bioluminescent reaction.

II

SUMMARY

The feasibility of a unique method to guard against the spacecraft crew health problem of potable water contamination has been successfully demonstrated. The new method detects total microbial contamination of water on a basis sufficiently broad, rapid, and sensitive to meet the requirements set forth by the Air Force in its statement of the problem. The specifications called for a method broadly applicable to all microbial species with a capability of detecting as few as ten to one hundred cells per milliliter of water within an elapsed assay time of 30 seconds. Results obtained in the one-year research project reported herein strongly indicate that the method developed can meet these most stringent conditions.

The method is based upon two factors:

1. The high sensitivity of the firefly bioluminescent reaction for the quantitative assay of adenosinetriphosphate (ATP);

2. The fact that ATP occurs in all living cells, including microorganisms.

Biochemical procedures were developed in a manner to make routine spacecraft use of the method by non-scientific personnel possible. The reaction mixture required for the test may be prepared in the laboratory and placed in frozen storage aboard the spacecraft where portions can be activated just prior to use. The active ingredients extracted from the firefly tail are the enzyme, luciferase; the substrate, luciferin; and magnesium. When a solution of ATP is introduced into a solution of the reaction mixture in the presence of dissolved oxygen (normally present to excess in both solutions), light is emitted. The amplitude of light response is directly proportional to the quantity of ATP present. The assay of known standard ATP solutions gives the slope of the calibration line, from which the amount of ATP in an unknown sample can be determined.

The use of dimethylsulfoxide (DMSO) was found to be highly satisfactory as a solvent to extract cellular ATP. Thus, the manner in which the system would operate aboard the spacecraft would be as follows:

> An ampoule of lyophilized reaction mixture would be removed from frozen storage. The ampoule would be broken and the contents dissolved in stored Tris buffer. The water sample to be tested would be treated with DMSO to release any intracellular ATP present. A portion of the reaction mixture would be placed in the cuvette of the photodetection instrument. An aliquot of the treated sample would be removed by syringe and

injected into the cuvette. The amplitude of the light emitted would be recorded. Based on results obtained in the same manner with standard ATP solutions, the amount of ATP in the sample would be determined.

In the course of this research project, studies were conducted with the following twelve different species of microorganisms: (a) bacteria Escherichia coli, Corynebacterium striatum, Bacillus subtilis, Clostridium sporogenes, Streptococcus salivarius, Thiobacillus novellus, Staphylococcus epidermidis, and Pseudomonas fluorescens; (b) fungi Saccharomyces cerevisiae and Aspergillus niger; (c) alga Chlorella pyrenoidosa; and (d) protozoa Tetrahymena pyriformis. ATP was detected sufficiently from each species to indicate feasibility for meeting the Air Force requirements. In addition, responses from the bacterial species suggest the possibility of making the test quantitative for total numbers of bacteria. Results with E. coli tested in various stages of growth indicate that growth phase differences are not large enough to interfere with quantitation of the method. The foregoing is based on the belief that bacterial quantitation to an accuracy of one order of magnitude is adequate for the desired test purposes. The ability to obtain such accuracy across the species tested was demonstrated with the exception of one species, Corynebacterium striatum. However, a plausible explanation for the variability with this organism lies in the difficulty in obtaining accurate cell counts by standard methods. The potential for developing a quantitative total bacteria test was demonstrated.

The feasibility study was conducted with the use of an instrument constructed especially for this purpose. Essentially, the instrument consisted of a light-tight reaction chamber which could be rotated to confront or oppose a photomultiplier tube. A cuvette containing the reaction mixture was loaded into the reaction chamber which was then rotated to confront the photomultiplier tube. An aliquot of the sample to be tested was injected into the cuvette by means of a syringe inserted through a light-tight rubber stopper. The response from the reaction was monitored on an oscilloscope and manually recorded.

The results of the feasibility project strongly indicate that membrane filtration of as little as ten milliliters of spacecraft water and the extraction of ATP from the bacteria so removed can satisfy the maximum Air Force requirement of detecting as few as ten microorganisms per milliliter of spacecraft water. This is possible irrespective of whether the microorganisms are bacteria, algae, fungi, or protozoa. Moreover, with a suitable means for separating the bacterial species from the larger microorganisms, such as through the use of differential filtration, it may be possible to develop the method as a quantitative assay for total bacteria. Inasmuch as sample extraction can be adequately accomplished in 15 seconds and the reaction time is approximately 0.5 second, no problem is foreseen in meeting the Air Force requirement for completing the assay in 30 seconds.

Finally, the prospects are most favorable for the development of suitable instrumentation, either manual or automatic, for spacecraft use. Further research and development in biochemistry and engineering designed to meet the Air Force requirements is recommended on the basis of the promising results obtained in this program.

SECTION III

BIOCHEMISTRY

1. MICROORGANISMS USED FOR TESTING

The following microorganisms were chosen for testing the ATP-luciferase bioluminescence technique because they represent a wide range of metabolically diversified species, many of which can be potential spacecraft contaminants:

	Organism[1]	Energy Source	O_2 Relationship
Bacteria	Escherichia coli (ATCC 4157)	Heterotroph	Facultative
	Corynebacterium striatum (ATCC 6940)	Heterotroph	Aerobic
	Bacillus subtilis (ATCC 6633)	Heterotroph	Aer. and Facul. strains
	Clostridium sporogenes	Heterotroph	Anaerobic
	Streptococcus salivarius (ATCC 9222)	Heterotroph	Facultative
	Thiobacillus novellus (ATCC 8093)	Autotroph	Facultative
	Staphylococcus epidermidis (ATCC 12228)	Heterotroph	Facultative
	Pseudomonas fluorescens (ATCC 13525)	Heterotroph	Aerobic
Algae	Chlorella pyrenoidosa (7-11-05)[2]	Autotroph	Aerobic
Fungi	Saccharomyces cerevisiae (ATCC 4111)	Heterotroph	Facultative
	Aspergillus niger (ATCC 6275)	Heterotroph	Aerobic
Protozoa	Tetrahymena pyriformis (ATCC 9357)	Heterotroph	Facultative

[1]All the ATCC type cultures were ordered directly from American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland.

[2]Indiana type culture collection

In addition, the following organisms were selected for extended studies (growth phase studies; cf, III.8):

a.	Escherichia coli	Heterotroph	Facultative
b.	Saccharomyces cerevisiae	Heterotroph	Facultative

2. PREPARATION OF ASSAY ENZYME AND REAGENTS

Aseptic and chemically clean techniques were applied in purifying all the reagents, including enzyme solutions, to avoid contamination from bacteria or chemicals, including ATP.

Fresh buffer was prepared and filtered through a membrane filter, 0.47 micron porosity. Disposable bottles were plugged with cotton, covered with aluminum foil after being filled with the buffer solution, and then autoclaved at 121° C for 30 minutes.

The injection needles and glass syringes were cleaned by soaking overnight in 0.1N HCl and rinsing with Tris buffer until no residual ATP response could be detected. All the nondisposable glassware and cuvettes used on reactants and samples were also autoclaved. The only other containers used were of the sterile, disposable-type.

a. Purification of Enzyme Luciferase

Although dehydrated firefly tails (or lanterns) are listed in a number of catalogs, there had initially been only one supplier who had any material available (Sigma Chemical Company), and this supplier had rationed severely the amount purchased by any laboratory. Hazleton Laboratories conducted an extensive firefly collecting campaign among the children in the area near the laboratory and succeeded in obtaining a supply adequate for this entire program. (They were placed in tubes with Silica Gel after capture and almost immediately thereafter into a deep freeze.) In addition, Hazleton Laboratories succeeded in obtaining another commercial source of dehydrated firefly tails (Carolina Biological Supply). This source will sell reasonable amounts of the enzyme source material to any laboratory. Furthermore, if they are informed of large requirements before the start of the collecting season (early July), they will arrange to collect and process any desired amount. The program may make use of this source should work continue beyond the present contract period.

The procedure used for processing fireflies starts with lyophilization of the whole firefly followed by removal of the lanterns. Acetone powders are prepared from this material by grinding the desiccated tails in a prechilled mortar with -20° acetone, filtering through a Buchner funnel having Whatman no. 2 filter paper and then air-drying

the powder. After completing the drying process in a vacuum desiccator, 1 gram of the powder is added to 5 ml of Tris buffer at 0° C and stirred for 10 minutes. An additional extraction with 5-ml buffer is made. A total of 10 ml is obtained. Insoluble material is removed by centrifugation at 230 x g for 10 min at 4° C. The potency of the material is determined at this point by assaying with 10^{-3} γ of ATP in the standard instrument system. Partial purification is effected by passing the material through a column of Sephadex G-100 eluted by Tris buffer. Each fraction is assayed for luciferase activity and the most potent ones from approximately four tubes, each containing 7 to 10 ml fractions, are combined. Activity of the pooled fraction (roughly 30 to 40 ml) is assayed. The pooled active fractions of enzyme solution collected off the preparative column are filtered through a 0.45 micron membrane filter. Two-ml aliquots of this material are then placed in lyophilizing bulbs and lyophilized for 2 hours. Prolonged lyophilization may reduce the enzyme activity. When lyophilization is complete, the bulbs are placed in a deep freeze (-5° C) and removed as required. The enzyme stored in this condition for 6 months still has the original activity. Prior to use, a bulb is removed and reconstituted with 2 ml of a solution containing 0.05 M Tris buffer, 0.01 M $MgSO_4 \cdot 7H_2O$ and luciferin (0.6 mg/ml). The reconstituted enzyme is again passed through a 0.45 micron membrane filter before use and kept in a capped disposable tube in an ice bath. It stays active for about 2 to 4 hours.

b. Luciferin

At the present time, it is not possible to purchase the substrate, luciferin [2-(6-hydroxy-2-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid], and all of the material used so far has been synthesized at Hazleton Laboratories.

The technique used for the synthesis of D-luciferin (only this optical isomer is active) is essentially the technique of White, et al (ref. 6), with the major exception that elution from the alumina column is effected with an equal (v/v) mixture of ether-petroleum ether. Criteria for acceptable D-luciferin are the following: mp = 189° ± 2°; $[\alpha]_D^{26}$ = -28° to -36° (in 1,2-dimethyl-formamide); UV max at 330 mμ, ε = 18,000; < 1% dehydroluciferin. As mentioned before, the luciferin is prepared at the concentration of 0.6 mg per ml in sterile distilled water.

c. Preparation of Reagents

The following reagents are used in standard procedures:

(1) Tris buffer: 0.05 M Tris, adjusted to pH 7.4 with concentrated HCl, autoclaved at 15 psi for 30 min and stored in refrigerator.

(2) $MgSO_4$: 0.01 M $MgSO_4$ prepared in Tris buffer.

(3) ATP: Stock solution contains 1 mg/ml of Tris. This is stored frozen and dilutions (in Tris buffer) are prepared as needed.

(4) Luciferin: 0.6 mg luciferin/ml of Tris buffer.

(5) Saline: 0.85% NaCl in H_2O.

(6) Reaction (R_x) mixture: Lyophilized luciferase is reconstituted by dissolving it in 2 ml of 0.05 M Tris buffer. To this is added 2 ml of $MgSO_4$ solution and 2 ml of the luciferin (0.6 mg/ml) solution.

(7) DMSO: 90% dimethylsulfoxide in 0.05 M Tris buffer, pH readjusted to 7.4 with HCl.

d. Preparation of Reaction Mixture

(1) Reconstitute a vial of lyophilized enzyme (approximately 50 mg dry weight stored in deep freeze) with 2 ml 0.05 M Tris buffer.

(2) Add 2 ml 0.01 M $MgSO_4 \cdot 7H_2O$ solution.

(3) Add 2 ml luciferin solution (0.6 mg/ml).

(4) Filter R_x through Swinny-13 sterile HA filter unit, 0.45 μ.

(5) Allow R_x to [illegible] at room temperature for about an hour in a capped, sterile plastic tube for inherent light to decrease.

(6) Dispense R_x in 100 λ aliquots into cuvettes before assay.

e. Preparation of Standard ATP Solution

A stock solution of standard ATP containing 1 mg/ml is made up and stored in 1-ml portions in capped, sterile plastic tubes in the freezer. A tube is removed and thawed out each time. Desired dilutions are made as follows:

Standard ATP Concentration	Procedure
10^{-1} γ/10 λ	0.1 ml stock ATP solution + 9.9 ml 0.05 M Tris buffer (100 x dilution)
10^{-3} γ/10 λ	0.1 ml 10^{-1} γ/10 λ solution + 9.9 ml 0.05 M Tris buffer (100 x dilution)
10^{-4} γ/10 λ	0.5 ml 10^{-3} γ/10 λ + 4.5 ml 0.05 M Tris buffer (10 x dilution)
10^{-5} γ/10 λ	0.5 ml 10^{-4} γ/10 λ + 4.5 ml 0.05 M Tris buffer (10 x dilution)
10^{-6} γ/10 λ	0.5 ml 10^{-5} γ/10 λ + 4.5 ml 0.05 M Tris buffer (10 x dilution)
10^{-7} γ/10 λ	0.5 ml 10^{-6} γ/10 λ + 4.5 ml 0.05 M Tris buffer (10 x dilution)

The standard ATP response curve obtained by assaying the solutions prepared in this way is discussed further in Section III.5.c., ASSAY TECHNIQUE.

3. QUANTITATION OF CELL COUNTS

The problem of obtaining accurate cell counts has been of great concern in the present assay program. Accurate counts are necessary to quantitate the ATP response. Cell counts should be particularly reliable in the most sensitive range of the ATP assay. Unfortunately, there is no available method capable of precise enumeration. For example the conventional plate count method uses a medium which cannot support growth of numerous species of organisms. Above all, there is no satisfactory correction factor for the so-called "clumping factor," each clump being counted as a single cell. The same organism may clump differently in different cultures.

Another way to estimate cell numbers is by chamber count. With this method, total cell number can be counted. However, it is difficult to get a sufficiently representative sample to assure accuracy. Moreover, dead cells are also counted. This method gives a higher count than the plate count method. Both methods were included in the present studies. The Chlorella and Tetrahymena organisms were estimated solely by chamber counts because of the inapplicability of plate counts.

Plate counts were initially made using pour plates and spread plates. The latter are easier to prepare and several experiments in which

both pour plates and spread plates were used yielded very similar results (see table I). Thereafter spread plates were routinely used.

4. FACTORS AFFECTING ATP EXTRACTION

a. Effect of pH on Extraction

Aliquots of Bacillus subtilis var globigii (BG) cells were extracted with 90% DMSO in 0.05 M Tris buffer adjusted to pH values varied from 6.0 to 8.5. After 5 minutes of extraction at room temperature, each system was centrifuged at 600 x g for 5 minutes at 0° C. The supernatant was diluted 1:10 with 0.05 M Tris buffer, pH 7.4. A volume of 0.1 ml of this diluted material was injected into 0.1 ml of a solution containing partially purified firefly luciferase, luciferin, and magnesium ion. As can be seen in the results shown in figure 2, decreasing the pH to 6.5 improves extraction, but decreasing the pH even further, to pH 6.0, has no additional effect. Furthermore, the difference in extractability between pH values of 6.0 and 7.5 is insufficient to warrant alteration of the procedure. In all the following experiments and in the standard method, ATP extraction was made by 90% DMSO in Tris buffer, pH 7.4.

b. Effect of Length of Extraction Time

Freshly grown cultures (18 hours) of Escherichia coli and BG were washed twice with saline and then resuspended in 0.05 M Tris buffer, pH 7.4. Nine-tenths ml of 90% DMSO - 10% 0.05 M Tris buffer, pH 7.4 - was added to a 0.1 ml portion of cell suspension (approximately 2 x 10^9 E. coli cells or 8 x 10^8 BG cells). After extraction for various periods at room temperature (24° C), the individual suspensions were centrifuged at 0° C and 600 x g for 5 minutes to remove cells. Two-tenths ml of the supernatant was diluted with 1.8 ml cold 0.05 M Tris buffer, pH 7.4. One-tenth ml was assayed with 0.1 ml enzyme solution. The results are presented in figure 3. Although there is considerable increase in the amount of ATP being extracted with longer extraction times, the major point of interest in this figure is the relatively large amount of ATP extracted in 15 seconds.

Because of the significance of rapid extraction in this program, the same procedure was repeated for shorter extraction times. Cells, after being washed with saline solution, were exposed to 90% DMSO for 15, 30, and 60 seconds and were immediately centrifuged at 0° C 600 x g for 5 minutes to remove cells. The supernatant was diluted 10 times with Tris buffer. An aliquot of this diluted ATP extract was assayed immediately. The other aliquot was assayed after being held for 30 minutes in an ice bath. As can be seen in table II, this 30-minute holding procedure resulted neither in the loss of ATP nor in the increase of ATP extracted. These responses were derived roughly from 2 x 10^7 cells for E. coli and 7 x 10^6 cells for BG.

Table I. Comparison Of Spread And Pour Plate Techniques For Estimating Cell Numbers.

Organisms	Dilution factor	Spread plate		Pour plate	
		Replicate plates (colonies)	Total no. cells per ml	Replicate plates (colonies)	Total no. cells per ml
E. coli	10^{-5}	46 60 58	5.5×10^6	63 58 43	5.5×10^6
	10^{-6}	7 7 4	6×10^6	5 6 3	5×10^6
B. subtilis	10^{-5}	73 76 85	7.8×10^6	82 87 88	8.6×10^6
	10^{-6}	5 7 —	6×10^6	6 7 6	6×10^6
S. salivarius	10^{-4}	191 209 230	2.1×10^6	171 163 220	1.9×10^6
	10^{-5}	20 20 17	1.9×10^6	18 13 15	1.5×10^6

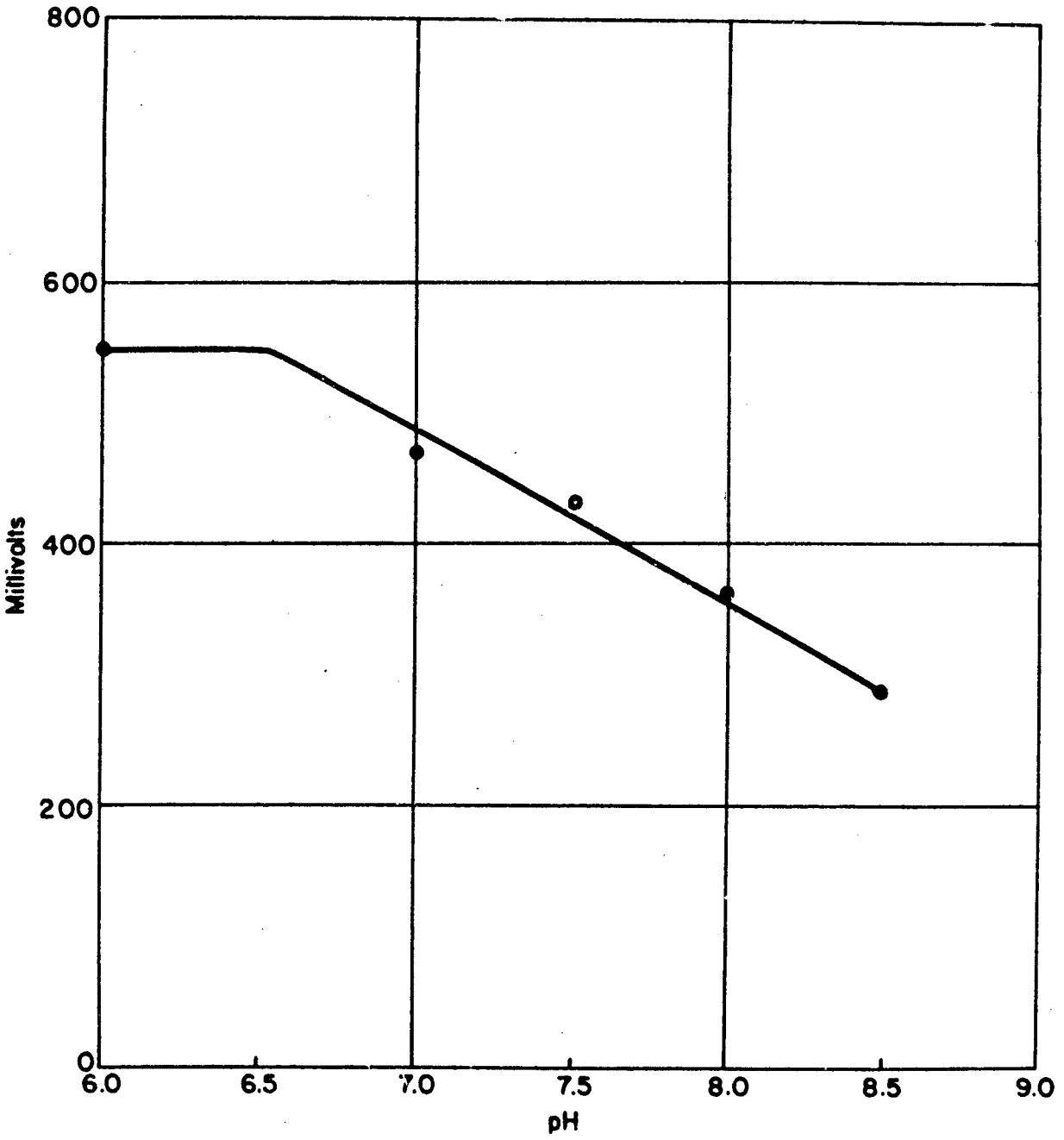


Figure 2 – Effect of pH on extraction of ATP from Bacillus subtilis var. globigii (3×10^8 cells) with 90% DMSO in 0.05 M tris buffer (pH 6.0 to 8.5) for 5 minutes at room temperature.

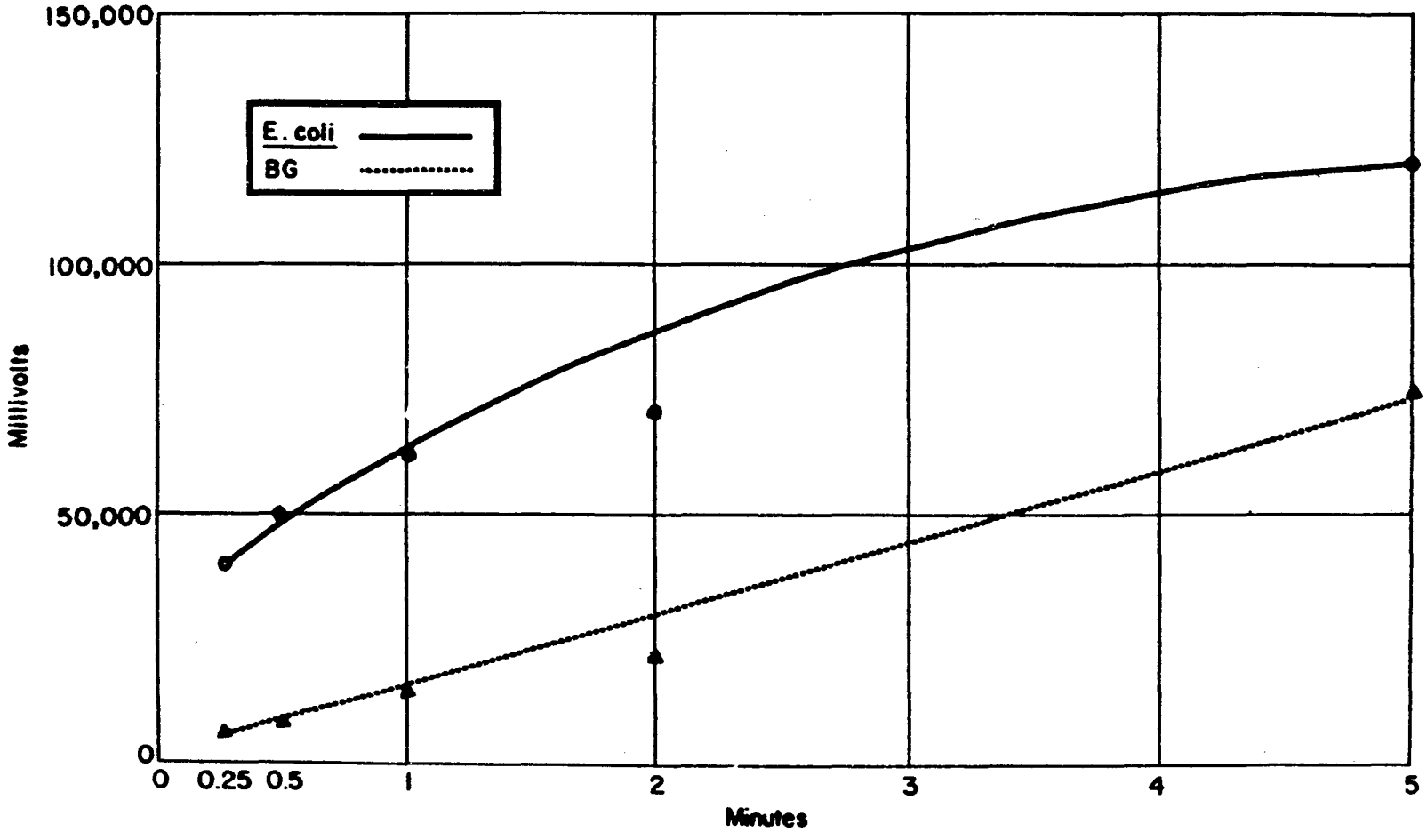


Figure 3 – Effect of time on extraction of ATP from E. coli (2×10^7 cells) and BG (8×10^6 cells) with 90% DMSO in 0.05M tris buffer (pH 7.4) at room temperature.

Table II. Effect Of Time On Amount Of ATP Extracted (Values In Millivolts).

System	Extraction time in seconds		
	15	30	60
E. coli (2×10^7 cells)			
Diluted extract assayed immediately	74,000	87,000	105,000
Diluted extract assayed after 30 minutes	70,000	82,000	97,000
BG (8×10^6 cells)			
Diluted extract assayed immediately	22,500	26,000	30,000
Diluted extract assayed after 30 minutes	23,000	25,000	29,000

Calibration of enzyme preparation: 10^{-3} γ ATP = 9000 mv

The next step in establishing a standard protocol was to apply the shorter extraction time procedure to relatively small numbers of cells. Using the same procedures as in the last paragraph, but decreasing the number of E. coli cells such that the injected extract represented 3.5×10^3, the results shown in figure 4 were obtained. The results shown for BG were obtained with 1.5×10^3 cells. Both cell numbers were obtained from approximate optical density measurements.

To be sure that the technique is generally applicable, ATP was extracted from Tetrahymena pyriformis. Using 1.5×10^5 cells, the response obtained after the 15-second extraction procedure was about 2000 millivolts, considerably above the lower limit of detection.

c. Elimination of Centrifugation

Since the whole procedure from extraction to assay must be completed as quickly as possible, an attempt was made to eliminate the 5-minute centrifugation in the hope that the suspended cells would not significantly interfere with the measurement of the light emitted during assay.

E. coli (10^4 cells) in 0.1 ml was extracted with 0.9 ml 90% DMSO in Tris buffer, pH 7.4. After a 5-minute extraction at room temperature, aliquots were diluted from two- to seven-fold with Tris buffer. Ten λ of each diluted material were injected into a cuvette containing 100 λ of luciferin-luciferase-magnesium. The results of ATP response measurements are shown in table III.

The best result was obtained from 5-minute extraction with 90% DMSO followed by a sixfold dilution with Tris buffer. A similar procedure was repeated to extract the same number of E. coli cells for a total of 15 seconds followed by sixfold dilution with Tris buffer. The ATP response was 172.5 mv, a value very close to that from the 5-minute extraction - 180 mv (see table III). Therefore, it was concluded that the ATP was extracted almost immediately upon addition of 90% DMSO to a cell suspension and the cells did not prevent detection with the assay when DMSO diluted to 13.5 (sixfold dilution) gave the best response.

5. STANDARD PROCEDURES

a. Cultivation and Preparation of Cells for Assay

Table IV lists culture media and conditions for growing the different types of tested organisms. All were grown either on broth or on agar. With the exception of Chlorella and Tetrahymena, inocula for broth cultures were grown under optimal conditions for 24 hours. One-tenth ml was then transferred to fresh broth medium (25 ml) in a nephelo culture

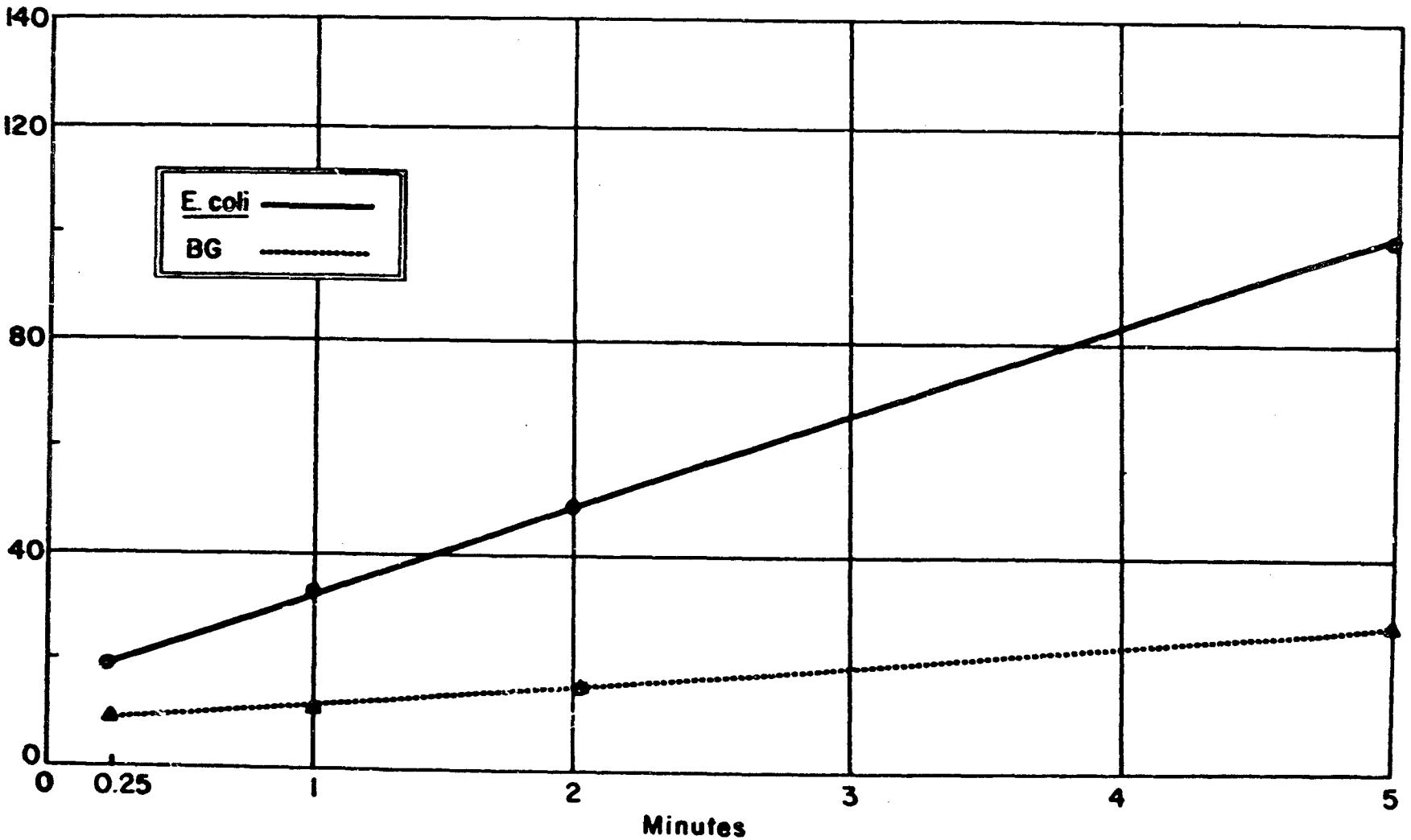


Figure 4 – Effect of time on extraction of ATP from E. coli (7×10^3 cells) and BG (3×10^3 cells) with 90% DMSO in 0.05M tris buffer (pH 7.4) at room temperature. This response is for smaller numbers of organisms than shown in figure 3.

Table III. ATP Response Affected By Various Dilutions With Tris Buffer On DMSO Extraction Of *E. coli* (100 Cells As Extrapolated From OD Curve).

DMSO extraction period	Dilution factor	Resulting percent DMSO	ATP response (mv)
5 minutes	2	40.5	0
5 minutes	3	27.0	66
5 minutes	4	20.3	92
5 minutes	5	16.2	135
5 minutes	6	13.5	180
5 minutes	7	11.6	115
15 seconds	6	13.5	172.5

Calibration of enzyme preparation: 10^{-2} γ ATP = 27,750 mv
Blank Tris buffer = 80 mv

Table IV. Cell Characteristics And Culture Systems For Test Organisms.

Test organisms	Morphology	Size (μ)	Preparation of test cultures: Media*	Inocula (hr)	Test culture (hr)	Approximate density (cells/ml)	Growth conditions	Cell count** Plate	Chamber	Oxygen requirements	Energy source
Escherichia coli (a)	Coccoid to rods	0.5 x 1-2	TSB	24	5	10^9	37° C (shaker)	TSA	PH	Facultative anaerobic	Heterotroph
Corynebacterium striatum (a)	Pleomorphic rods	0.3-0.5 x 2.0-3.0	TSB	24	5	10^7	37° C (shaker)	TSA	PH	Aerobic	Heterotroph
Bacillus subtilis (a)	Rods	0.7-0.8 x 2.0-3.0	TSB	24	5	10^9	37° C (shaker)	TSA	PH	Aerobic	Heterotroph
Clostridium sporogenes (a)	Rods and spores	0.6-0.8 x 3-7	FTG	24	24	10^9	37° C (static)	FTGA	PH	Anaerobic	Heterotroph
Streptococcus salivarius (a)	Cocci	0.8-1	TSB	24	5	10^{10}	37° C (shaker)	TSA	PH	Facultative anaerobic	Heterotroph
Staphylococcus epidermidis (a)	Cocci	0.5-0.6	TSB	24	5	10^8	37° C (shaker)	TSA	PH	Facultative anaerobic	Heterotroph
Pseudomonas fluorescens (a)	Rods	0.3-0.5 x 1-1.8	TSB	24	6	10^7	25° C (shaker)	TSA	PH	Aerobic	Heterotroph
Thiobacillus novellus (a)	Short rod	0.4-0.8 x 0.6-1.8	STA	-	96	-	25° C	NA	PH	Aerobic	Autotroph

Table IV. Continued

Test organisms	Morphology	Size (μ)	Preparation of test cultures: Media*	Inocula (hr)	Test culture (hr)	Approximate density (cells/ml)	Growth conditions	Cell count**: Plate	Cell count**: Chamber	Oxygen requirements	Energy source
Chlorella pyrenoidosa (b)	Spherical	4-6	NG	-	48	10^7	37° C (shaker)	-	P	Aerobic	Autotroph
Saccharomyces cerevisiae (c)	Oval	8-10 x 5-7	SDB	24	5	10^7	25° C (shaker)	SDA	PH	Facultative anaerobic	Heterotroph
Aspergillus niger (c)	Conidial spore	2.5-4	SDA	-	48-72	-	25° C	SDA	PH	Aerobic	Heterotroph
Tetrahymena pyriformis (d)	Pyriform	50 x 30	TB	-	72	10^6	25° C (shaker)	-	P	Aerobic	Heterotroph

* TSB: Tryptic Soy Broth. NG: Nitrate Glucose Broth. SDB: Sabouraud Dextrose Broth. FTG: Fluid Thioglycollate Broth. TB: Tetrahymena Broth. STA: Synthetic Thiobacillus Agar.

** TSA: Tryptic Soy Agar. FTGA: Fluid Thioglycollate Agar. SDA: Sabouraud Dextrose Agar. PH: Petroff-Hausser Chamber. P: Palmer Chamber.

a: bacteria b: algae c: fungi d: protozoa

flask and incubated for 5 hours to produce assay cells. The Thiobacillus and Aspergillus cultures were grown on agar media. The Thiobacillus cells were washed and the conidial spores of Aspergillus were collected from the agar plates for assay study. Since Chlorella and Tetrahymena cultures were slower growing organisms, they were grown in broth culture for 48 and 72 hours, respectively.

The cell suspension was centrifuged at 600 x g for 10 minutes at 0° C. After decanting and discarding the supernatant, the cells were washed with 5 ml of physiological saline. The saline was removed after another 5 minutes centrifugation and the pellet was resuspended in 10 ml of saline. This material, containing approximately 10^7 cells per ml, is the working suspension for both the determination of true cell count and for the extraction of ATP.

For the accurate determination of cell count, serial dilutions in saline were made of the working cell suspension. Appropriate dilution of the cell suspension was estimated by either pour plate or spread plate technique. A chamber count was also made by either Petroff-Hausser or Palmer chambers. The chamber count served as a guide for diluting the working cell suspension to desired cell number for ATP extraction.

b. Extraction Procedures

One-tenth ml of the appropriate number of cells in suspension was extracted with 0.9 ml 90% DMSO in Tris buffer at pH 7.4 by shaking approximately ten times. Sixfold dilution was made with Tris buffer immediately after the extraction and 10 λ of the extract was used for assaying ATP. If the original cell suspension contains 10^6 cells per ml, the final 10 λ of the extract will contain 170 cells.

c. Assay Technique

One hundred λ of the reaction mixture was placed in a tube cuvette and allowed to sit for about half an hour, to be sure that the solution had attained room temperature and inherent light had decreased. The inherent light is associated with enzyme preparation. Even though the enzyme has been partially purified, it still contains residual light "generating" compound, perhaps ATP. In every assay, the bioluminescent detector was adjusted so that a zero level could be seen on the oscilloscope (or the recorder) when no light source was in front of the phototube. The cuvette containing enzyme, luciferin, and magnesium ion was then placed in front of the phototube and the response noted - but the instrument was not readjusted to zero. The value obtained at this point constituted the inherent light of the system. Ten λ of the ATP solution either from cell extract or standard ATP was then injected into the cuvette and the maximum level obtained on the oscilloscope was recorded. The response was reported as the total increase (gross response) from the initially adjusted zero level including

inherent light. The inherent light level and the net response were also reported. As will be discussed later, there was evidence that net response is the better ATP index than the gross response. The type of data sheet used for assaying the ATP standard and the various microorganisms is shown in Appendix I.

Standard ATP solutions (10^{-7} or 10^{-6} γ to 10^{-3} γ) of at least three concentrations were assayed on each experimental day so that the unknown quantity of ATP in tested cells could be extrapolated from the curve. A typical standard curve in net response (mv) vs concentration of ATP is shown in figure 5. The linearity and slope of the curve are reproducible; however, due mainly to variation in enzyme activity, inherent light, etc., the curve shifts slightly from day to day. Therefore instead of mv response, the results are reported in γ of ATP extrapolated from a standard curve (mv vs ATP). Both features, linearity and parallelism of the curves, permit the use of a single point (or one ATP standard), if necessary, to establish a standard curve; this may be highly practical for space systems.

6. COMPLETED STUDIES ON TWELVE MICROORGANISMS

Tables V through XVI represent ATP assays from 12 stipulated microorganisms. At least four cultures at two levels of cells from each organism have been tested. The cell numbers by chamber count and corresponding plate counts were included and arranged in decreasing order based upon the chamber counts. The ATP net responses in millivolts represented gross response less inherent light. These values were converted to γ of ATP using the standard curve run on each experimental date. ATP content in γ was shown on the tables to indicate the net response values.

In determining the amount of ATP per cell the chamber counts were higher than the plate counts, as predicted. This was particularly so in the case of the spore former, Clostridium sporogenes. The ratio between plate and chamber count for this organism was 1 to 10 or 20. Generally, the number of cells was related to the amount of ATP. The lowest number of cells listed in each table produced a significant response.

The ATP content, in γ per cell, has been calculated on the basis of plate count as well as chamber count at each run. The order of magnitude for these values (based on either plate or chamber count) was, with but a few exceptions, consistent within each organism. The individual values (8 to 10 runs) from each organism were averaged and standard deviations were determined within the samples for each species. The average values are summarized in table XVII. The list of the organisms has been arranged in the order of increasing cell size - the smallest, Staphylococcus epidermidis, being the first and the largest, Tetrahymena pyriformis, the last (table XVII).

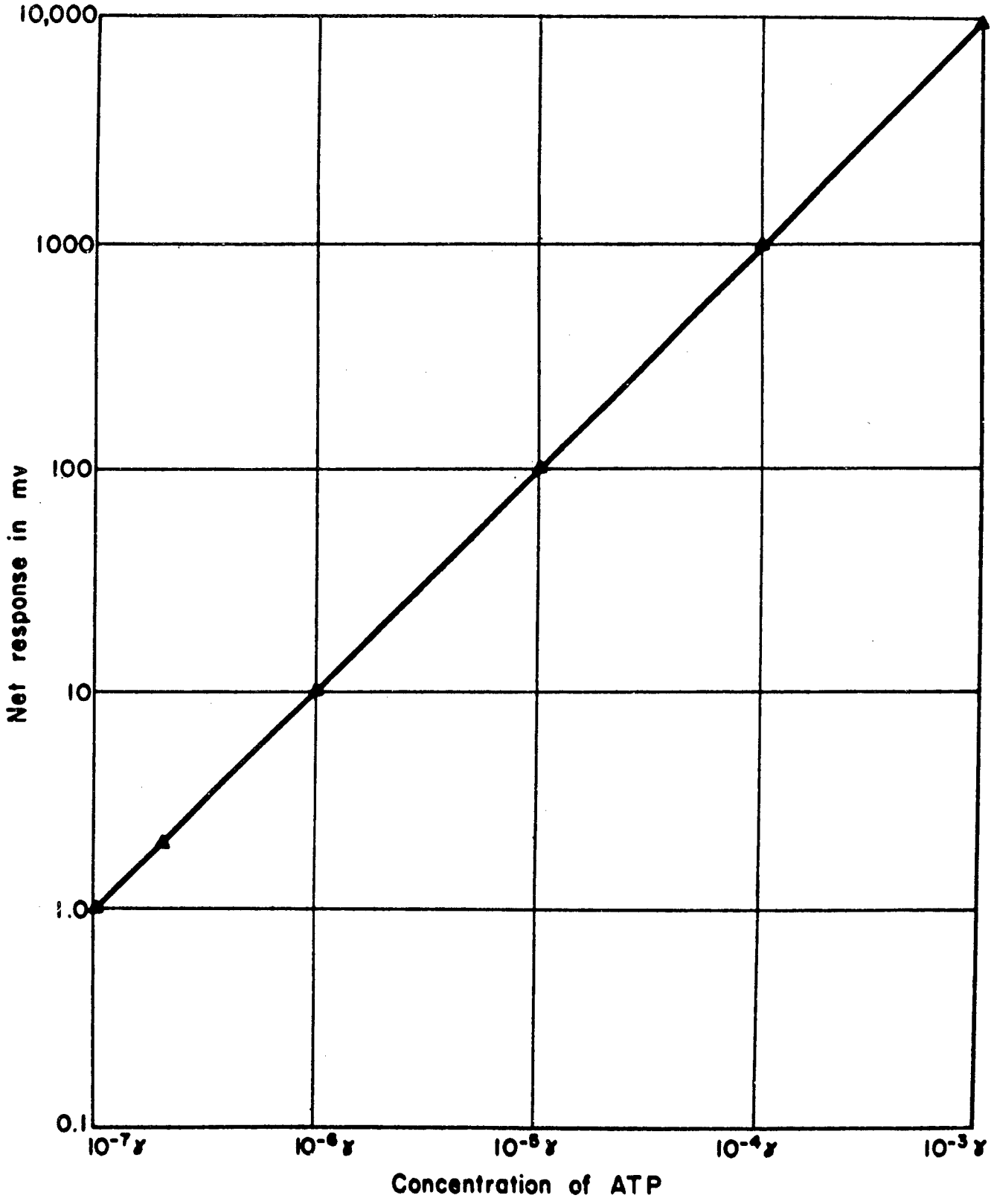


Figure 5 - Standard ATP curve on Bioluminescent detector.

Table V. ATP Response From Staphylococcus epidermidis (0.5 - 0.6 μ*)

Experiment	No. cells tested		ATP	ATP (γ/cell)	
	Plate count	Chamber count	(γ)	By plate count	By chamber count
(1)	20,000	35,000	9.4×10^{-6}	4.7×10^{-10}	2.7×10^{-10}
(2)	7,700	20,000	1.0×10^{-6}	1.3×10^{-10}	0.5×10^{-10}
(4)	3,800	7,300	1.4×10^{-6}	3.7×10^{-10}	1.9×10^{-10}
(1)	2,000	3,500	9.5×10^{-7}	4.8×10^{-10}	2.7×10^{-10}
(3)	1,000	1,700	4.3×10^{-7}	4.3×10^{-10}	2.5×10^{-10}
(2)	770	2,000	1.1×10^{-7}	1.3×10^{-10}	0.5×10^{-10}
(4)	380	730	2.0×10^{-7}	5.3×10^{-10}	2.7×10^{-10}
(3)	100	170	7.1×10^{-8}	7.1×10^{-10}	4.2×10^{-10}
			Average	4.1×10^{-10}	2.2×10^{-10}
			Standard Deviation	2.0×10^{-10}	1.2×10^{-10}

* Cell size of organism

Table VI. ATP Response From Pseudomonas fluorescens (0.3 - 0.5 x 1 - 1.8 μ).

Experiment	No. cells tested		ATP	ATP (γ/cell)	
	Plate count	Chamber count	(γ)	By plate count	By chamber count
(3)	1700	6600	3.0×10^{-6}	1.7×10^{-9}	4.4×10^{-10}
(4)	3200	6600	1.4×10^{-6}	0.4×10^{-9}	2.1×10^{-10}
(5)	470	3000	8.3×10^{-7}	1.8×10^{-9}	2.8×10^{-10}
(1)	850	1700	1.5×10^{-6}	1.8×10^{-9}	8.8×10^{-10}
(2)	500	1000	6.1×10^{-7}	1.2×10^{-9}	6.0×10^{-10}
(3)	170	660	3.3×10^{-7}	2.0×10^{-9}	5.2×10^{-10}
(4)	320	660	1.4×10^{-7}	0.4×10^{-9}	2.1×10^{-10}
(1)	35	170	2.6×10^{-7}	3.1×10^{-9}	15×10^{-10}
(5)	47	300	6.5×10^{-8}	1.4×10^{-9}	2.2×10^{-10}
(2)	50	100	9.5×10^{-8}	2.0×10^{-9}	10×10^{-10}
			Average	1.6×10^{-9}	5.9×10^{-10}
			Standard Deviation	0.8×10^{-9}	4.3×10^{-10}

Table VII. ATP Response From Streptococcus salivarius (0.8 - 1.0 μ).

Experiment	No. cells tested		ATP (γ)	ATP (γ/cell)	
	Plate count	Chamber count		By plate count	By chamber count
(2)	5000	10,000	4.5×10^{-6}	0.9×10^{-9}	4.5×10^{-10}
(1)	3700	7,300	4.2×10^{-6}	1.1×10^{-9}	5.8×10^{-10}
(3)	3500	7,000	4.5×10^{-6}	1.3×10^{-9}	6.4×10^{-10}
(5)	2000	4,000	1.5×10^{-6}	0.8×10^{-9}	3.8×10^{-10}
(4)	1700	3,300	1.7×10^{-6}	1.0×10^{-9}	5.1×10^{-10}
(2)	500	1,000	5.3×10^{-7}	1.1×10^{-9}	5.3×10^{-10}
(1)	370	730	4.3×10^{-7}	1.2×10^{-9}	5.9×10^{-10}
(3)	350	700	5.2×10^{-7}	1.5×10^{-9}	7.4×10^{-10}
(5)	200	400	2.8×10^{-7}	1.4×10^{-9}	7.0×10^{-10}
(4)	170	330	No response	-	-
			Average	1.1×10^{-9}	$5.[illegible] \times 10^{-10}$
			Standard Deviation	0.2×10^{-9}	1.2×10^{-10}

Table VIII. ATP Response From Escherichia coli (0.5 x 1 - 2 μ).

Experiment	No. cells tested		ATP	ATP (γ/cell)	
	Plate count	Chamber count	(γ)	By plate count	By chamber count
(3)	6000	6000	5.2×10^{-6}	8.7×10^{-10}	8.7×10^{-10}
(4)	4100	4100	1.8×10^{-6}	3.9×10^{-10}	3.9×10^{-10}
(2)	4000	4000	1.0×10^{-6}	2.5×10^{-10}	2.5×10^{-10}
(1)	3000	2700	2.1×10^{-6}	7.0×10^{-10}	7.8×10^{-10}
(3)	600	600	5.3×10^{-7}	8.8×10^{-10}	8.8×10^{-10}
(4)	410	410	1.4×10^{-7}	3.4×10^{-10}	3.4×10^{-10}
(2)	400	400	8.5×10^{-8}	2.1×10^{-10}	2.1×10^{-10}
(1)	300	270	2.6×10^{-7}	8.7×10^{-10}	9.6×10^{-10}
			Average	5.6×10^{-10}	5.8×10^{-10}
			Standard Deviation	3.0×10^{-10}	3.2×10^{-10}

Table IX. ATP Response From Thiobacillus novellus (0.4 - 0.8 x 0.6 - 1.8 μ).

Experiment	No. cells tested		ATP (γ)	ATP (γ/cell)	
	Plate count	Chamber count		By plate count	By chamber count
(1)	3300	3250	6.0×10^{-7}	1.8×10^{-10}	1.8×10^{-10}
(4)	670	1400	7.6×10^{-7}	1.1×10^{-9}	5.4×10^{-10}
(3)	420	1200	5.8×10^{-7}	1.4×10^{-9}	4.8×10^{-10}
(2)	334	830	4.2×10^{-7}	1.3×10^{-9}	5.0×10^{-10}
(1)	330	325	7×10^{-8}	2.1×10^{-10}	2.1×10^{-10}
(4)	67	140	8.0×10^{-8}	1.2×10^{-9}	5.7×10^{-10}
(3)	42	120	6.0×10^{-8}	1.4×10^{-9}	5.0×10^{-10}
(2)	33.4	83.0	4.0×10^{-8}	1.2×10^{-9}	4.8×10^{-10}
(1)	33	32.5	6×10^{-9}	1.8×10^{-10}	1.8×10^{-10}
(4)	6.7	14	-	-	-
(3)	4.0	12	-	-	-
(2)	3.4	8.3	-	-	-
			Average	9.1×10^{-10}	4.0×10^{-10}
			Standard Deviation	5.5×10^{-10}	1.6×10^{-10}

Table X. ATP Response From Bacillus subtilis (0.7 - 0.8 x 2.0 - 3.0 μ).

Experiment	No. cells tested		ATP	ATP (γ/cell)	
	Plate count	Chamber count	(γ)	By plate count	By chamber count
(5)	11,000	11,000	4.4×10^{-5}	4.0×10^{-9}	4.0×10^{-9}
(3)	4,150	8,300	2.0×10^{-5}	4.8×10^{-9}	2.4×10^{-9}
(2)	7,700	7,700	1.1×10^{-5}	1.4×10^{-9}	1.4×10^{-9}
(4)	2,850	5,700	8.6×10^{-6}	3.0×10^{-9}	1.5×10^{-9}
(1)	3,000	3,000	8.9×10^{-6}	3.0×10^{-9}	3.0×10^{-9}
(5)	1,100	1,100	4.3×10^{-6}	3.9×10^{-9}	3.9×10^{-9}
(3)	415	830	1.8×10^{-6}	4.2×10^{-9}	2.1×10^{-9}
(2)	770	770	1.1×10^{-6}	1.4×10^{-9}	1.4×10^{-9}
(4)	285	570	9.2×10^{-7}	3.2×10^{-9}	1.6×10^{-9}
(1)	300	300	7.6×10^{-7}	2.5×10^{-9}	2.5×10^{-9}
			Average	3.1×10^{-9}	2.4×10^{-9}
			Standard Deviation	1.1×10^{-9}	1.0×10^{-9}

Table XI. ATP Response From Corynebacterium striatum (0.3 - 0.5 x 2.0 - 3.0 μ).

Experiment	No. cells tested		ATP	ATP (γ/cell)	
	Plate count	Chamber count	(γ)	By plate count	By chamber count
(4)	550	1100	4.3×10^{-6}	7.8×10^{-9}	3.9×10^{-9}
(2)	350	700	2.0×10^{-6}	5.7×10^{-9}	2.9×10^{-9}
(1)	325	650	6.3×10^{-6}	19.0×10^{-9}	9.7×10^{-9}
(5)	265	530	8.0×10^{-7}	3.0×10^{-9}	1.5×10^{-9}
(4)	55	110	5.2×10^{-7}	9.5×10^{-9}	4.7×10^{-9}
(3)	50	100	1.8×10^{-7}	3.6×10^{-9}	1.8×10^{-9}
(2)	35	70	2.0×10^{-7}	5.7×10^{-9}	2.9×10^{-9}
(1)	33	65	1.0×10^{-6}	30.0×10^{-9}	15.0×10^{-9}
(5)	27	53	8.5×10^{-8}	3.2×10^{-9}	1.6×10^{-9}
(3)	5	10	No response	-	-
			Average	9.7×10^{-9}	4.9×10^{-9}
			Standard Deviation	9.1×10^{-9}	4.6×10^{-9}

Table XII. ATP Response From Clostridium sporogenes (0.6 - 0.8 x 3.0 - 7.0 μ).

Experiment	No. cells tested		ATP	ATP (γ/cell)	
	Plate count	Chamber count	(γ)	By plate count	By chamber count
(3)	4000	50,000	1.3×10^{-5}	3.3×10^{-9}	2.6×10^{-10}
(4)	4700	45,000	4.5×10^{-6}	1.0×10^{-9}	1.0×10^{-10}
(2)	4500	38,000	9.0×10^{-6}	2.0×10^{-9}	2.4×10^{-10}
(1)	1600	31,000	5.0×10^{-6}	3.1×10^{-9}	1.6×10^{-10}
(3)	400	5,000	1.6×10^{-6}	4.0×10^{-9}	3.2×10^{-10}
(4)	470	4,500	4.5×10^{-7}	1.0×10^{-9}	1.0×10^{-10}
(2)	450	3,800	1.0×10^{-6}	2.2×10^{-9}	2.6×10^{-10}
(1)	160	3,100	5.5×10^{-7}	3.4×10^{-9}	1.8×10^{-10}
(3)	40	500	1.6×10^{-8}	4.0×10^{-9}	3.2×10^{-10}
(4)	47	450	5.0×10^{-8}	1.1×10^{-9}	1.1×10^{-10}
(2)	45	380	1.1×10^{-7}	2.4×10^{-9}	2.9×10^{-10}
(1)	16	310	4.2×10^{-8}	3.6×10^{-9}	1.4×10^{-10}
			Average	2.5×10^{-9}	2.1×10^{-10}
			Standard Deviation	1.1×10^{-9}	0.8×10^{-10}

Table XIII. ATP Response From Conidial Spores Of Aspergillus niger (2.5 - 4 μ).

Experiment	No. cells tested		ATP	ATP (γ/cell)	
	Plate count	Chamber count	(γ)	By plate count	By chamber count
(3)	180	600	5.4×10^{-5}	3.0×10^{-7}	9.0×10^{-8}
(4)	155	500	4.1×10^{-5}	2.6×10^{-7}	8.2×10^{-8}
(1)	63	67	2.4×10^{-6}	0.4×10^{-7}	3.6×10^{-8}
(3)	18	60	5.6×10^{-6}	3.1×10^{-7}	9.3×10^{-8}
(2)	53	53	1.6×10^{-6}	0.3×10^{-7}	3.0×10^{-8}
(4)	15	50	4.1×10^{-6}	2.7×10^{-7}	8.2×10^{-8}
(1)	6.3	6.7	2.6×10^{-7}	0.4×10^{-7}	3.9×10^{-8}
(3)	1.8	6	6.0×10^{-7}	3.7×10^{-7}	10×10^{-8}
(2)	5.3	5.3	1.4×10^{-7}	0.3×10^{-7}	2.6×10^{-8}
(4)	1.5	5	4.8×10^{-7}	3.2×10^{-7}	9.6×10^{-8}
(1)	0.63	0.67	2.3×10^{-8}	0.4×10^{-7}	3.4×10^{-8}
(2)	0.53	0.53	1.4×10^{-8}	0.3×10^{-7}	3.0×10^{-8}
			Average	1.7×10^{-7}	6.1×10^{-8}
			Standard Deviation	1.4×10^{-7}	3.1×10^{-8}

Table XIV. ATP Response From Saccharomyces cerevisiae (8.0 - 10 x 5.0 - 7.0 μ).

Experiment	No. cells tested		ATP (γ)	ATP (γ/cell)	
	Plate count	Chamber count		By plate count	By chamber count
(2)	73	400	1.1×10^{-5}	1.6×10^{-7}	3×10^{-8}
(4)	48	273	5.2×10^{-6}	1.1×10^{-7}	1.9×10^{-8}
(3)	47	266	5.6×10^{-6}	1.2×10^{-7}	2.1×10^{-8}
(1)	65	220	9.1×10^{-6}	1.4×10^{-7}	4.1×10^{-8}
(2)	7.3	40	1.1×10^{-6}	1.5×10^{-7}	2.8×10^{-8}
(3)	4.7	27	5.1×10^{-7}	1.1×10^{-7}	1.9×10^{-8}
(4)	4.8	27	6.2×10^{-7}	1.3×10^{-7}	2.3×10^{-8}
(1)	6.5	22	8.9×10^{-7}	1.4×10^{-7}	4.1×10^{-8}
			Average	1.3×10^{-7}	2.8×10^{-8}
			Standard Deviation	0.19×10^{-7}	0.91×10^{-8}

Table XV. ATP Response From Chlorella pyrenoidosa (4.0 - 6.0 μ).

Experiment	No. cells tested / Chamber count	ATP (γ)	ATP (γ/cell) By chamber count
(3)	2000	4.2×10^{-5}	1.4×10^{-8}
(2)	1450	4.5×10^{-5}	3.1×10^{-8}
(4)	610	3.5×10^{-5}	5.6×10^{-8}
(5)	290	1.6×10^{-5}	5.5×10^{-8}
(3)	200	3.8×10^{-6}	1.9×10^{-8}
(2)	145	4.8×10^{-6}	3.3×10^{-8}
(1)	91	4.2×10^{-6}	4.6×10^{-8}
(4)	61	4.2×10^{-6}	6.9×10^{-8}
(5)	29	1.5×10^{-6}	5.2×10^{-8}
(3)	20	4.2×10^{-7}	2.1×10^{-8}
(2)	14.5	5.2×10^{-7}	3.6×10^{-8}
(1)	9.1	5.0×10^{-7}	5.5×10^{-8}
(4)	6.1	4×10^{-7}	6.6×10^{-8}
(5)	2.9	1.5×10^{-7}	5.2×10^{-8}
(1)	0.9	9.5×10^{-8}	11×10^{-8}
(4)	0.6	0.5×10^{-7}	8.3×10^{-8}
(5)	0.3	No response	-
		Average	5.0×10^{-8}
		Standard Deviation	2.5×10^{-8}

Table XVI. ATP Response From Tetrahymena pyriformis (30 - 50 μ).

Experiment	No. cells tested Chamber count	ATP (γ)	ATP (γ/cell) By chamber count
(1)	11.4	2.2×10^{-5}	1.9×10^{-6}
(3)	8.7	2.0×10^{-5}	2.3×10^{-6}
(2)	6.8	2.0×10^{-5}	2.9×10^{-6}
(4)	6.2	1.8×10^{-5}	2.9×10^{-6}
(1)	1.1	3.8×10^{-6}	3.4×10^{-6}
(3)	0.9	2.1×10^{-6}	2.3×10^{-6}
(2)	0.7	2.8×10^{-6}	4.0×10^{-6}
(4)	0.6	1.5×10^{-6}	2.5×10^{-6}
		Average	2.8×10^{-6}
		Standard Deviation	0.68×10^{-6}

Table XVII. Summarized Results Of ATP Response By Lowest Number Of Cells Tested And Average ATP (γ) Per Cell.

Test organisms	Cell size (μ)	Responsive minimum number of cells tested: Cell counts: Plate count	Responsive minimum number of cells tested: Cell counts: Chamber count	ATP (γ)	Average ATP (γ) per cell*: Plate count	Average ATP (γ) per cell*: Chamber count
Staphylococcus epidermidis	0.5 - 0.6	100	170	7.1×10^{-8}	4.1×10^{-10}	2.2×10^{-10}
Pseudomonas fluorescens	0.3 - 0.5 x 1 - 1.8	50	100	9.5×10^{-8}	1.6×10^{-9}	5.9×10^{-10}
Streptococcus salivarius	0.8 - 1	200	400	2.8×10^{-7}	1.1×10^{-9}	5.7×10^{-10}
Escherichia coli	0.5 x 1 - 2	300	270	2.6×10^{-7}	5.6×10^{-10}	5.8×10^{-10}
Thiobacillus novellus	0.4 - 0.8 x 0.6 - 1.8	33	32.5	6.0×10^{-9}	9.1×10^{-10}	4.0×10^{-10}
Bacillus subtilis	0.7 - 0.8 x 2 - 3	300	300	7.6×10^{-7}	3.1×10^{-9}	2.4×10^{-9}
Corynebacterium striatum	0.3 - 0.5 x 2 - 3	27	53	8.5×10^{-8}	9.7×10^{-9}	4.9×10^{-9}
Clostridium sporogenes	0.6 - 0.8 x 3 - 7	16	310	4.2×10^{-8}	2.5×10^{-9}	2.1×10^{-10}
Saccharomyces cerevisiae	8 - 10 x 5 - 7	4.7	27	5.1×10^{-7}	1.3×10^{-7}	2.8×10^{-8}
Aspergillus niger	2.5 - 4	0.53	0.53	1.4×10^{-8}	1.7×10^{-7}	6.1×10^{-8}
Chlorella pyrenoidosa	4 - 6	-	0.6	5.0×10^{-8}	-	5.0×10^{-8}
Tetrahymena pyriformis	30 - 50	-	0.6	1.5×10^{-6}	-	2.8×10^{-6}

* Average for all counts of 8-12 tests per organism

ATP content per cell generally correlated with the size of the tested organism. Even more surprising and significant from the standpoint of the objective of this project, however, is the relatively small variation between bacterial species in the per cell content of ATP. The mean ATP content per cell for bacteria varied between species only slightly more than one order of magnitude. This was reflected in the statistical analysis of variation in ATP per cell within each bacterial species, as well as among bacterial species. The mean ($\bar{X}$), standard deviation (S), and coefficient of variation ($S/\bar{X}$) based on plate count were found to be as follows:

	$\bar{X} \pm S$ ($\times 10^{-10}$)	Coefficient of Var. ($S/\bar{X}$) %
S. epidermidis	4.1 ± 2.0	49
E. coli	5.6 ± 3.0	54
T. novellus	9.1 ± 5.5	60
S. salivarius	11.0 ± 2.0	18
P. fluorescens	16.0 ± 8.0	50
C. sporogenes	26.0 ± 11.0	42
B. subtilis	31.0 ± 11.0	35
C. striatum	97.0 ± 91.0	94
All the above species	25.0 ± 93.0	370
All the above species except C. striatum	14.7 ± 18.8	128

The coefficient of variation for the individual organisms ranged from a low of 18% in S. salivarius to a high of 94% in C. striatum. The pleomorphic cell structure of the latter organism was believed to be the cause for such a high coefficient of variation.

The mean, standard deviation and coefficient of variation among all the bacterial species tested were calculated and listed above with and without C. striatum. The variation was reduced to one order of magnitude ($S = 18.8$). Because the C. striatum possesses such a unique cell structure, quantitation of this organism as a basis for comparison of the ATP assay with that of other organisms may be especially inaccurate by conventional counting techniques.

Based on chamber count, the mean value of ATP (γ) per cell in Staphylococcus epidermidis (0.5 - 0.6 micron) was $2.2 \times 10^{-10}\ \gamma$; the Bacillus subtilis (0.7 - 0.8 x 2-3 micron), 2.4×10^{-9}; and the Tetrahymena pyriformis (30 to 50 micron), 2.8×10^{-6}. Clostridium sporogenes, however, did not follow in line. Despite the cell being larger, the ATP per cell was smaller than in Corynebacterium. The Clostridium is an anaerobe and the fact that the fermentative pathway produces less ATP per molecule of

carbohydrate consumed may account for the difference. Also, the cell population included a considerable number of spores which may contain less ATP than the vegetative cells.

Despite the fact that autotrophic Thiobacillus novellus was grown in a strict autotrophic medium, the ATP content in this organism was in line with the other heterotrophs in regard to the relationship between cell size and relative ATP content.

Because of difficulty in quantitating mycelium for the fungi Aspergillus, the conidial spores instead of mycelium have been selected for ATP determination. More ATP is expected to be found in the mycelium than in the spores. Nevertheless, the conidia did contain considerable amount of ATP (6.1×10^{-8} γ per spore by chamber count), the value being comparable to vegetative cells of Saccharomyces cerevisiae. This organism is also a fungi, but it can be quantified.

The Chlorella, on the other hand, was smaller in size than the Saccharomyces, yet it contained more ATP per cell than the latter. This is possible because the Chlorella is a photosynthetic organism. The ATP generating mechanism is different from that of yeast and other heterotrophic organisms (ref. 7).

The net ATP response from the minimum numbers of cells tested for each organism is included in table XVII. These values are not the smallest number of cells detectable, but merely the smallest numbers tested. In most cases, the responses were still considerably higher than the smallest levels detectable at the highest sensitivity setting of the instrument. Thus, there is little doubt that the minimal detectable cell number for most of the organisms tested is 100 or less.

7. COMPARISON OF NET AND GROSS RESPONSE AS AN INDEX FOR ATP

As described in "ASSAY TECHNIQUE " (p. 23), the ATP response can be expressed in two magnitudes, i.e., gross and net responses. The difference between these two responses is the inherent light. Inherent light is a background light observed when a cuvette is loaded with the reaction mixture and placed in front of the photomultiplier without adding ATP. The gross response then represents the total light response from the initially adjusted zero reading of the instrument caused by the inherent light and the ATP reaction. The net response is the gross response less inherent light. No evidence to the effect that the two types of light were truly additive had been obtained heretofore. Thus, the question as to which response is a better ATP index had to be studied. Since the inherent light, and consequently the gross response, showed a higher variability than the net response, the latter was thought to be a better measure of ATP content. Furthermore, the net response was always found to be log linear,

i.e., a straight line when plotted on log log paper, while the gross response was curvilinear on log log paper. This characteristic of net response simplified calibration.

As mentioned earlier, a standard dose-response curve constructed from three to four points with ATP concentrations ranging from 10^{-7} to 10^{-3} γ was made at each experimental date. Even though the absolute values of the responses for the standard curves were different from day to day, their shape and slope were preserved. The preservation of shape and slope was found to exist throughout all 21 experiments. As an exercise to demonstrate the qualities of shape and slope, the net and gross responses were adjusted, compared, and then averaged. The resulting mean values were then tabulated in table XVIII and plotted in figure 6. This procedure has been used only to show the consistency of the qualitative characteristics from day to day during the research and is not part of the routine assay procedure. Table XVIII represents summarized data of 21 standard curves made at different dates. The original data are shown in the "N" (non-adjusted) columns. The values at 10^{-6} γ ATP for net response ranged from 1.8 mv (Experiment No. 4) to 27 mv (No. 11), and for gross response, from 10 mv (No. 2) to 63 mv (No. 7). Normalization of the data can be accomplished by using the mean values of all 21 experiments. Because of convenience, however, the values in experiment No. 20 were selected for this exercise since they did not alter the characteristics of the analysis. The factors which were used to adjust net and gross response to 11 and 18 mv, respectively, at 10^{-6} γ ATP dosage were used to adjust the net and gross responses at 10^{-7}, 10^{-5}, 10^{-4}, and 10^{-3} γ ATP concentration. The correction factors for each response on each experiment are also included in the table. The adjusted values are listed under the columns labeled "A." Figure 6 shows the dose response curve of these adjusted values vs ATP concentration. The shape of the adjusted net and gross response curves as seen in figure 6 is similar to that seen on a day-to-day basis. Therefore, this exercise (normalization of the data) validates the choice of the net response as the index of ATP content.

8. ATP CONTENT IN VARIOUS GROWTH PHASES OF MICROORGANISMS

Estimation of the amount of ATP in cells of 12 studied organisms has been done only in cells of the log growth phase. Although literature (ref. 8) has indicated that ATP levels in microbial cells are rather stable, some studies to verify this observation were conducted. E. coli and S. cerevisiae cultures were selected for the test. One-tenth ml of the 16-hour cultures of E. coli in TSB and the 24-hour cultures of S. cerevisiae in SDB were inoculated into 25 ml of the appropriate growth media in nephelo culture flasks. The former was incubated at 37° C and the latter, at 30° C on the shaker bath. After the desired time interval, the cell density (optical density) was determined on the Klett photometer. The OD vs time curves were constructed for both organisms and are shown in

Table XVIII. Comparison Of Adjusted And Non-Adjusted Net And Gross Responses As ATP Index.

Experiment	Correction factor		10^{-7} γ ATP				10^{-6} γ ATP				10^{-5} γ ATP				10^{-4} γ ATP				10^{-3} γ ATP			
	Net	Gross	Net		Gross		Net		Gross		Net		Gross		Net		Gross		Net		Gross	
			A*	N*	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N
1	5.50	1.52					11	2.0	18	12	115.5	21	45	30	1265	230	364	240	13,800	2,500	3,690	2,500
2	3.15	1.82					11	3.5	18	10	110	35	75	41	1145	365	672	370	11,450	3,650	6,630	3,650
3	4.40	1.33					11	2.5	18	13.8	106	24	40	30.5	1035	235	305	235	11,000	2,500	3,250	2,500
4	6.05	1.10					11	1.8	18	16.3	116	19	35	31.5	1160	190	220	200	12,500	2,050	2,250	2,050
5	5.50	1.16					11	2.0	18	15.5	104.5	19	34	29.5	1130	205	238	205	13,800	2,500	2,900	2,500
6	2.75	0.93					11	4.0	18	19.5	110	40	48	51.5	1100	400	370	400	11,000	4,000	3,700	4,000
7	0.79	0.29					11	14	18	63	111	155	56	195	1220	1550	456	1550	11,200	14,250	4,200	14,250
8	5.50	0.84					11	2.0	18	21.5	107.5	19.5	27	32	1075	195	164	195	10,600	2,100	7,600	2,100
9	2.20	0.65					11	5.0	18	28	94.5	43	43	67	945	430	290	450	9,600	4,350	2,800	4,350
10	0.85	0.52					11	13	18	35	89	105	64	125	1060	1250	645	1250	10,800	12,700	6,550	12,700
11	0.41	0.46					11	27	18	39	111	273	134	290	1100	2700	1240	2700	11,400	28,000	13,000	28,000

Table XVIII. Continued.

Experiment	Correction factor		10^{-7} γATP				10^{-6} γATP				10^{-5} γATP				10^{-4} γATP				10^{-3} γATP			
	Net	Gross	Net		Gross		Net		Gross		Net		Gross		Net		Gross		Net		Gross	
			A*	N*	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N
12	0.48	0.45					11	23	18	40	110	230	112	250	1080	2250	1010	2250	11,200	23,500	10,600	23,500
13	1.05	0.80					11	10.5	18	22.5	108	103	91	114	1020	980	783	980	11,700	11,200	8,950	11,200
14	1.05	0.76					11	10.5	18	23.5	97.5	93	83	108	1040	990	756	990	11,300	10,700	8,200	10,700
15	0.54	0.41	1.2	2.3	12.4	28	11	20.5	18	44	108	200	94	230	995	1850	760	1850	10,200	19,000	7,800	19,000
16	0.85	0.64	1.1	1.3	10.3	16	11	13	18	28	92	109	79	122	990	1170	756	1170	10,200	12,000	7,750	12,000
17	0.51	0.60	1.0	2.0	7.5	12.5	11	21.5	18	30	102	200	112	210	1085	2125	1138	2125	10,500	20,500	12,400	20,500
18	0.79	0.41	1.2	1.5	7.4	18	11	14	18	44	122	155	68	165	1280	1625	666	1625	12,600	16,000	6,557	16,000
19	0.85	0.52	1.3	1.5	12.4	24	11	13	18	35	106	125	75	145.5	1035	1220	640	1240	11,400	13,500	7,000	13,500
20	1.00	1.00	1.5	1.5	9.0	9	11	11	18	18	91	91	98	98	1070	1070	1070	1070	11,100	11,100	11,100	11,100
21	1.00	0.57	1.0	1.0	13.4	23.5	11	11	18	31.5	102	102	69	121	1010	1010	578	1010	10,700	10,700	6,100	10,700
Average	2.15	0.80	1.16		10.3		11		18		105.4		70.6		1088		625		11,336		6,812	

* A = Adjusted response
N = Nonadjusted response

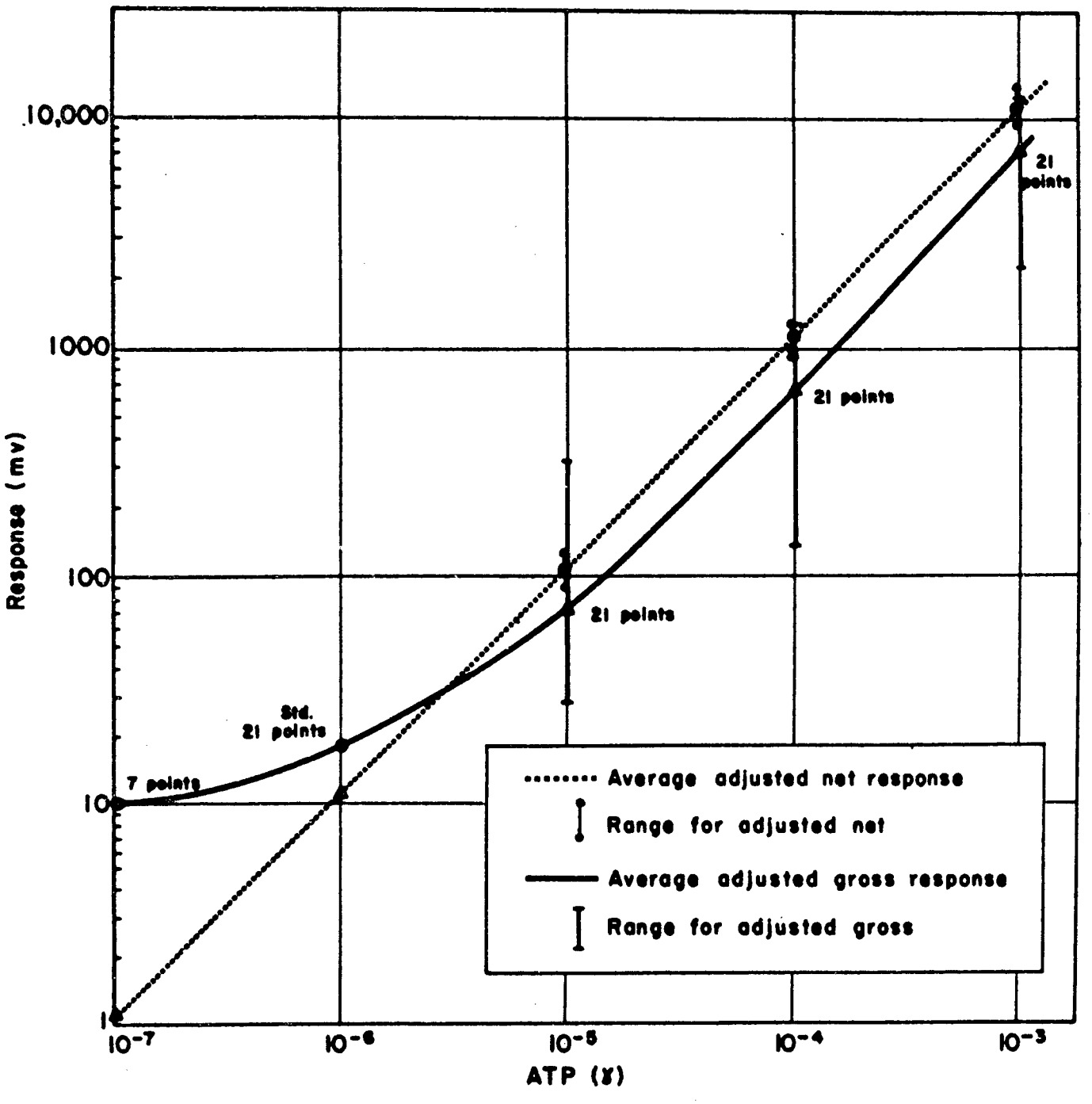


Figure 6 — Adjusted response versus ATP standards of all experiments. Each point is a calculated mean value (see text).

figures 7 and 8. The ATP determinations were made at the points of growth phase indicated on the figures and the results are shown in table XIX for E. coli and in table XX for S. cerevisiae.

The ATP in γ per cell, based on chamber counts for E. coli at various phases (table XIX) ranged from 5.4×10^{-11} in the stationary phase to 6.6×10^{-10} in the acceleration phase. Since the cells in the stationary phase are composed of old and dead cells, they were lowest in ATP content. There was approximately a factor of 10 difference in ATP content per cell between the two phases.

A similar observation was also made in S. cerevisiae cultures when the γ of ATP per cell in the stationary phase was 7×10^{-8}, while in the log phase the value was 2.5×10^{-7}, over three times higher than the former value (table XX).

In addition to growth phase studies, a pilot study was undertaken to compare the ATP content of viable and dead cells (formaldehyde-treated). Preliminary results indicate that no appreciable amount of ATP was detected in the dead cells of E. coli and S. cerevisiae. Further work is necessary, however, to confirm this observation.

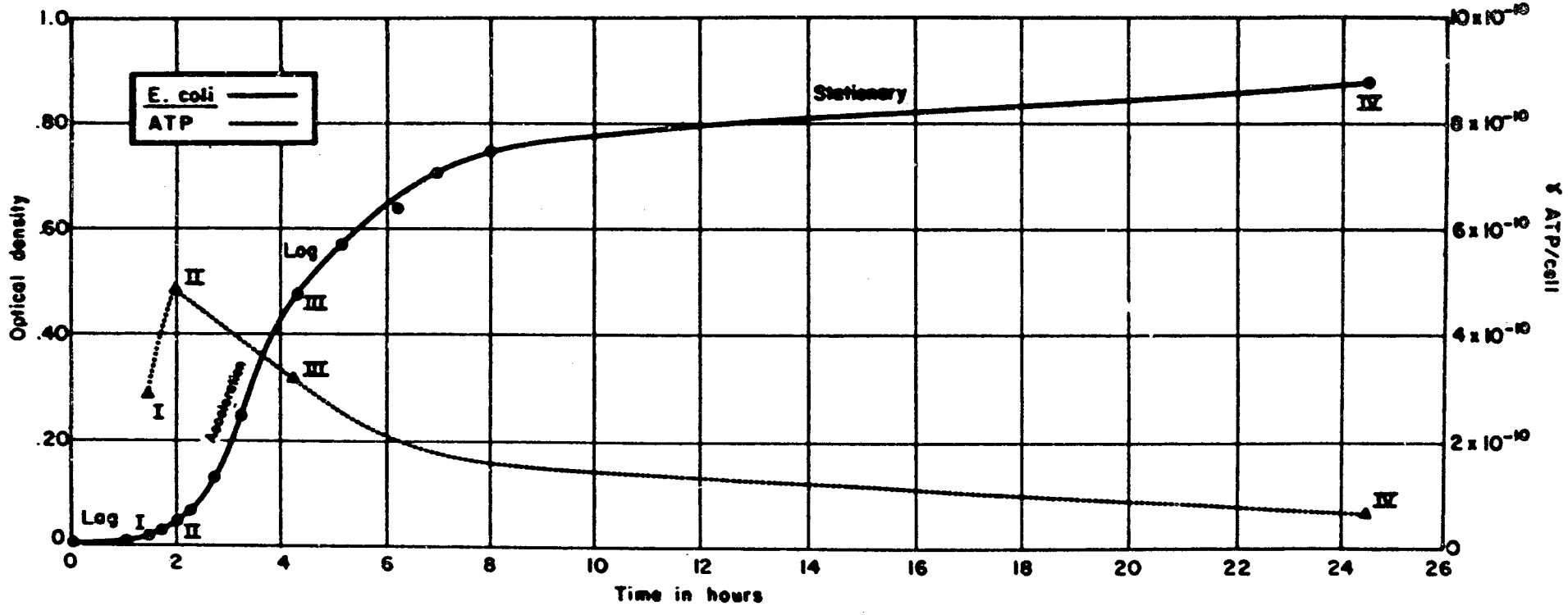


Figure 7 – Growth curve of E. coli and ATP determination at various growth phases

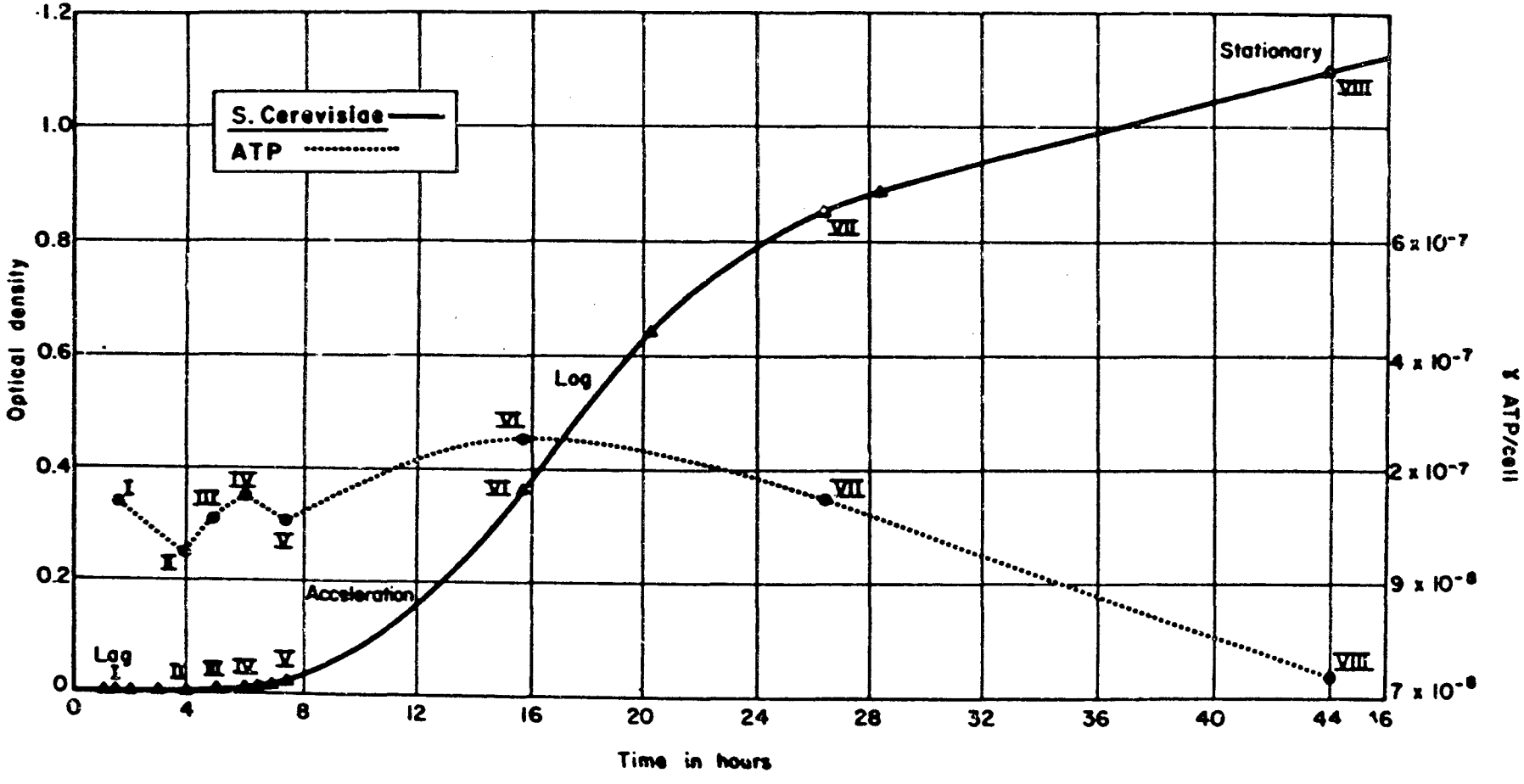


Figure 8 – Growth curve of S. Cerevisiae and ATP determination at various growth phases

Table XIX. ATP Content Of E. coli Grown At Various Growth Phases.

Growth phase*	Cell no. in 10 λ sample		Net response (mv)	ATP (γ)	ATP (γ per cell)	
	Plate	Chamber			Plate count	Chamber count
Lag (I)	2×10^{3}	3.2×10^{3}	8	7.9×10^{-7}	4.0×10^{-10}	2.5×10^{-10}
	2×10^{2}	3.2×10^{2}	1	1.0×10^{-7}	5.0×10^{-10}	3.1×10^{-10}
	2×10^{1}	3.2×10^{1}	0	-	-	-
Acceler-ation (II)	1.4×10^{4}	1.5×10^{4}	75	7.3×10^{-6}	5.2×10^{-10}	4.9×10^{-10}
	1.4×10^{3}	1.5×10^{3}	7	6.9×10^{-7}	4.9×10^{-10}	4.6×10^{-10}
	1.4×10^{2}	1.5×10^{2}	1	1.0×10^{-7}	7.1×10^{-10}	6.6×10^{-10}
Log (III)	6.3×10^{3}	6.3×10^{3}	18.8	1.9×10^{-6}	3.0×10^{-10}	3.0×10^{-10}
	6.3×10^{2}	6.3×10^{2}	2.0	2.0×10^{-7}	3.2×10^{-10}	3.2×10^{-10}
	6.3×10^{1}	6.3×10^{1}	0	-	-	-
Station-ary (IV)	1.36×10^{4}	1.28×10^{4}	8	6.9×10^{-7}	5.1×10^{-11}	5.4×10^{-11}
	1.36×10^{3}	1.28×10^{3}	1	1.0×10^{-7}	7.2×10^{-11}	7.8×10^{-11}
	1.36×10^{2}	1.28×10^{2}	0	-	-	-

* See Microbiology, M. J. Pelczar and R. D. Reid, p. 69. McGraw-Hill, 1958.

Table XX. ATP Content Of *Saccharomyces cerevisiae* Grown At Various Growth Phases.

Growth phase*	Cell no. in 10 λ sample		Net response (mv)	ATP (γ)	ATP (γ/cell)	
	Plate	Chamber			Plate count	Chamber count
Lag (I)	107	134	124	1.9×10^{-5}	1.8×10^{-7}	1.4×10^{-7}
	10.7	13.4	11	1.9×10^{-6}	1.8×10^{-7}	1.4×10^{-7}
	1.1	1.3	1.25	1.9×10^{-7}	1.8×10^{-7}	1.4×10^{-7}
Lag (II)	380	350	200	3.3×10^{-5}	8.7×10^{-8}	9.5×10^{-8}
	38	35	21	3.4×10^{-6}	9.0×10^{-8}	9.8×10^{-8}
	3.8	3.5	2.0	3.2×10^{-7}	8.4×10^{-8}	9.2×10^{-8}
Lag (III)	475	530	345	5.6×10^{-5}	1.2×10^{-7}	1.1×10^{-7}
	47.5	53	31	5.0×10^{-6}	1.1×10^{-7}	9.4×10^{-8}
	4.75	5.3	3.5	5.6×10^{-7}	1.2×10^{-7}	1.1×10^{-7}
Lag (IV)	41.7	50	47	7.6×10^{-6}	1.8×10^{-7}	1.5×10^{-7}
	4.2	5.0	4.5	7.2×10^{-7}	1.7×10^{-7}	1.4×10^{-7}
	0.42	0.5	0.5	7.7×10^{-8}	1.8×10^{-7}	1.5×10^{-7}

Table XX. Continued

Growth phase*	Cell no. in 10 λ sample		Net response (mv)	ATP (γ)	ATP (γ/cell)	
	Plate	Chamber			Plate count	Chamber count
Acceler-ation (V)	460	480	305	4.9×10^{-5}	1.1×10^{-7}	1.0×10^{-7}
	46	48	30	4.9×10^{-6}	1.1×10^{-7}	1.0×10^{-7}
	4.6	4.8	3.0	4.9×10^{-7}	1.1×10^{-7}	1.0×10^{-7}
Log (VI)	808	800	1240	2×10^{-4}	2.5×10^{-7}	2.5×10^{-7}
	80.8	80	123	2×10^{-5}	2.5×10^{-7}	2.5×10^{-7}
	8.08	8.0	12.5	1.95×10^{-6}	2.4×10^{-7}	2.4×10^{-7}
	0.8	0.8	13	2×10^{-7}	2.5×10^{-7}	2.5×10^{-7}
End of log (VII)	1700	1900	3150	3×10^{-4}	1.8×10^{-7}	1.6×10^{-7}
	170	190	275	2.7×10^{-5}	1.6×10^{-7}	1.4×10^{-7}
	17	19	28	2.7×10^{-6}	1.6×10^{-7}	1.4×10^{-7}
	1.7	1.9	2.7	2.7×10^{-7}	1.6×10^{-7}	1.4×10^{-7}
Station-ary (VIII)	4000	4170	6400	3.0×10^{-4}	7.5×10^{-8}	7.2×10^{-8}
	400	417	600	2.9×10^{-5}	7.3×10^{-8}	7.0×10^{-8}
	40	41.7	64	3.1×10^{-6}	7.8×10^{-8}	7.4×10^{-8}
	4	4.2	6.3	3.2×10^{-7}	8.0×10^{-8}	7.6×10^{-8}

* See Microbiology, M. J. Pelczar and R. D. Reid, p. 69. McGraw-Hill, 1958.

SECTION IV

INSTRUMENTATION

1. SYSTEM DESCRIPTION

Figure 9 is a block diagram of the instrument system. The bioluminescence detector is an assembly consisting of a sensitive photomultiplier tube, the voltage divider network which feeds the appropriate supply voltages to the photomultiplier dynodes, signal processing circuitry, a rotating mount for standard 6 mm x 50 mm test tubes which serve as reaction chambers, and a light-tight housing. High voltage for the dividing network has been normally supplied by a power supply, John Fluke model 412B.

A Tektronix model 503 oscilloscope has been used to display the instrument output. A simple device has been constructed for balancing out the photomultiplier dark current and thus allowing the oscilloscope trace to be indexed at any desired vertical position.

2. BIOLUMINESCENCE DETECTOR

An exploded isometric view of the bioluminescence detector is shown in figure 10. Photographs of the components are shown in figure 11.

The assembled detector is a rectangular light-tight housing for the photomultiplier. The glass envelope of the PM tube is contained within the PM tube chamber (Part No. 2). The socket of the PM tube extends into the base chamber (Part No. 1), and it is here that the components of the voltage divider and signal processing electronics are mounted. Holes to dissipate heat generated by resistance losses are provided in the base chamber. The end plate (Part No. 6) blanks off the end of the base chamber and provides a mount for a UG-931 MHV chassis connector and a UG-88 chassis connector to make the high voltage and signal output connections respectively. The sample chamber is connected to the PM tube chamber in front of the photocathode and has a light-tight revolving drum in which is seated the cuvette or test tube reaction chamber.

The sample drum (Part No. 4) is designed so that two cuvettes can be mounted in opposing recessed chambers and can be rotated into the sample chamber, one at a time, without exposing the photocathode to the ambient light in the laboratory. A replaceable, light-tight rubber plug is mounted in the top of the sample chamber directly over each cuvette to allow the introduction of reagents by syringe needle after the cuvette or test tube has been rotated into the sample chamber.

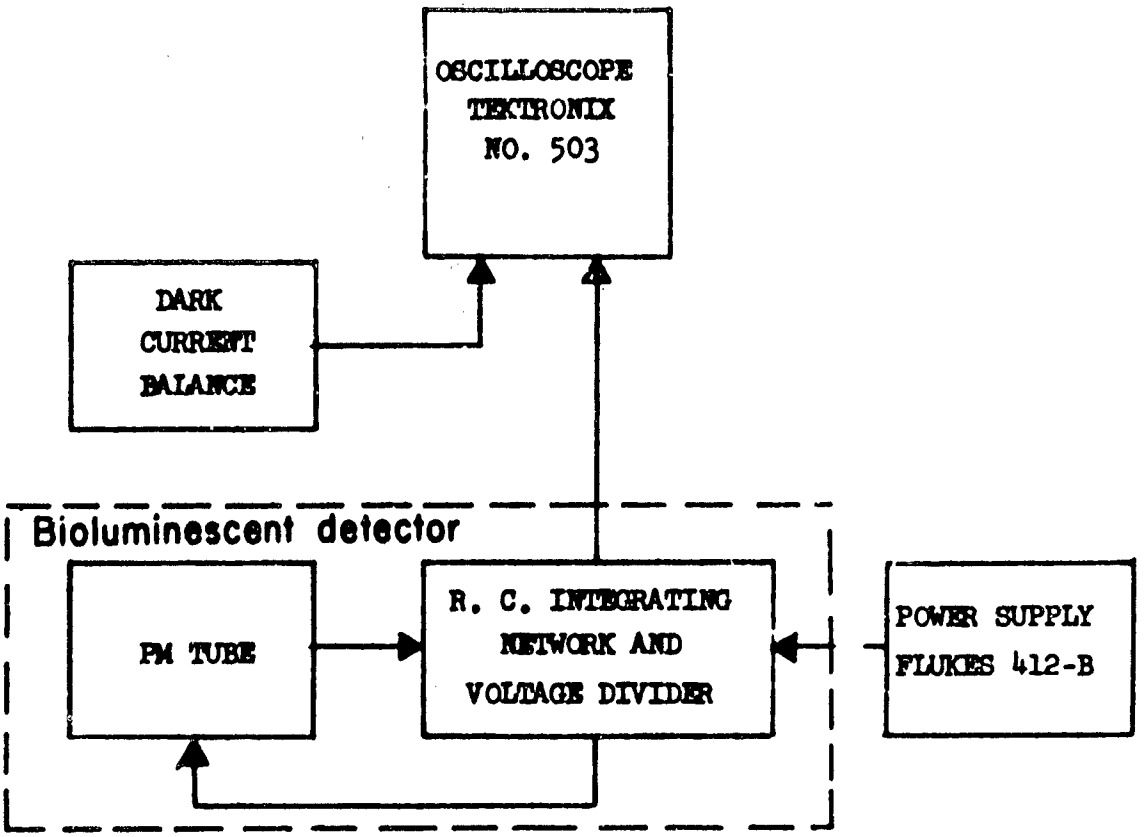


Figure 9 – Bioluminescent detector – block diagram

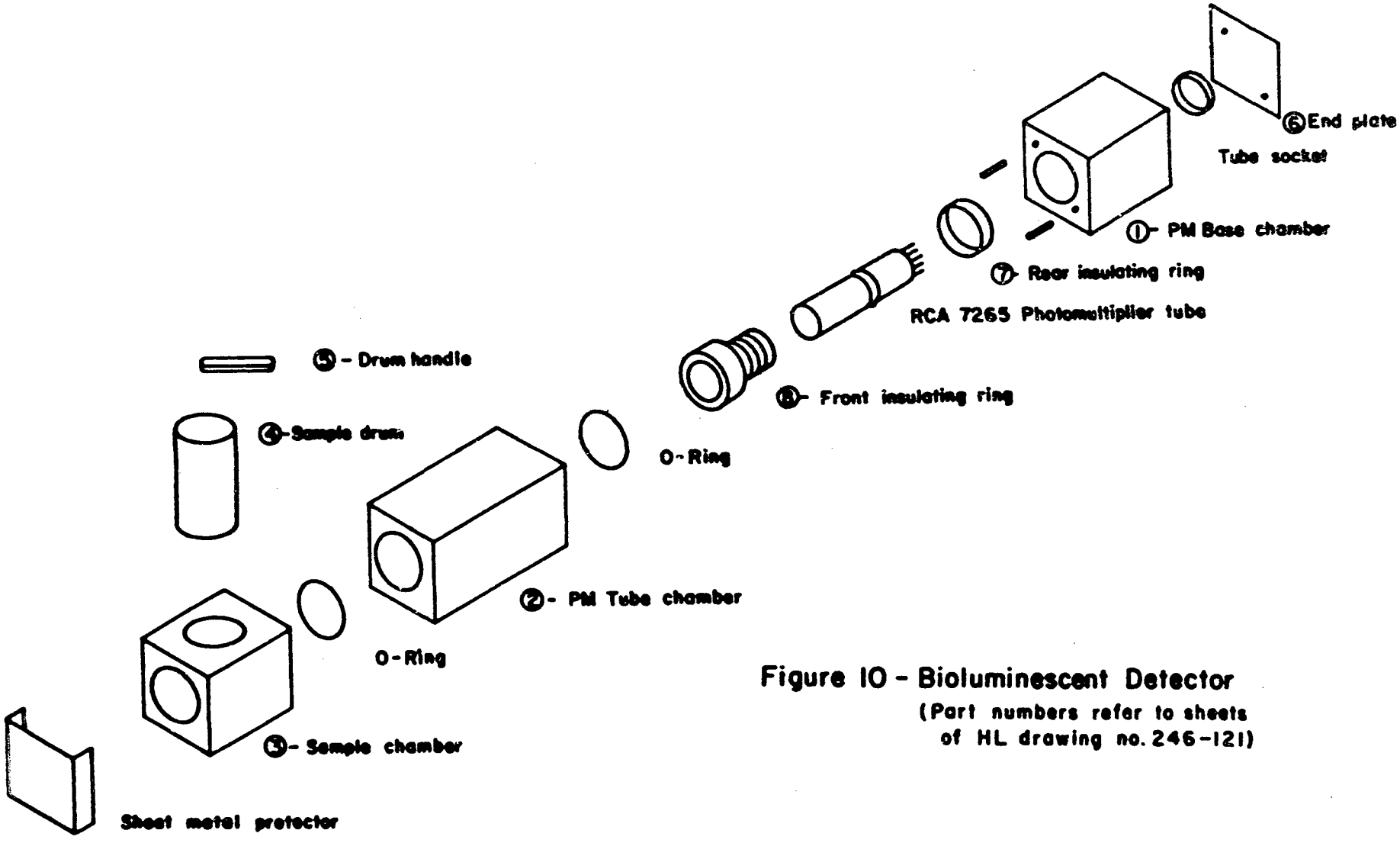
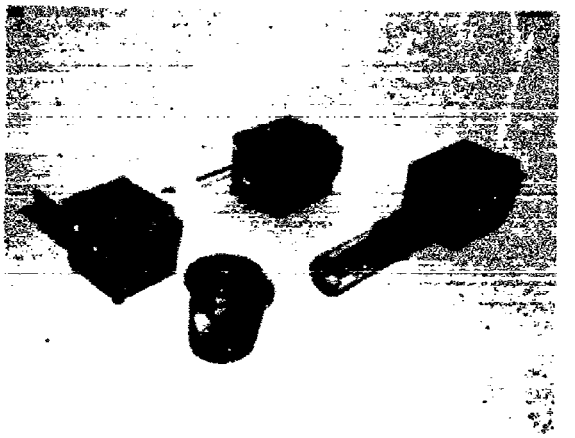


Figure 10 - Bioluminescent Detector
(Part numbers refer to sheets of HL drawing no. 246-121)

A.



B.

Figure II – Disassembled bioluminescence detector

A – Components separated

B - Components aligned (but not fully assembled)

A ring assembly consisting of two insulating plastic, threaded and mated rings fastens around the flanged metal collar on the photomultiplier tube. This ring insulates the collar, which shapes the focusing field for the photocathode, from the instrument casing and supports the photomultiplier in its chamber. The resistors and capacitors of the divider network and signal processing electronics are mounted directly on the tube socket in the PM base chamber. A sheet metal cover protects the face of the sample chamber when the instrument is not in use.

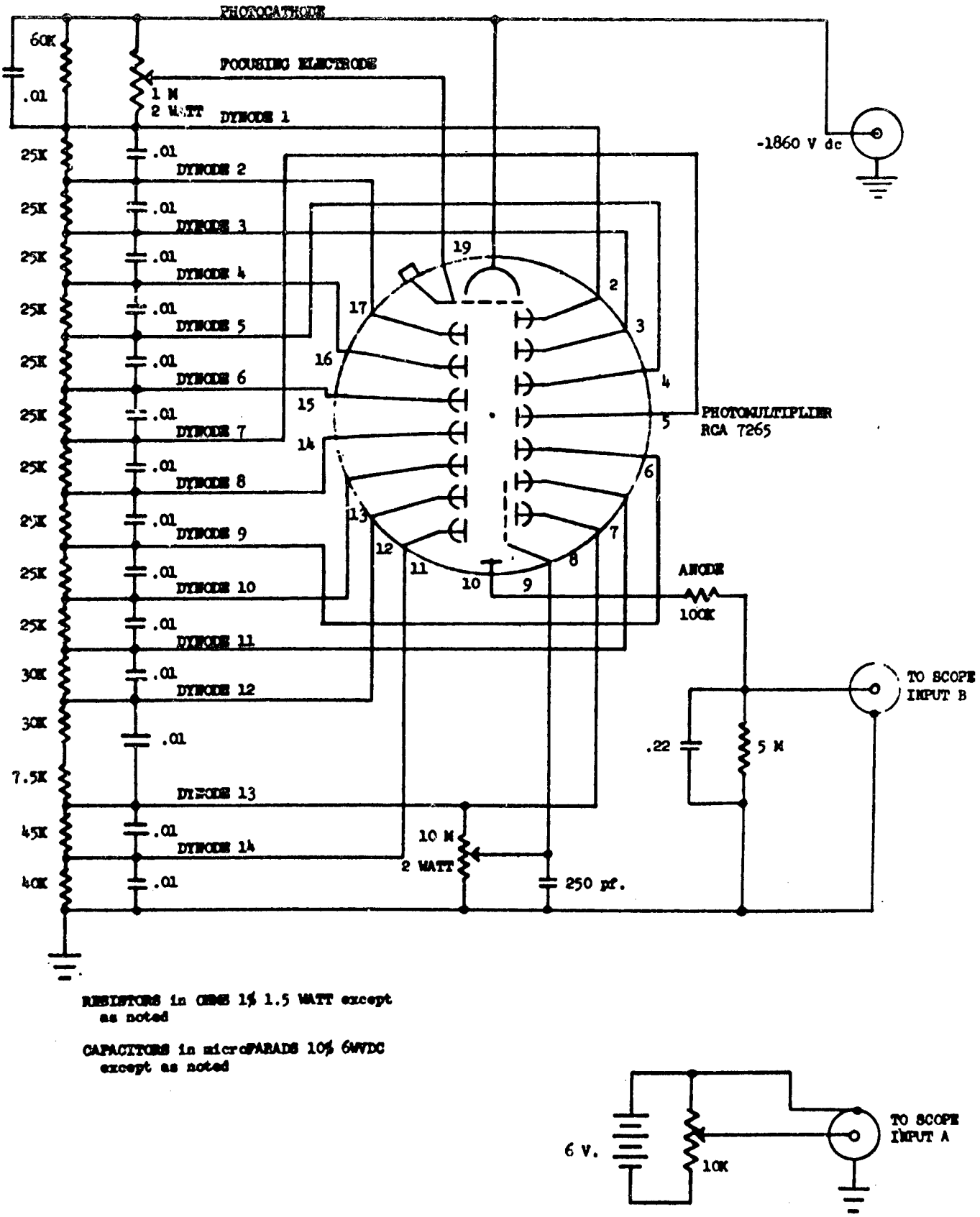
The photomultiplier used in this instrument is an RCA 7265. This tube has 14 dynodes, a focusing electrode, an accelerating electrode, an anode, and a photocathode. The manufacturer's specifications for this tube are given in reference 9.

The voltage divider design and the supply voltage have been chosen for "continuous luminous excitation and d-c anode current" (see reference 9). The circuit diagram is shown in figure 12. A simple RC network has been included in the output circuit to inhibit the response to high signal frequencies. This further adapts the photomultiplier for detection of continuous luminous excitation since it has the effect of filtering high frequency variations in signal output due to pulsations in the light being detected and also due to noise.

When the oscilloscope is operated in its most sensitive ranges (mv/cm), the photomultiplier dark current is sometimes so large that the vertical deflection index control on the oscilloscope will not bring the trace to the center of the cathode ray tube. When this occurs, an additional bias can be introduced into the vertical deflection circuitry from an external source. If the oscilloscope is designed for dual inputs, the external bias can be introduced on the second input connector by setting the input mode switch to detect the difference between the two inputs. A simple voltage divider potentiometer across a dry cell can be used as a bias voltage source. The details of a simple dark current, balance circuit are shown in figure 12 along with the photomultiplier circuitry. This control was constructed and mounted in the accessory box delivered with the instrument.

3. POWER SUPPLY

The power supply, John Fluke model 412B, was chosen to provide the high voltage (-1860 v d-c) for the photomultiplier voltage divider because of the low ripple content, and excellent voltage regulation. Stability of the power supply is extremely important since the percent variation in input voltage will be reflected in the output signal as that percentage times the number of dynodes. In this instrument, for example, a fluctuation of 0.1% in the supply voltage will cause a change of 1.4% in the output (voltage or current if the load is resistive) that is not due to a change in the light being measured.



RESISTORS in OHMS 1% 1.5 WATT except as noted

CAPACITORS in microFARADS 10% 6WVDC except as noted

Figure 12 - Phototube circuitry

The controls for the power supply consist of five rotary switches used to select the output voltage, a polarity reversing switch, a power switch and indicator light, and a high voltage switch and indicator lamps (see figure 13). There is also a kilovoltmeter in the panel to provide a gross check of output voltage.

The first rotary switch selects increments of 500 volts from 0 to 1500 (see figure 13). The second selects increments of 100 volts to be added to the first. The third switch selects additional increments of 10 volts, the fourth increments of 1 volt, and the fifth increments of 1/10 volt. Thus, to select -1860 volts, the first switch would be set at 1500, the second at 300, the third at 60, and the fourth and fifth at zero. Output is delivered through a coaxial connector. If the polarity switch is set on "+," the center conductor is positive with respect to the outside conductor. If the polarity switch is set on "-," the center conductor is negative with respect to the outside conductor. When the power supply is connected to the bioluminescence detector with the coaxial cable provided, the polarity switch must be set on "-."

The power switch applies 115 volt 60 cps energy to the instrument and is distributed to vacuum tube filaments and indicator light to allow the instrument to warm up and be on standby for use. After a suitable warm-up time (approximately 1 minute), the "standby" indicator light comes on and the high voltage switch can be set to "on" to apply power to the photomultiplier.

4. DISPLAY

There is a considerable latitude in the choice of a display for the output of the bioluminescence detector. The choice of a display will depend to a great extent on the output range (in millivolts) in which the instrument is expected to be used, as well as the convenience, desired format, and need for permanent records. The instrument has been used successfully with oscilloscopes and oscillographs and could be adapted for use with an electrometer or many types of analog read-out or recording instruments. The display used most frequently in the biochemical work has been a Tektronix type 503 oscilloscope. Since an oscilloscope provides a readily available and versatile display, the operating description which follows describes operation with the Tektronix 503.

5. CONNECTIONS

In order to operate the instrument, the bioluminescence detector, power supply, oscilloscope, and dark current balance must be connected as shown in figure 14. Interconnecting cables provided with the equipment are coaxial, using mil-std. connectors as labeled in figure 14. Two UG-273/U

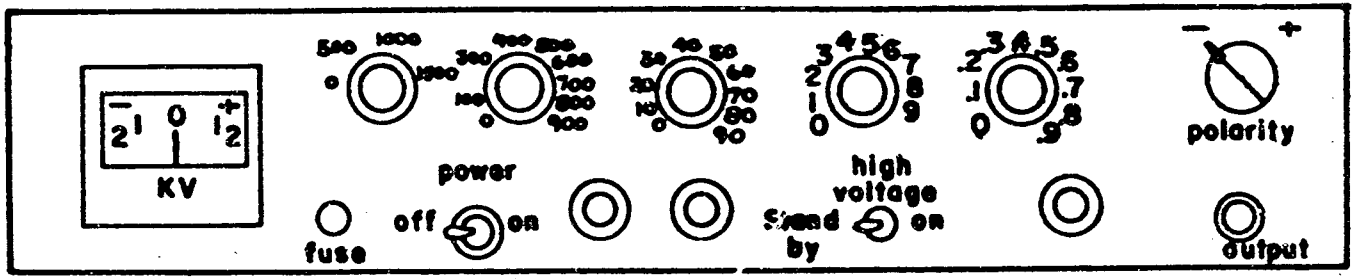


Figure 13 – Power supply

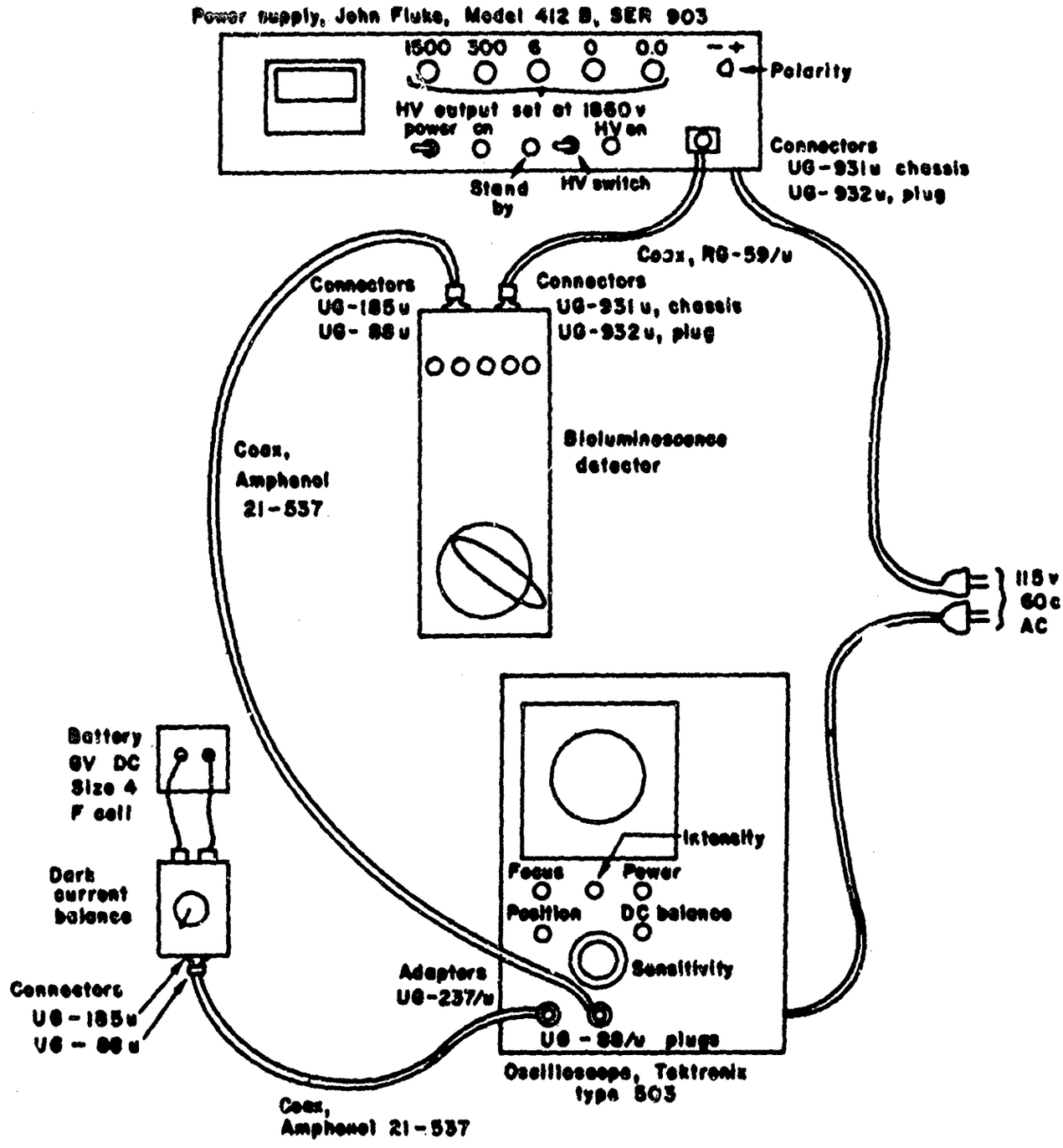


Figure 14 - Equipment connections and controls.

adapters are used to allow the connection of the signal line from the bioluminescence detector (Amphenol 21-537 cable with UG-88/U plugs) and the output line from the dark current balance (also Amphenol 21-537 with UG-88/U plugs) to the oscilloscope. The high voltage output of the power supply is distributed to the bioluminescence detector through a length of RG-59/U coaxial cable with UG-932/U MHV plug connectors. Any 6-volt battery can be used to supply the dark current balance but the larger cells will have a longer operating life.

6. OPERATION

After the equipment has been set up and interconnected, it is operated as described below. There are certain fixed settings for controls on each unit that should be checked each time the instrument system is used. These will be described first.

a. Fixed Settings

(1) Oscilloscope (if Tektronix 503 is used)

(a) The "SLOPE" switch should be set at "+."

(b) The "COUPLING" switch should be set at "AC."

(c) The "SOURCE" switch should be set at "INT."

(d) The "LEVEL" control should be set full counter-clockwise to "AUTO."

(e) The "SWEEP TIME/CM" control should be set at "0.2 m SEC."

(f) The "HORIZONTAL DISPLAY" control should be set at "SWEEP NORMAL (X1)."

(2) Power Supply

(a) The "HIGH VOLTAGE" selectors should be set to deliver an output of 1860.0 volts. This can be effected by setting the first switch at "1500," the second at "300," the third at "60," and the last two at "0."

(b) The "POLARITY" selector must be set at "-" (minus).

(3) Dark Current Balance - The battery should be connected just prior to using the instrument. Since the potentiometer, used to divide the battery voltage, puts a

continuous drain on the battery while it is being used, the battery should be disconnected at the end of each extended operating period to conserve its life.

b. Adjustments

(1) After having checked the fixed settings described above, switch on the "POWER AND SCALE ILLUM" control on the oscilloscope. Allow the oscilloscope to warm up for at least 5 minutes.

(2) After a 5-minute warm-up period, adjust the "INTENSITY" and "FOCUS" controls on the oscilloscope for the desired brightness and resolution.

(3) Move "POWER" switch to "ON" on the power supply and allow a warm-up period.

(4) When the power supply has stabilized at the operating temperature, the "STANDBY" pilot light will come on. When this happens, move the "HIGH VOLTAGE" switch from "STANDBY" to "ON."

(5) After the oscilloscope and power supply have been energized, index or "balance" the vertical channel of the oscilloscope as follows:

(a) Set the "INPUT" switches to "GND" position.

(b) Set the "SENSITIVITY VARIABLE" control full clockwise to the "CALIBRATED" position.

(c) Set the "SENSITIVITY" control to the ".2 VOLTS/CM" position.

(d) Move the sweep to the center of the cathode ray tube with the "POSITION" control.

(e) Set the "SENSITIVITY" control to the "1 MV/CM" position. With the "DC BAL" control, move the sweep back to the center of the cathode ray tube (CRT) and continue to adjust until there is no sweep movement as the "SENSITIVITY" switch is moved from the ".2 VOLTS/CM" to "1 MV/CM" positions. The "SENSITIVITY" switch should be positioned to a scale that allows accurate readings in the range of the response expected. For low values (10^{-6} - 10^{-8} γ) of ATP this would be "1 MV/CM" or

".2 VOLTS/CM" but for higher amounts of ATP a higher scale setting may be required.

(f) Return the "INPUT" switches to "DC" position. Note: The vertical position of the sweep can be controlled by the "VERTICAL POSITION" control on the oscilloscope or with the dark current balance potentiometer. For most test situations, the "VERTICAL POSITION" control should be sufficient to keep the sweep centered. Where high inherent light levels are encountered in certain reagent mixtures, it may be necessary to use both controls to center the sweep.

(6) Test the instrument output by means of a standard light source such as a cuvette loaded with a compound of C^{14} suspended in a liquid scintillator. Carbon 14 is preferred, since it has a very long half life, thereby providing a convenient repeatable light output.

(7) Load a test tube, previously filled with all assay reagents except ATP (100 λ), into the cuvette holder on the drum and rotate the test tube into the readout position.

(8) Insert a Hamilton glass syringe loaded with 10 λ standard ATP solution through the rubber stopper in the injection port on top of the drum and inject. The injection speed should be fast and consistent to obtain reproducible results. If an abnormal light response is observed, three things should be checked: (1) a bent needle tip; (2) improper positioning of the syringe may cause the injected ATP solution to run down the side of the cuvette and repositioning of the syringe may be necessary; (3) dry atmospheric conditions may allow development of static on the drum and cause abnormal response.

(9) Record the maximum deflection in millivolts reached by the oscilloscope sweep as the reaction progresses.

(10) Repeat the operation on the standards to obtain at least three points from 10^{-7} to 10^{-3} γ ATP.

(11) Repeat steps (8) and (9) on all extracted test samples, substituting them for the standards.

(12) Recheck instrument with standard light source.

(13) Upon completion of the operation of the instrument, turn the power supply "HIGH VOLTAGE" switch to "STANDBY" and "POWER" switch to "OFF." Turn the oscilloscope "POWER AND SCALE ILLUM" switch to "PWR OFF." Disconnect the 6-volt battery from the dark current balance control.

SECTION V

DISCUSSION

The present investigation has produced promising results toward development of a simple, sensitive and rapid technique for detecting microorganisms. The system has demonstrated its ability to detect many varieties of microorganisms including heterotrophic, autotrophic, aerobic, anaerobic, and spore-forming bacteria, yeast, and fungi, as well as algae and protozoa, in less than the stipulated 30-second time limit.

With all species tested, several hundred or fewer cells were readily detected. With the filtration system as originally proposed, no problem exists in meeting the specified sensitivity limit of 10 to 100 cells per ml of water.

For all the tested bacterial species, whether autotrophs or heterotrophs, the amount of ATP per cell consistently fell between 10^{-9} to 10^{-10} γ per cell. The variation was less when γ ATP per cell was based on chamber count than on plate count. The fungi, algae and protozoa were larger in cell size and their γ ATP per cell was higher than for bacteria. Therefore, a "differential filtration" system should be emphasized in the future to isolate bacterial cells for the ATP assays. Until such a system is developed, however, it may be necessary to consider expressing the amount of ATP for all microorganisms in terms of biomass rather than cell number.

As far as the reproducibility of results of the standard ATP assay is concerned, a consistency in producing a linear dose-response curve has been shown as represented by net response in mv vs ATP at various concentrations ranging from 10^{-7} γ to 10^{-3} γ. There have been a few exceptional cases where a lower cell number of a given species gave a higher ATP response than a sample of higher cell number. This was presumably due to inadequacy in quantitation of microbial cells as was mentioned before. This occurred only in comparing the ATP content at lower cell numbers. Generally the accuracy of ATP determination was good for cells in the 10^{-6} or higher concentrations, but less in levels below 10^{-6}, due in part to errors in dilution.

The feasibility of the enzymatic assay technique has been amply demonstrated, and the next stage in its development toward actual use is to decide how to implement it. This reduces roughly to two choices - an automated instrument and a manual assay, although there is some question about the degree of automation desirable. Although no specific design studies were conducted, research to date has shown no reason why complete automation would not be possible. A fully automated water supply assay instrument might be quite complex, but justifiable because of the resulting accuracy, repeatability, and operational simplicity.

The approach requiring the simplest equipment would be the manual assay. In such a system, the only instrumentation required would be one of the new, miniature, ruggedized photomultiplier tubes in a light-tight housing; some provision for introducing test specimens into the light-tight chamber without exposing the photocathode; an integrated, miniaturized, d-c, medium high voltage power supply; and a miniaturized display such as an oscilloscope with a 3-inch cathode ray tube or a d-c millivoltmeter. This equipment could be mounted on a single chassis with a very small volume and weight (perhaps as small as 12" x 8" x 5" and less than 10 lbs weight). Reagents would be prepackaged in sealed, sterile containers with rubber stoppers to facilitate extraction, allow fluid containment in a weightless environment, and prevent contamination. For example, the enzyme, substrate, and $MgSO_4$ would be stored lyophilized and frozen (by the ambient temperature in space) in cuvettes sealed with rubber stoppers. Tris buffer could be prepackaged in fluid form in bottles or possibly in crystalline form mixed with the enzyme, substrate, and $MgSO_4$ in the reaction cuvette. If this latter storage method could be used, it would only be necessary to add distilled water to the cuvette to reconstitute the reagent mixture. Standard solutions of ATP for calibration could be premixed and frozen. Since the dose-net response curve has consistently been linear, thus only one point of ATP standard (10^{-4} γ ATP per 10 λ) is required to establish the standard curve.

An operational sequence for such a system might then be conducted as follows:

1. Remove 2 preloaded reaction cuvettes and at least one standard ATP vial from frozen storage.

2. Remove the capped bottle of distilled water and the bottle of DMSO from heated storage.

3. Draw the correct volume of distilled water to reconstitute the reagent mixture into a syringe.

4. Inject the distilled water into the two reaction cuvettes and allow them to stand until they come to capsule temperature.

5. Draw a test sample from the water supply into an automatic pipetting device such as an Adams Autopet syringe attached to a Swinny filter. The volume of water passed through the DMSO-resistant membrane filter can then be measured by the Autopet and the organisms collected on the membrane filter.

6. Remove the filter from the water system.

7. Draw the correct volume of DMSO for ATP extraction into a syringe.

8. Place the Swinny filter with the test sample on the DMSO syringe with the needle mounted on the filter outlet.

9. Force the DMSO through the filter into an empty, stoppered bottle.

10. Draw the correct volume of distilled water for dilution of DMSO into a syringe.

11. Inject the distilled water into the bottle with the DMSO extracted sample in it.

12. Draw 10 λ of standard ATP solution into a syringe.

13. Place one reaction cuvette into the instrument.

14. Read and record the inherent light level.

15. Inject the standard ATP solution into the reaction cuvette.

16. Read and record the response. One point will be enough to establish a calibration curve whose slope is known. At most, two points will be sufficient to establish the instrument system calibration.

17. Draw the test sample into a syringe.

18. Place the second cuvette into the instrument.

19. Read the inherent light.

20. Inject the test sample into the reaction cuvette.

21. Read and record the response.

One problem is readily apparent from this hypothetical assay sequence. This is the relatively large number of syringes that would be required, particularly for a flight of long duration. This suggests that, if a manual assay is chosen, some development should be undertaken to produce a reagent packaging concept that would allow sterile fluid transfers under weightless conditions. Roughly, this means to store the reagents in the syringes or containers with suitable valving and couplings from which, and into which, the fluids can be forced. In either case, whether automated or manual, the instrument system requires further engineering development before an operational system for spacecraft use results.

APPENDIX I

Sample Data Sheet for ATP Assay of Microorganism

Date

1. Name of Microorganism

2. Method of Propagation

3. Cell Determination by O.D. Method

   O.D. Approximate Cell No. (Figure on p. Notebook)

4. Cell Determination by Plate and Chamber Count

   a. Plate Count (Pour Plate or Spread Plate) b. Chamber Count ( )

Dilution Factor	No.	Average	No. per ml.	Fields	Average

Clumping

Other

5. Preparation of Cell Extraction and Assay

6. Enzyme

7. Light Response

Systems	Setting (mv/cm)	Reading (cm)	Inherent Light (cm)	Response (mv) (Gross) (Av.)	(Net) (Av.)
a. Light Source I					
b. Light Source II					
c. 0.05 M Tris Buffer					
d. 1% DMSO in Buffer					
e. Enzyme Activities 1×10^{-3} ATP					
1×10^{-4} ATP					
1×10^{-5} ATP					
1×10^{-6} ATP					
1×10^{-7} ATP					
f. Cell No. ( )					
g. Cell No. ( )					

APPENDIX II

MATERIALS AND SUPPLIES

A. Media

1. Tryptic Soy Broth (TSB) - 30.0 grams of dehydrated Difco Bacto Tryptic Soy Broth dissolved in one liter distilled water.

2. Tryptic Soy Agar (TSA) - 40.0 grams of dehydrated Difco Bacto Tryptic Soy Agar dissolved in one liter distilled water.

3. Sabouraud Dextrose Broth (SDB) - 30.0 grams of dehydrated Difco Bacto Sabouraud Dextrose Broth dissolved in one liter distilled water.

4. Sabouraud Dextrose Agar (SDA) - 65.0 grams of dehydrated Difco Bacto Sabouraud Dextrose Agar dissolved in one liter distilled water.

5. Fluid Thioglycollate Broth (FTG) - 29.8 grams of dehydrated Difco Bacto Fluid Thioglycollate Medium dissolved in one liter distilled water.

6. Fluid Thioglycollate Agar (FTGA) - 20.0 grams of Difco Bacto Agar added to one liter Fluid Thioglycollate Broth.

7. Tetrahymena Broth (TB) - 27.0 grams of dehydrated Difco Bacto Tetrahymena Broth dissolved in one liter distilled water.

8. Nutrient Agar (NA) - 23.0 grams of dehydrated Difco Bacto Nutrient Agar dissolved in one liter distilled water.

9. Synthetic Thiobacillus Agar (STA)

$Na_2S_2O_3 \cdot 5H_2O$	8 grams
KH_2PO_4	4 grams
K_2HPO_4	4 grams
NH_4Cl	0.5 gram

$MgSO_4 \cdot 7H_2O$	0.8	gram
Metal mixture*	10	ml
Agar	20	grams
Distilled water	1	liter

*Metal mixture:

EDTA	5	grams
$MgSO_4 \cdot 7H_2O$	2	grams
$CaCl_2$	0.554	gram
$MnCl_2 \cdot 4H_2O$	0.506	gram
$FeSO_4 \cdot 7H_2O$	0.499	gram
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.110	gram
$CuSO_4 \cdot 5H_2O$	0.157	gram
$CoCl_2 \cdot 6H_2O$	0.161	gram
Distilled water	100	ml

Note: The thiosulfate and the phosphates are autoclaved separately and then added to the other components.

10. Nitrate Glucose Broth (NG)

$MgSO_4 \cdot 7H_2O$	1.0 grams
KNO_3	3.4 grams
KH_2PO_4	0.25 gram
Minor Elements Chelate solution*	1.0 ml
Iron Sequestive solution**	1.0 ml
Glucose	5.0 grams
Distilled water	1 liter

All media mentioned above are sterilized by autoclaving for 15 minutes at 15 lb. pressure (121° C).

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*Minor Elements Chelate solution (10x strength)

EDTA Mn	189 mg %
EDTA Cu	56 mg %
EDTA Co	60 mg %
EDTA Zn	54 mg %

Dilute this solution 1:10 with distilled water prior to use and take 1 ml to prepare 1 liter of Nitrate Glucose Broth.

**Iron Sequestive solution

EDTA·Na·Fe	40 mg per ml distilled water

B. Chemicals and Reagents

Items	Description
Sephadex G 100	100 g bottle
$MgSO_4 \cdot 7H_2O$	1 lb
DMSO	1 qt
Acetone (ACS)	1 qt
Tham (Tris)	500 g bottle
ATP	1 g bottle
Luciferin	
Firefly luciferase	
Media	

C. Glassware and Disposable Supplies

Items	Description
Sephadex column	2.5 x 45 cm
Cooling jacket (for the column)	2.5 x 45 cm
50 µl syringe with needle	
Dispos. test tube with cap	17 x 100 mm
Dispos. steel pipette	1 ml (1/100th)
Petri dish	100 x 15 mm
Yale disposable needle	22G, 1-1/2"
B-D Tuberculin syringe	1 cc
B-D dispos. syringe	5 cc
Cuvette (culture tube)	6 x 50 mm ExAx

Items	Description
Polycarbonate conical graduated test tube	15 ml
Dispo. pipette	5 ml
Tear drying bulb	5 ml
Pasteur pipette	145 mm
Nalgene Filter unit, disposable	
Swinny Filter unit (Swinnex 13)	HA 0.45 μ
Millipore filter	HA 0.45 μ (13 mm)
Micro slides	25 x 75 mm
Nephelo culture flask	14 x 130 mm
Petroff-Hausser chamber	
Palmer chamber	

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13. ABSTRACT Research has been conducted and reported herein toward development of a sensitive, biologically nonspecific assay technique which uses the firefly bioluminescent reaction to detect ATP extracted from microorganisms present in water supplies. An instrument using a photomultiplier to detect the light output from the bioluminescent reaction has been designed, constructed, and tested, and is described in this report. Extraction of ATP from cells is done chemically with DMSO. Tests were conducted to find the optimum concentration of DMSO and the simplest extraction procedures. Cultures of a wide variety of organisms were grown, both heterotrophic and autotrophic, and included numerous species of bacteria, algae, fungi, and protozoa. Assays of ATP extractibility and response to measured numbers of cells were conducted using the bioluminescent detector. These tests have shown the ability to detect concentrations of only a few hundred cells of any of the test species and in many cases less than 100 cells in less than 30 seconds. The ATP response is related to number of cells and cell size and can be used as a measure of the number of cells per volume of sample if something is known of the cell size. Development of a differential filtering technique may be necessary to obtain highest accuracy in quantitation if cells of varying size are encountered. The inherent light level of the enzyme solution without ATP added varies from day to day and consequently the net response of the instrument - gross response less inherent light - is the best measure of ATP level. The assay technique may have applicability to terrestrial water supplies - particularly under hazardous or difficult conditions - as well as to spacecraft waters.

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14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Microorganism Detection						
Firefly Bioluminescence						
Adenosinetriphosphate						
Enzymatic Detection of Microorganisms						
Luciferase						
Luciferin						