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APPLICATIONS OF LUMINESCENT SYSTEMS TO INFECTIOUS DISEASE METHODOLOGY

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GSFC —— **GODDARD SPACE FLIGHT CENTER** ——
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INTRODUCTION

Management of infectious disease continues to be dependent on primary isolation and culturing of organisms infecting biological fluids. The time consumed in these processes does not allow for immediate and informed treatment of the patient. In the past several years, much attention has been directed toward the development of bioluminescent and chemiluminescent systems as rapid diagnostic tools. Instrumentation and procedures compatible with automation now allow for the sensitive detection of as few as 10,000 bacteria/ml in biological fluids. Quantitation is immediate, accurate, reproducible, and independent of overnight culturing.

The medical applications of luminescent systems, which will be described, are a direct outgrowth of the National Aeronautics and Space Administration (NASA) effort to develop methods for detecting extraterrestrial life. The firefly luciferase assay for adenosine triphosphate (ATP) was a candidate for remote detection of microbes on other planets, such as Mars, as ATP is found in all living organisms on Earth. An indication of the presence of ATP on another planet would suggest the existence of extraterrestrial life forms similar to those we know (Reference 1).

The firefly luciferase ATP assay involves an enzymatic reaction specific for ATP that results in the production of light (References 2, 3). The reaction mechanism for light emission can be summarized as follows (Reference 4):

$$E + LH_2 + ATP \xrightarrow{Mg^{++}} E \cdot LH_2 \cdot AMP + PP \qquad (1)$$

$$E \cdot LH_2 \cdot AMP + O_2 \longrightarrow Oxyluciferin + CO_2 + AMP + h\nu \qquad (2)$$
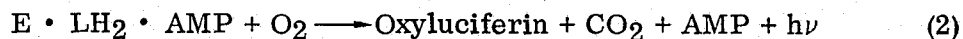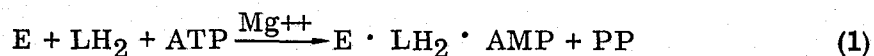
where:

E	= firefly luciferase
LH^2	= luciferin (reduced state)
ATP	= adenosine triphosphate
$E \cdot LH_2 \cdot AMP$	= luciferase-luciferyl-adenylate complex (active intermediate)
oxyluciferin	= luciferin (oxidized state)
PP	= pyrophosphate

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$$\text{AMP} \quad = \text{ adenosine} - 5' \text{ monophosphate}$$

$$h\nu \quad = \text{ light at an emission maxima 550 nm}$$

When ATP is added to luciferase, luciferin, and magnesium ion, the light produced is in direct proportion to the ATP concentration. The relevance of this assay to the field of infectious disease methodology derives from the ability to subsequently relate concentrations of bacterial ATP to bacteria/ml in a biological fluid, such as urine or blood. Procedures using the firefly luciferase ATP assay have been developed for selective measurement of soluble ATP or mammalian cell ATP in body fluids, detection and quantitation of bacteriuria and bacteremia, and testing of antimicrobial susceptibilities.

Applications of this versatile assay are not limited to the medical field, but include monitoring levels of microorganisms in drinking water, sewage treatment plant effluent, industrial effluents, activated sludge (Reference 5), industrial effluents, freshwater streams, ocean sites (References 6, 7), food, beverages, pharmaceuticals, gasolines, oils, etc.

A second bioluminescent system that offers quantitative information on bacterial levels in body fluids involves the specific reaction of bacterial luciferase with flavin mononucleotide (FMN) to produce light. The overall reaction sequence for the in vitro reaction may be simplified as follows:

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{light} + \text{products} \qquad (3)$$
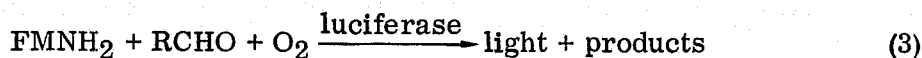
where:

$$\text{FMNH}_2 \quad = \text{ reduced FMN}$$

$$\text{RCHO} \quad = \text{ saturated long-chain aldehyde } (C_6 \text{ to } C_{14})$$

$$\text{O}_2 \quad = \text{ oxygen}$$

$$\text{luciferase} \quad = \text{ bacterial luciferase}$$

(The reaction intermediates and products remain to be established definitively.) The bacterial luciferase FMN assay is similar to the firefly luciferase ATP assay in that light emission is proportional to the limiting component FMN when all others are in excess. FMN concentration can be related to bacterial levels or used directly in the evaluation of abnormal flavin concentrations which might then relate to metabolic disorders (Reference 8).

Chemiluminescent methods, including luminol chemiluminescent procedures, offer some advantages over bioluminescent enzymatic systems that must incorporate precautions to ensure enzyme stability. In the case of the firefly luciferase ATP assay, dependence on firefly lantern availability and chemical synthesis of luciferin (LH_2) add significantly to costs. Luminol, the synthetic chemical responsible for light production in one chemiluminescent system, is readily available in large quantities at low cost. The light produced is a result of luminol reacting with bacterial iron porphyrins in the presence of hydrogen perioxide (Reference 9):

$$\text{Luminol} + H_2O_2 \xrightarrow[\substack{\text{or Metallic} \\ \text{Ions}}]{\text{Porphyrins}} \text{Excited Luminol} \longrightarrow \text{Products} + \underset{h\nu\ 470\ nm}{\ } \qquad (4)$$
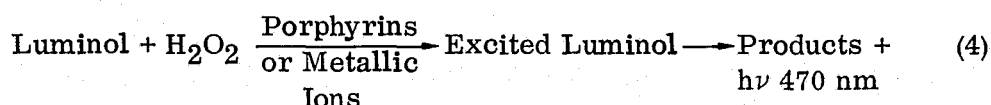
Specificity of this luminol system approaches that of the bioluminescent systems when luminol activators other than intra-bacterial porphyrins are effectively eliminated. The sensitivity is comparable or better in most cases.

This chapter seeks to provide the information necessary to use luminescent systems as analytical tools in the management of infectious disease programs. Applications of the firefly luciferase ATP assay are the central focus, as substantial effort has been expended in the development and clinical testing of the various techniques employed. Instrumentation, reagents, and procedures are described in considerable detail with emphasis on practical considerations. The references at the end of the report may provide more background information, discussion of the principles involved, and additional data evolved in the course of methodology development.

INSTRUMENTATION

Any photometric instrument to be used in measuring the low light levels encountered in bioluminescent and chemiluminescent reactions must be able to provide sensitive, linear, and reproducible results. Such results will be dependent in large part on the quality of the photomultiplier tube and the ability of the instrument to null background noise and endogenous light, and to yield a high signal-to-noise ratio. Instruments suitable for this purpose include commercially available photometers sold by E. I. du Pont de Nemours Company, Inc.*, Scientific Applications Inc. (SAI)†, and the American

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*E. I. du Pont de Nemours Company, Inc., Instrument Products Division, Wilmington, Delaware 19898

†SAI Technology, Inc., 4060 Sorrento Valley Blvd., San Diego California 92121

Instrument Company (Aminco)*; a newly developed photometer to be marketed by Vitatect Corporation**; and standard liquid scintillation counters.

Most photometers follow a basic design similar to the one schematically diagrammed in figure 1. A high voltage supply provides power to the photomultiplier tube which is positioned so that light from the reaction strikes the photocathode. The signal from the photomultiplier tube is amplified and a readout device such as a digital display, a meter, or a rapid response recorder assigns a numerical value. Background noise, which must be nulled before measurement of the sample, emanates from the endogenous light of the reactant and/or from the dark current resulting from thermal electron emissions from the photocathode.

The light flash produced in luminescent reactions can be measured in terms of either the peak light response or the integrated area under the light reaction curve. In theory, integration should provide the most sensitive measurement of light production. However, in the case of the firefly luciferase ATP assay (and other enzymatic luminescent reactions), sensitivity is negated by blank light levels produced by the chemical environment of the ATP sample—enzyme mixture, and by endogenous light levels which emanate from the enzyme itself, and which are believed to be due to the release of ATP from nonactive sites on the luciferase molecule. In addition, ATP produced from contaminating nucleotide phosphates by diphosphonucleotide kinases reaches a peak light level at a much slower rate than in situ ATP; therefore, instantaneous peak height is a more valid measurement of the original ATP in the sample in this case. Some of these problems associated with the firefly luciferase ATP assay that affect the accuracy and sensitivity of integrating the light signal can be mitigated by using highly purified luciferase preparations (Reference 10).

CHARACTERISTICS OF COMMERCIALLY AVAILABLE PHOTOMETERS

Bioluminescent and chemiluminescent research at Goddard Space Flight Center (GSFC) has involved the use of three commercially available photometers: the Du Pont 760 Luminescence Biometer; the SAI model 2000 ATP Photometer, and the Aminco Chem-Glow Photometer. Each instrument has been modified to allow injection of liquid into liquid in a light-tight environment. A hollow metal tower (11 x 105 mm) that can house a disposable tuberculin syringe (1 cc) has been fitted into the instrument injection port to prevent light from

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*American Instrument Company, Division of Travenol Laboratories, Inc., 8030 Georgia Avenue, Silver Spring, Maryland 20910

**Vitatect Corporation, P. O. Box 31108, Temple Hills, Maryland 20031
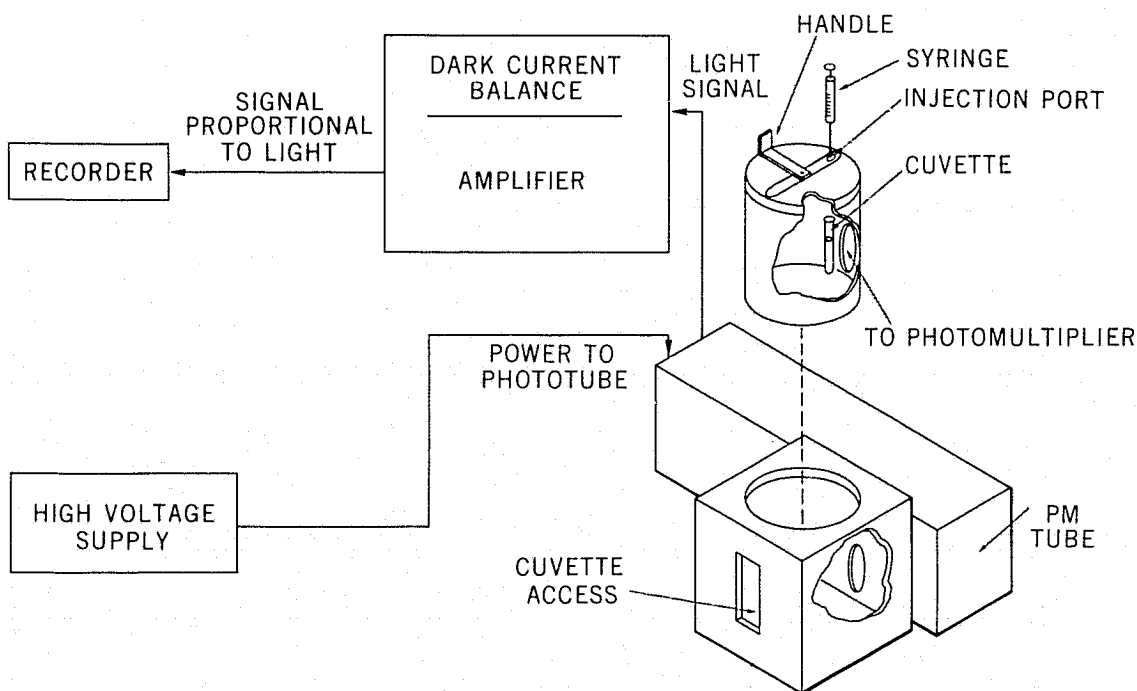
Figure 1. Basic Photometer Design

entering the reaction chamber via the injection syringe. A soft rubber diaphragm fits into the bottom of the tower to be pierced by the injection needle (22-gauge, 1 1/2"). After repeated injections the diaphragm offers little resistance to the penetrating needle and light may leak into the reaction chamber. The diaphragm should be replaced as needed to avoid light leakage.

Accuracy in measuring light responses from luminescent reactions can be affected by inconsistencies in the speed of syringe injections. A protracted injection results in a lower peak light response due to the instantaneous nature of luminescent reactions, while a quick and forceful injection creates undesirable air bubbles that interfere with light from the reaction mixture reaching the photocathode. A technician can master a deliberate, medium injection speed for maximum and consistent light values. Several companies, including Shandon Southern Instruments, Inc., Aminco, and the Hamilton Company, manufacture mechanical injection devices designed to eliminate fluctuations in injection speed and volume (Reference 11).

Each photometer can be effectively calibrated and checked for stability using radioactive light sources with their constant light output and long half-lives. A convenient standard consists of a radioactive material such as C^{14}-glucose

in a liquid scintillator. This is sealed into a glass cuvette (Standard CS-1 from Johnston Laboratories in Cockeysville, Md.). The Du Pont Biometer requires a light standard of 10 microcuries per cuvette for calibration, although it can also be calibrated by setting an internal lamp to an ATP standard as suggested in the Du Pont manual. SAI and Aminco instruments can use either 10 or 1 microcurie light standards. Aminco provides a 1 microcurie light standard with each meter.

Du Pont 760 Luminescence Biometer

The Du Pont Biometer shown in figure 2 is specifically designed to measure the peak light response of luminescent reactions. It has proven to be a successful tool in such varied applications as microbial detection (Reference 12), water quality assessment (Reference 13) and the detection of penicillin in milk (Reference 14). The instrumental design provides for amplification of an analog signal from the photomultiplier tube and subsequent charging of a "memory" capacitor. The peak accumulates for 3 seconds before conversion into a convenient digital display incorporating an automatic range change over 5 decades. The instrument also furnishes an automatic zeroing switch to null background noise.
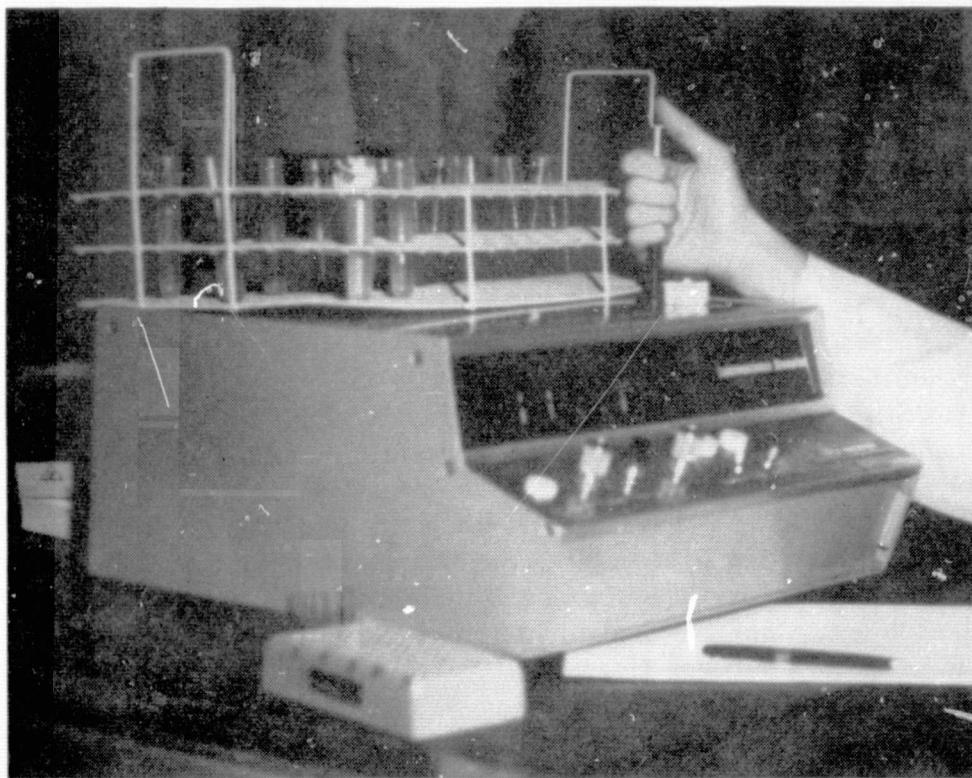
Figure 2.   Du Pont Biometer

The reaction chamber, diagrammed in figure 3, is housed in a cylindrical drum which, when rotated, exposes to the photomultiplier tube the luminescent reactant contained in a 6- × 50-mm glass cuvette (available from Du Pont). A total of 0.2 ml post-injection reaction mixture is visible to the photocathode for light-response measurement. Depression of the read button reveals the numerical interpretation of the peak light signal.
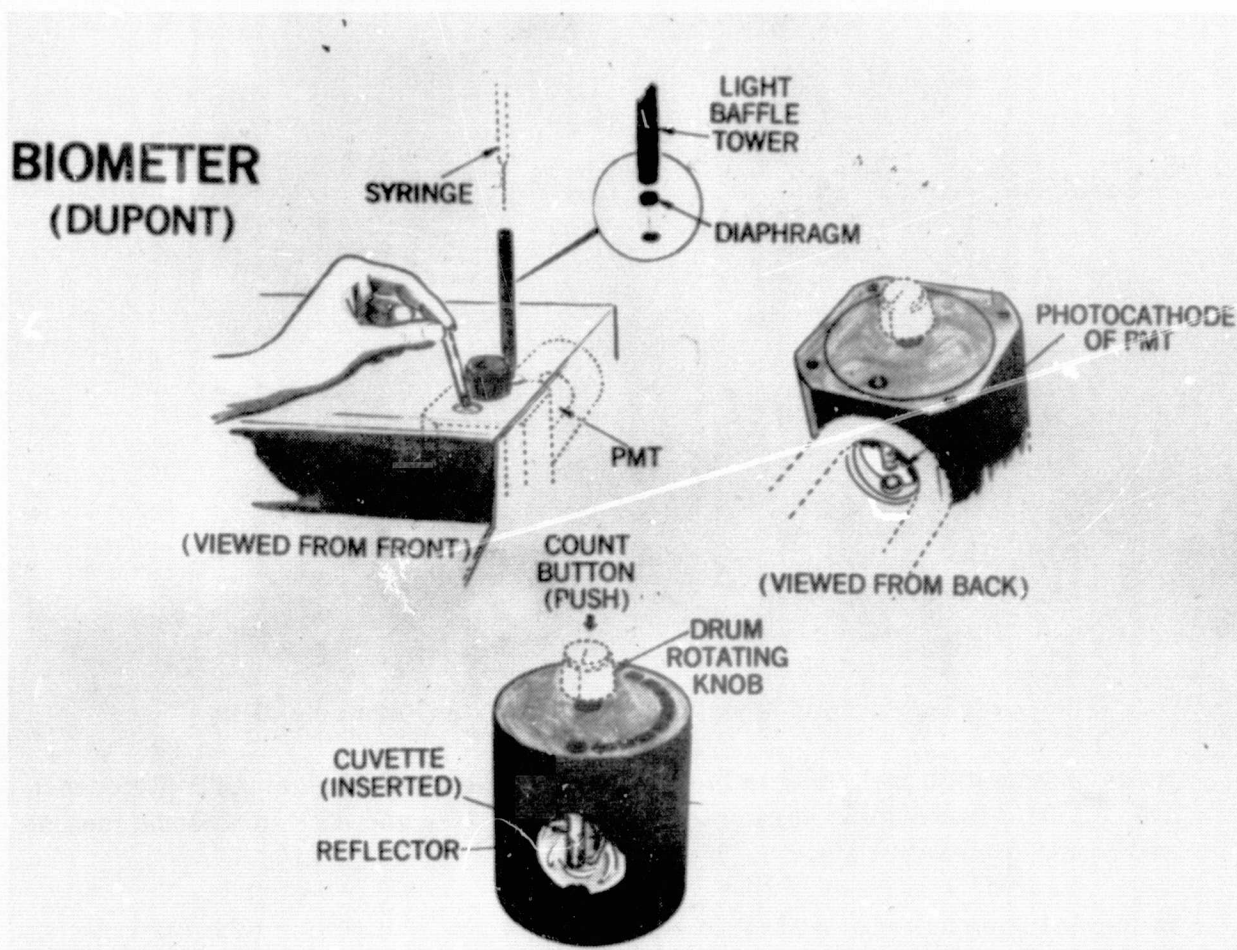
Figure 3. Du Pont Biometer, Reaction Chamber

SAI Model 2000 Photometer

The SAI photometer shown in figure 4 measures luminescent reaction response in either peak mode, requiring 6 seconds of assay time, or integrated mode, requiring 60 seconds. SAI's latest model 3000 photometer offers an added option for varying time of integration. Successful and varied applications of
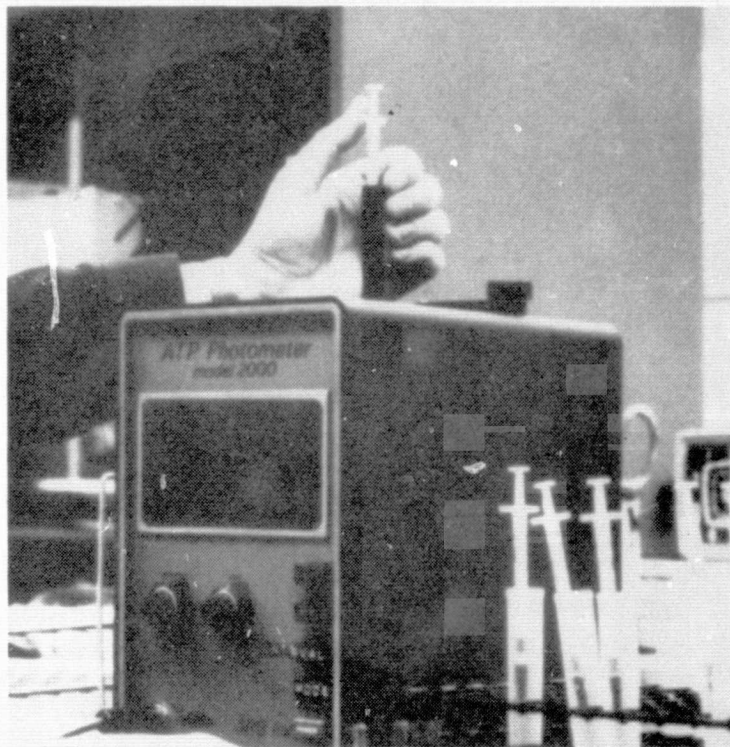
Figure 4.   SAI Model 2000 Photometer (Formerly JRB)

these photometric instruments include the measurement of algal ATP (Reference 15), oil field bactericide parameters (Reference 16), and ATP in soil and marine sediments (Reference 17).

In the SAI photometers, a shutter separates the photomultiplier tube from the reaction chamber as indicated in figure 5. Opening the shutter exposes the reaction mixture to the photocathode. A maximum volume of 2.0 ml contained in a standard scintillation vial may be assayed using the reflectorized conical sample holder provided by the company. An additional reflectorized conical holder has been fabricated at Goddard to accommodate a 6 x 50 mm cuvette. SAI also markets an automated injector kit which precludes the need to mix re-actants in the vial before placement in the reaction chamber. An assay button when pressed provides a maximum 6-digit readout of the peak or integrated light response.
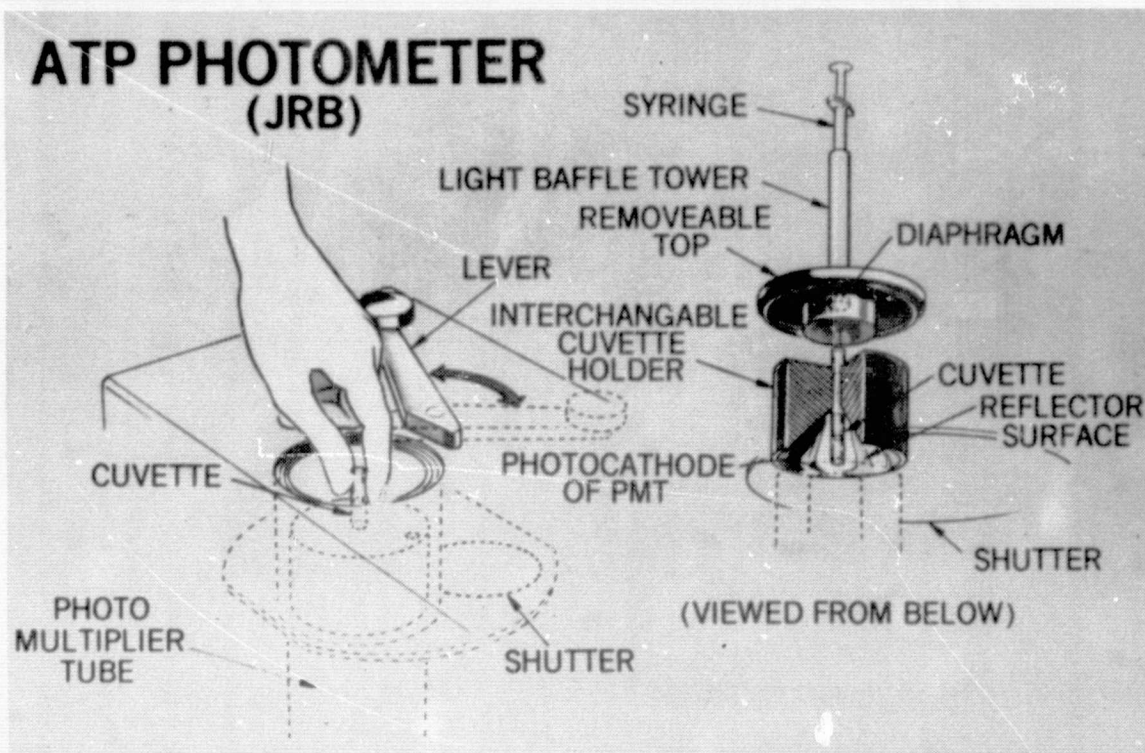
Figure 5.   SAI Photometer, Reaction Chamber

Aminco Chem-Glow Photometer

The Chem-Glow Photometer shown in figure 6 registers peak light response on
a meter with a manual range selection of 4 decades. Readout for this photometer,
as well as for the Biometer and SAI models, can also be monitored by oscillo-
scope or by a strip chart recorder, such as the Hewlett Packard (Moseley) x-y
recorder providing an x axis speed of 0.25 to 50 seconds per cm. An Aminco
integrator attachment measures total light flux for periods between 1 and 60
seconds with digital display of 3 decades. With integrator, recorder, and
automatic injector, the peak and total light response can be read simultaneously.
The Chem-Glow has been used successfully in such applications as the
determination of ATP content in germinating seeds (Reference 18), quantifi-
cation of algal ATP (Reference 19), and physiological studies of calcinosis
(Reference 20).

A rotating drum (see figure 7) houses 6- × 50-mm glass cuvettes with maximum
exposure of 0.4 ml of reaction mixture to the photocathode. Use of 12 × 35 mm
cuvettes in a larger optional reaction head exposes a 1.7 ml sample volume.

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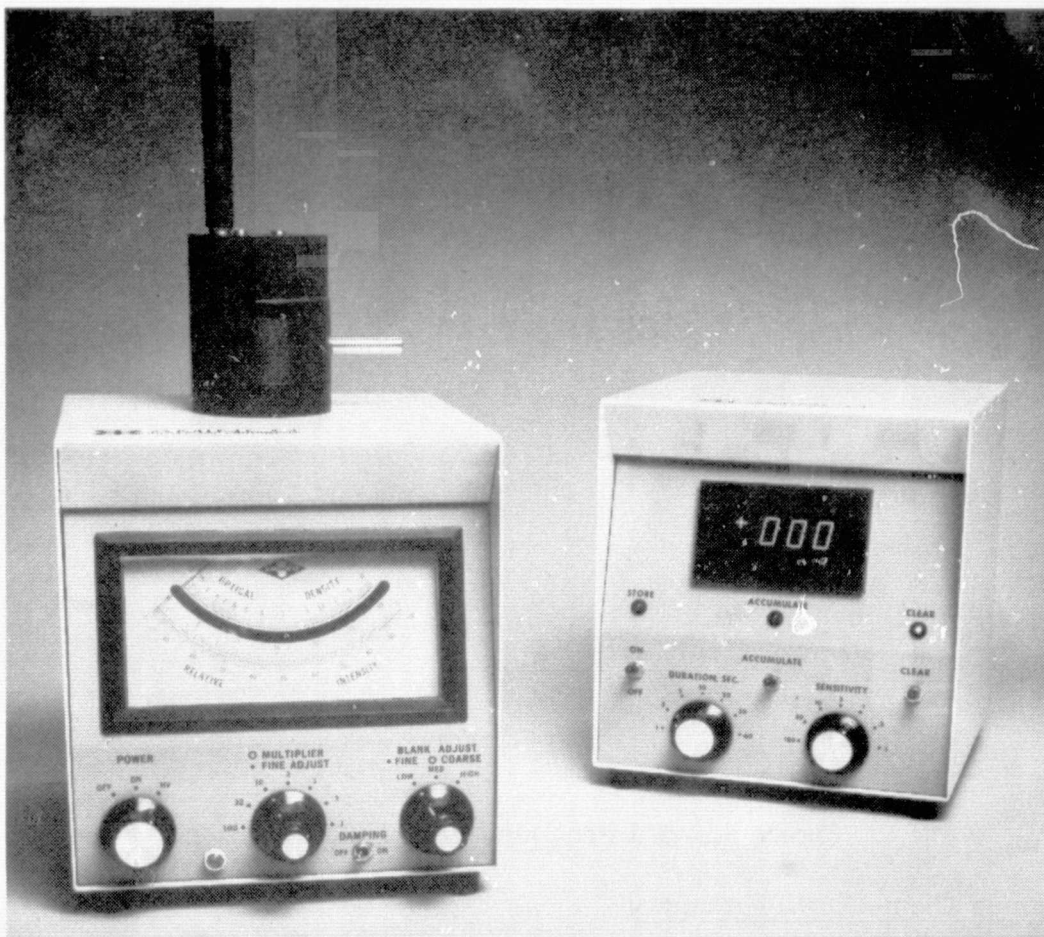
Figure 6. Aminco Chem-Glow Photometer and Integrator

COMPARISON OF PHOTOMETERS

The commercially available photometers discussed in this section have been tested and compared on the basis of functional sensitivity, linearity, and reproducibility. Tests include measured responses to a series of radioactive light standards varying by factors of ten, to serial dilutions of purified chemical ATP using the firefly luciferase ATP assay, and to extracted ATP from serial dilutions of E. coli using the same assay (Reference 21).

Adjusting the photomultiplier tube gain so that a particular radioactive light standard is set to the same percentage of the total range for each photometer, allows full use of the range of each instrument independent of the others.

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CHEM-GLOW
(AMINCO)

LIGHT BAFFLE TOWER

CUVETTE

SYRINGE

LEVER

ROTATING DRUM

DIAPHRAGM

CUVETTE

INNER SHIELD OPENING

ROTATING DRUM

PHOTOCATHODE OF PMT
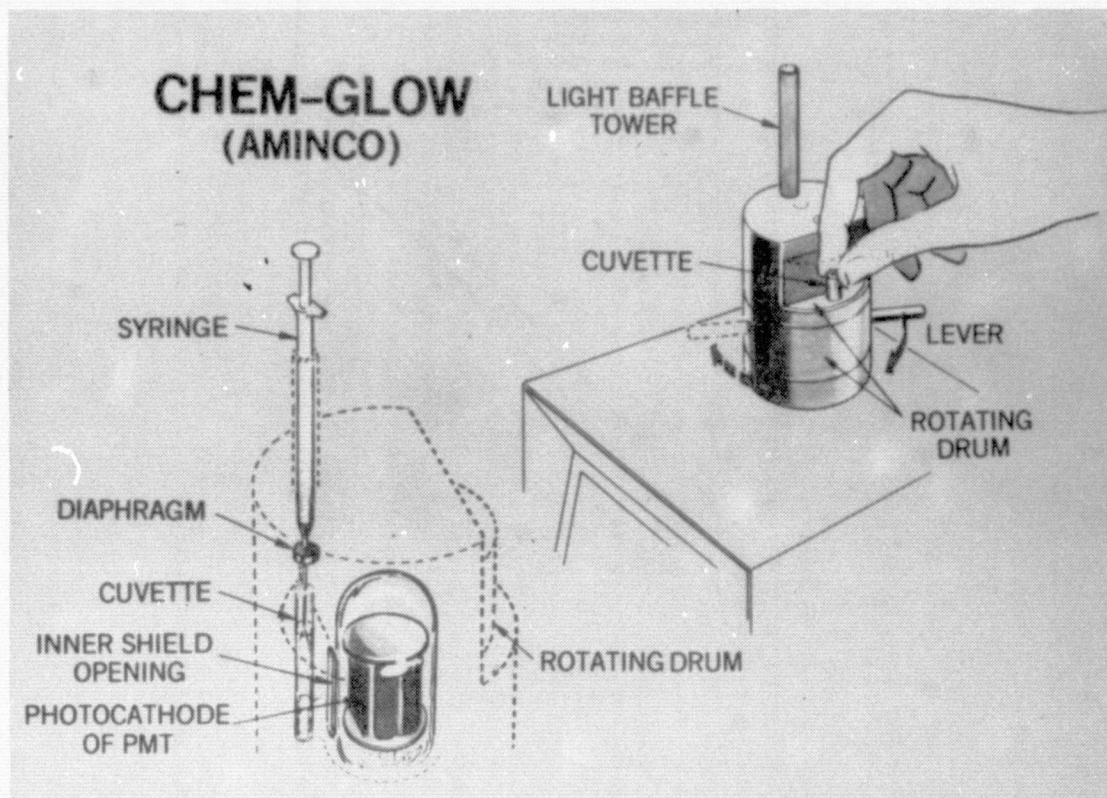
Figure 7.   Chem-Glow Photometer, Reaction Chamber

Choice of arbitrary "y" values for any particular curve permits all curves to be shown on the same graph. Therefore, figures 8 through 12 do not indicate absolute relationships between the photometers.

Figure 8 represents readings obtained from a series of four radioactive light standards. Linearity and reproducibility appear to be comparable in all cases. It is necessary, however, to use the integrator attachment with the Chem-Glow photometer to achieve sensitivity comparable to the Du Pont and SAI model photometers.

Figure 9 represents standard ATP curves produced by the three photometers. Linearity and sensitivity are quite comparable. Reproducibility begins to fail below a sensitivity level of 10^{-6} micrograms of injected ATP, as indicated by the extended Chem-Glow curve. Light responses from standard ATP diluted in nitric acid to simulate bacterial ATP extraction are graphed in figure 10. Figure 11 represents ATP curves at varying integration times, using the Chem-Glow photometer and integrator attachment. These data reveal that linearity is not dependent on integration time.

11
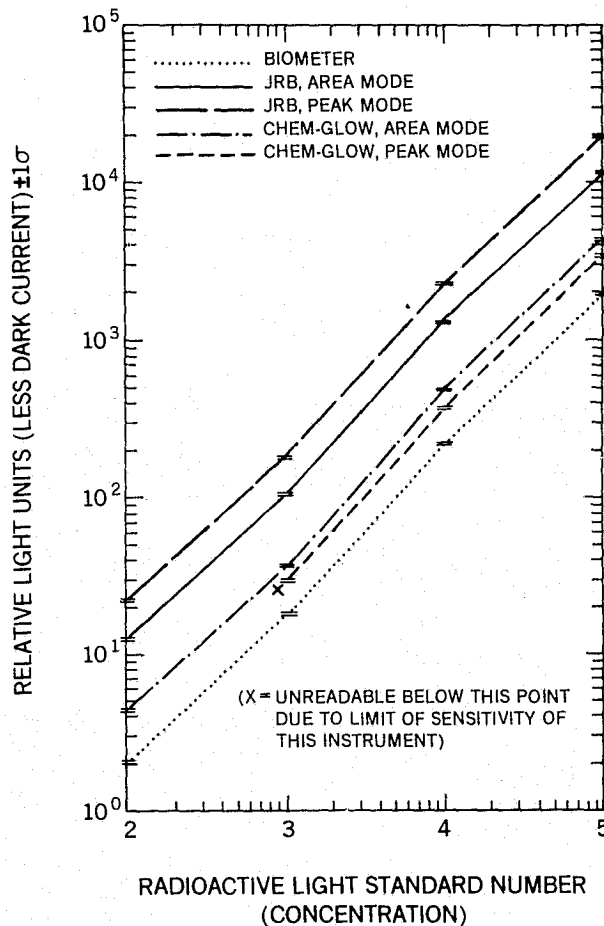
Figure 8. Comparison of Radioactive Light
Standards

Light responses to serial dilutions of ATP extracted from E. coli with nitric acid are shown in figure 12. About 10^4 bacteria injected can be detected reliably with all three instruments in peak and integrated modes.

VITATECT II

Vitatect Corporation is in the process of marketing an instrument that provides a movable filter tape for concentrating and processing the sample, a unique feature among photometers. In the detection of microorganisms using the firefly luciferase ATP assay, the concentration of bacteria, extraction of ATP, and reaction with luciferase all occur on the filter tape inside the photometer.
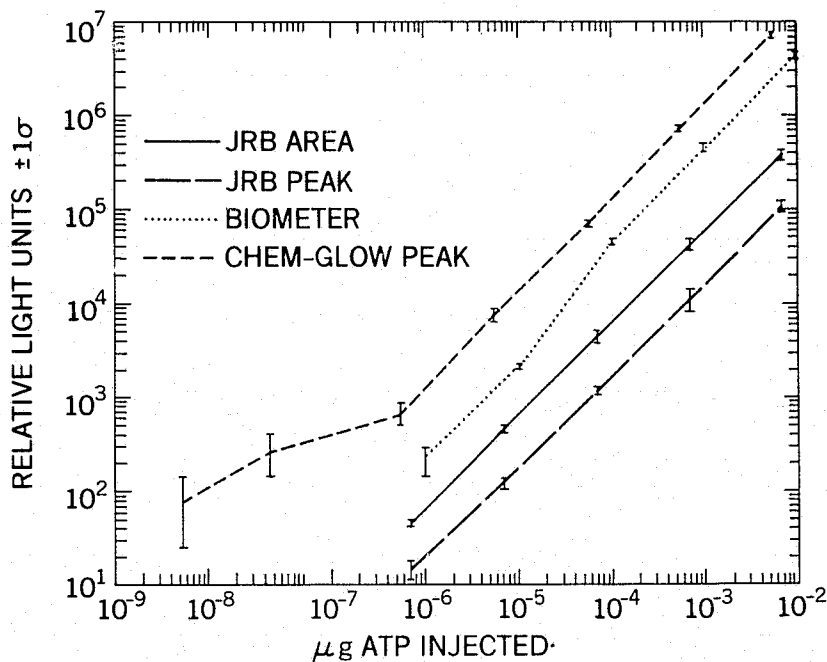
Figure 9. ATP Concentration Curve, Without Nitric Acid,
Comparing Three Photometers

Vitatect II, a prototype instrument shown in figure 13 and evaluated at GSFC,
consists of a reaction system incorporating the movable 0.22μ filter tape and
a photometer with a ten-stage head-on photomultiplier tube operated at 2000
volts. Momentary peak light emissions register on a log decade meter and inte-
gration values ranging from 0 to 99 seconds, with a selected 0- to 99-second
delay, concurrently appear in digital display over a range of 7 decades
(Reference 22).

The reaction system schematically diagrammed in figure 14 incorporates three
separate injection ports: one for the initial application of the sample to the
filter tape; one for optional treatment of materials held by the filter with
washing solutions or extracting agents; and one for addition of the light-producing
reagent. An indexed knob moves the (extracted) sample-impregnated-area
of the filter into reaction position above the photocathode where a transparent
cellophane tape forms a backing for the filter tape and protects the surface of
the photomultiplier tube. The light-producing reagent is injected in a volume
barely sufficient to wet the impregnated area and penetrate the back of the
filter tape. Light emission is then registered in terms of peak and integrated
values (Reference 22).
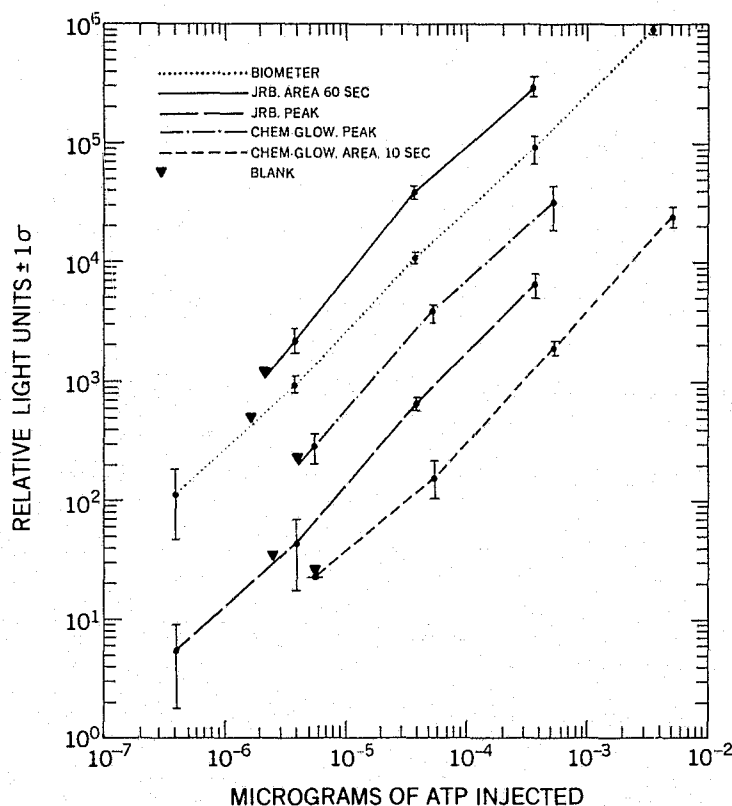
Figure 10. ATP Concentration Curve, with Nitric Acid,
Comparing the Three Photometers

An evaluation of the performance characteristics of the Vitatect II included
tests similar to those used in comparative studies of the other commercially
available photometers. The radioactive light standards when exposed to the
photocathode confirm linearity within and between range settings as well as
reproducibility at each combination. The results of serial dilutions of ATP
applied to the filter tape and reacted with firefly luciferase above the photo-
cathode demonstrate a sensitivity of 10^{-6} micrograms of injected ATP which
is similar to the sensitivity of other photometers. A concentration curve meas-
ured by the firefly luciferase ATP assay confirms overall linearity, repro-
ducibility, and sensitivity provided by this instrument. Table 1 lists bacteria
per ml as determined by an initial Coulter count, light response to each
dilution minus a reagent blank reading, and coefficients of variation. The
results taken at 30-second integration counts appear to be linear from 7×10^8
to about 10^4 bacteria per ml.

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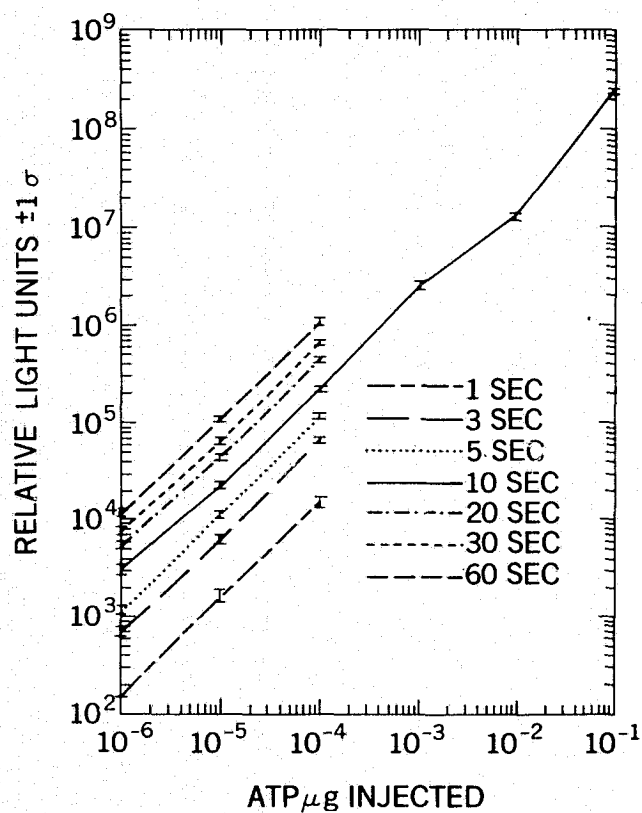
Figure 11. ATP Concentration Curve, without
Nitric Acid, Using the Chem-
Glow with Integrator at
Various Integration
Times
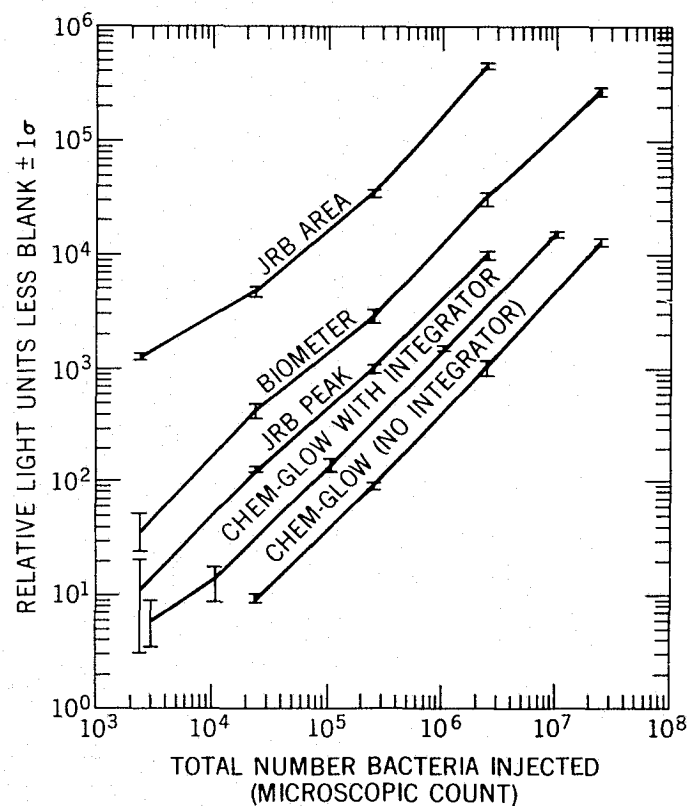
Figure 12. Bacterial Curve, Using Nitric
Acid on Three Photometers
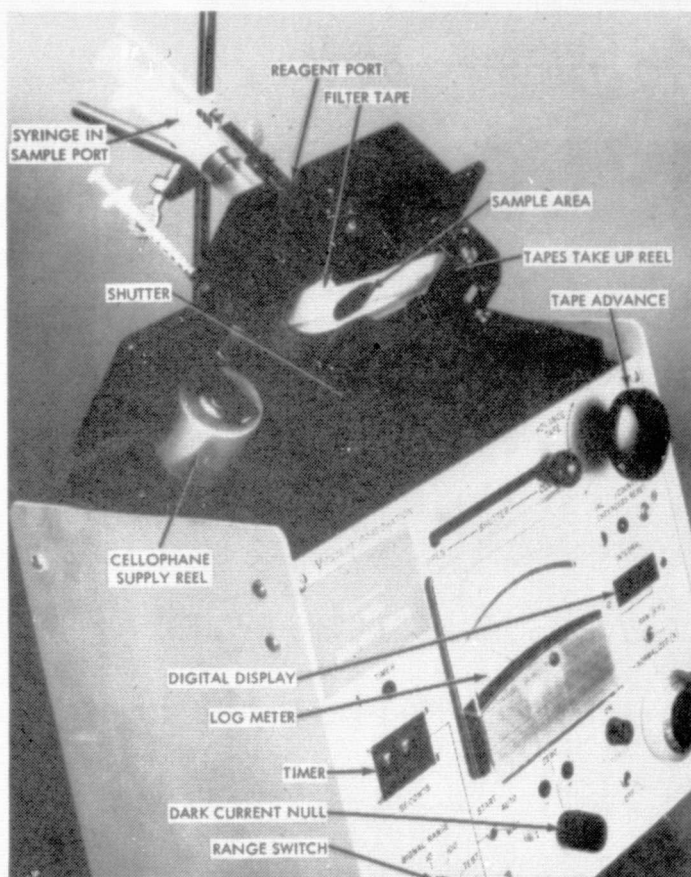
Figure 13. Vitatect II Prototype Instrument

LIQUID SCINTILLATION COUNTERS

In addition to these photometers, liquid scintillation counters designed to measure radioactivity, and spectrophotometers (Reference 23) have been used successfully to measure low level light reactions. In luminescent reactions the enzyme or chemiluminescent compound is the light producer instead of the liquid scintillator. The resultant light flash is measured in photons per second. Scintillation counters utilize two photomultiplier tubes and coincidence circuitry to reduce false counts given by extraneous sources. Coincidence circuitry should not be used with luminescent light emissions since only single photon events occur, and a loss in signal will result in the case of a low concentration of unknown sample (ATP, for example). To use a scintillation counter effectively with luminescent reactions, simply disconnect one of the photomultiplier tubes prior to assay or deactivate the coincidence circuits (Reference 24). These photomultiplier tubes are of particularly high quality, and are often cooled to further reduce dark current.
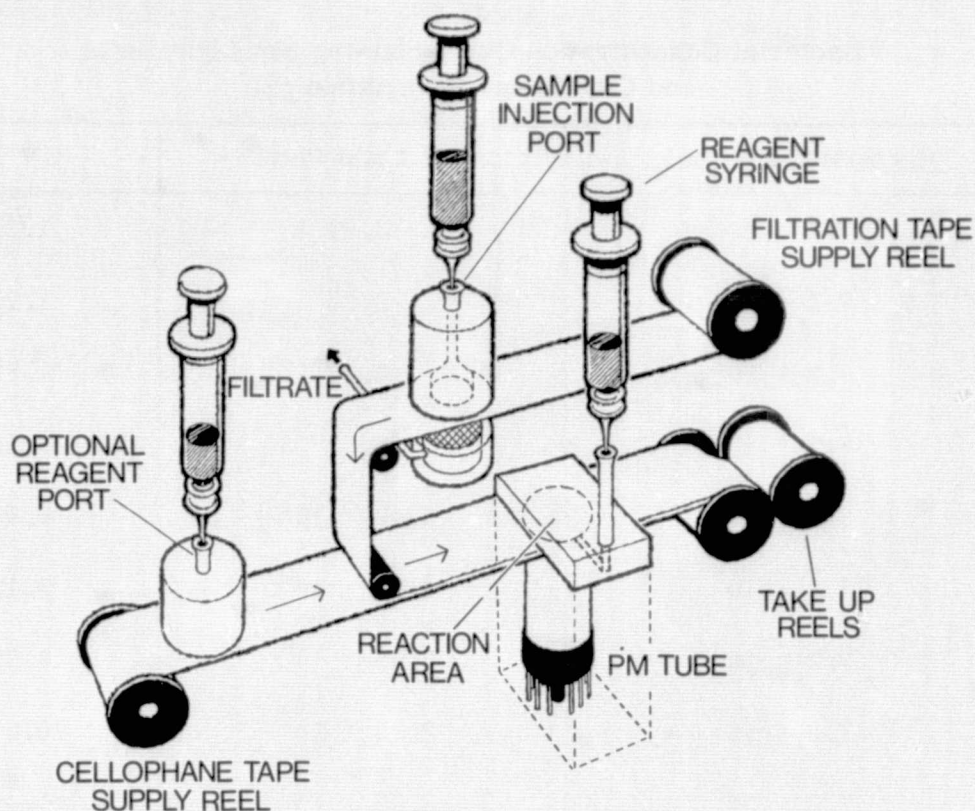
Figure 14.   Vitatect II, Reaction System

The additional sensitivity achieved with the use of high quality photomultiplier tubes in liquid scintillation counters may actually prove to be a disadvantage when measuring bioluminescent and chemiluminescent reactions. Inherent light levels due to the luciferase enzyme or to the chemiluminescent reactant, and blank light levels produced by all reagents in the sample except the factor being measured, may register high readings. Blank sample readings, including inherent light of the reactant, should be subtracted from sample readings for the unknown factor in order to reflect true sample values. High blank levels will also limit the available range for sample measurement above the blank value. Another disadvantage is that the configuration of the liquid scintillation instrument does not lend itself to reactions requiring addition of one liquid to another in a light-tight environment. Unless the instrument has been mechanically modified, sample and reactant must be premixed outside of the instrument and then inserted into the scintillation counter chamber. Because luminescent reactions are instantaneous, seconds lost in mixing and positioning the reactants may result in a measurement that misses the peak light response.

Table 1
Bacterial Concentration Curve Showing Net Light Units
and Coefficient of Variation (%)

Bacteria/Filter/ml	Net Light Units	CV %
7.2×10^0	3.43	1.22
7.2×10^1	2.25	7.7
7.2×10^2	3.68	1.89
7.2×10^3	6.00	8.16
7.2×10^4	3.42×10^1	4.99
7.2×10^5	4.00×10^2	3.13
7.2×10^6	2.95×10^3	4.38
7.2×10^7	3.25×10^4	10.2
7.2×10^8	3.48×10^5	1.44

FIREFLY LUCIFERASE ADENOSINE TRIPHOSPHATE (ATP) ASSAY

REAGENTS

All reagents are prepared in sterile, distilled, deionized water, from chemicals of the highest purity available. It is advisable to use disposable plasticware or glassware that has been acid cleaned (soaked in 1.0 N HCl for 30 minutes), and thoroughly rinsed in distilled H_2O, to remove residual ATP. Avoid the use of potassium salts as these are more inhibitory to luciferase than sodium salts; therefore, pH adjustments should be made with NaOH and HCl. Reagent solutions must be stored at $10^\circ C$ or lower (refrigerated) to inhibit bacterial growth.

Adenosine Triphosphate (ATP)

Determination of the light response from a known quantity of ATP in a defined reagent environment allows the conversion of light units to micrograms (μg) of ATP for a sample assayed in the same luciferase preparation under the same defined reagent conditions.

Adenosine 5' Triphosphate: Equine Muscle Disodium Salt, crystalline form

molecular weight (MW) 614.2

Sigma Chemical Company

Each preweighed vial contains 30 mg ATP which is 99 percent pure and contains 3.5 H_2O per mole (the amount of H_2O of hydration is variable).

Store at -20°C

Preparation of Stock—

1000 μg ATP/ml

MW pure ATP = 507.2

MW Sigma disodium salt = 614.2

.milligrams of pure ATP in 30 mg Sigma ATP at 99% purity =

$\frac{507.2}{614.2}$ x 30 mg x 0.99 = 24.53 mg

24.53 mg ATP + 24.53 ml H_2O ⟶ 1000 μg ATP/ml

- Add 24.53 ml H_2O to contents of one vial Sigma ATP and mix well: Rinse vial with ATP solution to collect any remaining ATP. Mix final solution well.

- Dispense in 1000 μg (1 ml) aliquots and store at -20°C.

Preparation of ATP Dilutions—

- Dilute stock serially 1:10 to desired concentration using diluent appropriate for application (H_2O, saline, HNO_3, and so on).

- ATP is unstable and cannot be stored at room temperature or refrigerated without a steady decline in activity. It is therefore necessary to prepare fresh standard ATP from frozen stock for use with each assay. Alternatively, a 1:10 dilution of stock in M_gSO_4 (1×10^{-2} M) and ethylenediamine tetraacetic acid (EDTA) (1×10^{-3} M) can be stored at 25°C for an 8-hour period without significant decay of ATP and used as a stock for further dilution throughout the day. Be sure to dilute Mg^{+2}/EDTA-treated ATP before use as a standard assay because this concentration of Mg^{+2} inhibits the luciferase response by about 30 percent.

Apyrase

Soluble ATP, which may contaminate a sample to be assayed for bacterial ATP can be removed by the addition of apyrase, an ATP hydrolyzing enzyme.

The pH for optimum apyrase activity is 6.0. The lowest pH for activity is 3.0. The apyrase must be inactivated before extracting the bacterial ATP to be assayed. This is done with the extractant, nitric acid, by lowering the pH to below 3.0. Apyrase can be reactivated if the pH is again raised, so care must be taken with the extracted samples to keep the pH below 3.0, until injection into the luciferase. Since Ca^{+2} is required as a cofactor at an acting concentration of 5×10^{-3} M, apyrase is extracted in the appropriate molarity of $CaCl_2$.

Apyrase is very stable and extremely active. It contains varying amounts of ATPase, ADPase, and AMPase activity. Because there may be phosphorylating enzymes associated with the luciferase enzyme preparation, the presence of ADP and AMP can interfere with quantitation of the amount of ATP when using the luciferase reaction. Each lot of apyrase should be assayed for its ability to produce a low blank with a high concentration of ATP after a constant time. The concentration of the new lot should be adjusted to give a constant activity; for example, removal of 100 μg ATP in 15 minutes to two times the blank level.

When using concentration procedures, different lots of apyrase may result in varying amounts of particulates that interfere with extraction and mixing by clogging a filter or settling in a pellet in the centrifuge tube. In such cases, 10 to 100 mg/ml of apyrase are extracted in $CaCl_2$ and centrifuged (10,000 rpm for 5 minutes) or filtered. Apyrase may then be lyophilized for storage and reconstituted to the desired activity when needed.

Apyrase: 5' ATPase-2.5 units per mg, 5' ADPase-0.46 units per mg, 5' AMPase-

0.03 units per mg.

Potato purified-grade 1

Sigma Chemical Company

Store dry below 0°C

2,000 units per vial based on 5'-ATPase activity

Calcium Chloride (CaCl$_2$)

Within the concentration range of apyrase used (acting concentration 1 to 10 mg/ml), calcium is required as a cofactor at 5×10^{-3} M. Apyrase is rehydrated in the appropriate molarity of CaCl$_2$ (or CaCl$_2$ – 0.6 percent TX) to maintain an acting concentration of approximately 5×10^{-3} M Ca^{+2}.

A stock solution of 3 M CaCl$_2$ is stored at -20°C.

> Calcium Chloride (Anhydrous)
>
> MW 110.99
>
> Analytical reagent
>
> Mallinckrodt Laboratory Chemicals
>
> Store at room temperature

Preparation of 100 ml of 0.03 M CaCl$_2$, 0.6-percent Triton X-100 (TX-100):

> (1)   Combine 1 ml stock 3 M CaCl$_2$ and 10 ml 6-percent Triton X-100.
>
> (2)   Add H$_2$O to produce 100 ml and mix well.

Cleland's Reagent

Cleland's reagent is a sulfhydryl protecting compound. Since luciferase contains sulfhydryl groups at its active sites, a solution of 1×10^{-3} M Cleland's in the rehydrating Tris (hydroxymethyl) aminomethane ('TRIS) buffer will maintain maximum luciferase activity for 8 hours with refrigeration, and will minimize activity decay during an assay period.

Dithioerythritol or Dithiothreitol

> MW 154.3
>
> Sigma Chemical Company
>
> Store desiccated at 0° to 5°C

Ethylenediamine Tetraacetic Acid (EDTA)

The chelating agent EDTA may be used at a concentration of 1×10^{-3} M to stabilize both standard ATP and luciferase for 24 hours and to limit inhibition of the reaction by metallic ions.

Ethlenediamine Tetraacetic Acid: Disodium Salt

    MW 336.2

    Sigma Chemical Company

    Store at room temperature

Luciferin-Luciferase

Dessicated firefly lanterns may be extracted in buffer to yield a crude luciferin-luciferase preparation. The enzyme luciferase is apparently a protein of MW 50,000 which commonly occurs in an aggregrate (dimer) form. There is evidence that luciferase is actually a heterogeneous group of proteins with slightly different substrate binding properties (Reference 25). Each protein unit has several free sulfhydryl groups, of which two are essential for luciferin binding. The cofactor magnesium is required at 1×10^{-2} M for formation of the activated enzyme-substrate (luciferin MW 280.33) complex.

Crude extracts of firefly lanterns are available from Calbiochem, Antonik, Inc., Sigma Chemical Company, and Worthington Biochemical Corporation at a cost of less than $0.10 per assay. Procedures for further purification require the addition of synthetic luciferin (approximately $3.00 per mg) which increases the cost of the assay but significantly improves sensitivity (approximately 100 fold). Boehringer Mannheim and E. I. Du Pont offer purified enzyme preparations.

The Du Pont purified luciferin-luciferase has been utilized for our procedures development. Each reagent kit consists of 20 vials of lyophilized enzyme-substrate, 1 vial morpholinopropane sulfonic acid (MOPS) buffer tablets, and reaction cuvettes (6 x 50 mm) at a cost of about $300 (as of 1976). The enzyme is stored at -20°C. Du Pont recommends rehydration of one vial in 3.0 ml of the supplied MOPS buffer. To obtain maximum sensitivity, we suggest rehydrating in 1.5 ml TRIS-Mg^{+2} buffer, which yields 300 assays per reagent kit (0.1 ml enzyme-substrate per assay).

The salt required for solubility of the globulin luciferase is supplied by the rehydration buffer TRIS, as is the cofactor magnesium. The addition of

1×10^{-3} M Cleland's reagent (in TRIS buffer) promotes enzyme stability by protecting the sulfhydryl groups. Rehydrated enzyme, stored in the dark at 10°C, will remain stable (negligible activity loss) for 4 hours. This period is increased to 8 hours in Cleland's reagent, and with the addition of 1×10^{-3} M EDTA, it is stable for 24 hours. EDTA is not necessary for short-term storage and need not be used unless pooled enzyme is prepared for use over an entire workday, or metallic contaminants are expected in the sample.

After rehydration, the luciferase mixture emits light without the addition of any ATP. This inherent light may be due to small amounts of ATP in the enzyme mixture that are not removed during purification. Incubation of rehydrated enzyme at room temperature, for at least 45 min. prior to assay, will reduce the level of inherent light.

An alternative method for obtaining highly purified luciferase (at reduced cost) involves preparation from dessicated firefly tails as follows:

Preparation of Acetone Powder--

- Grind firefly lanterns (Sigma Chemical Company, Antonik, Worthington, or Calbiochem--\$20 per gram) and a small quantity of sand with mortar and pestle, keeping the mixture cold with liquid nitrogen or an acetone-dry ice mixture. Grind to a fine powder.

- Extract with cold acetone (4°C) for 10 minutes to remove lipids (at least 100 ml per 5 grams).

- Filter (Buchner) and wash with cold acetone. Dry completely and store at -20°C.

Preparation of Crude Extract of Luciferase-Luciferin--

- Add 10 ml cold 0.05 M TRIS, pH 7.75, with 1×10^{-3} M Cleland's reagent per gram acetone powder. Mix gently at 10°C for 30 minutes.

- Centrifuge at 10,000 relative centrifugal force x gravity (RCF x G) for 10 minutes. Collect supernate and discard precipitate.

Purification of Luciferase--

- At room temperature bring to 30 percent $(NH_4)_2 SO_4$, and discard precipitate; bring to 70 percent $(NH_4)_2 SO_4$, and collect precipitate.

- Wash luciferase (70 percent precipitate) in 70 percent $(NH_4)_2 SO_4$ pH 7.75.

- Dissolve washed pellet in TRIS buffer: 0.05 M, pH 7.75, 1×10^{-3} M Cleland's reagent, 1×10^{-2} M $MgSO_4$ (minimum volume 2.5 ml/g acetone powder). Centrifuge to clarify. Apply enzyme to Sephadex G-200 column and elute with the same TRIS buffer. Assay fractions by mixing small portion 1:1 with luciferin in TRIS buffer.

- Pool fractions with high activity and low inherent light. (One gram of acetone powder should produce about 20 ml of enzyme with activity equal to the Du Pont product.) Dilute pool (with same TRIS buffer) to desired activity level. Add 0.1 mg luciferin per 1 ml diluted fraction pool. Aliquot and lyophilize enzyme. Store dessicated at -20°C.

- Rehydrate in H_2O (for 0.05 M TRIS, pH 7.75) or 0.20 M TRIS, pH 8.2 (for 0.25 M TRIS, pH 8.20).

Magnesium Sulfate ($MgSO_4$)

Magnesium is a cofactor required in the luciferase reaction at a concentration of 10^{-2} M. This is added to TRIS buffer to reach a final concentration of 0.01 M $MgSO_4$ (1.0 ml stock 1 M $MgSO_4$ plus 99 ml TRIS buffer). $MgSO_4$ in the same concentration may be used to stabilize standard ATP.

Magnesium Sulfate (Anhydrous) -Certified ACS

MW 120.37

Fisher Scientific Company

Store at room temperature.

A stock solution of 1 M $MgSO_4$ is stored at -20°C.

Malate Buffer

Contaminating ATP in a sample to be assayed for bacterial ATP may be bound to surfaces and large molecules, such as membrane fragments and proteins, that protect it from hydrolysis by apyrase. Release of bound ATP occurs if the pH of the specimen is dropped to approximately 4.25, where the binding of ATP by salt linkages is depressed and apyrase remains marginally active.

Malic acid buffers effectively in this pH range with a pK of 3.4.

    Malic Acid

    MW 134.1

    Sigma Chemical Company

    Store at room temperature.

Nitric Acid (HNO_3)

Nitric acid inactivates apyrase and ruptures the bacterial cells, releasing bacterial ATP. The optimal nitric acid concentration is the lowest necessary for complete ATP extraction. For 10 bacterial urinary pathogens tested, 0.1 N HNO_3 was found to be the optimal extracting concentration, added directly to the pellet or filter for extraction. If the bacteria are not concentrated in a negligible volume, a stronger nitric acid concentration must be added to account for acid dilution by sample volume and to achieve the final extracting concentration of 0.1 N. The pH of the nitric-treated sample should be at or below 1.2.

Nitric Acid: Reagent ACS

    Fisher Scientific Company

    16 Normal, 71 percent by weight

    Store at room temperature.

Preparation of Stock

    (1)   1.5 N: 9.37 ml 16 N HNO_3 per 100/ml

    (2)   1.0 N: 6.25 ml 16 N HNO_3 per 100/ml

    (3)   Stock solution should be stored at $10^\circ C$ or lower.

Rhozyme-41

Rhozyme-41 is a mixture of proteases from Aspergillis orizae. The enzyme is active over pH 6 through 9, with the optimum for degradation of urine glycoproteins and mammalian cell membranes being about pH 8.

Rhozyme-41 Concentrate

Rohm and Haas Company

Store at 10°C or lower

Preparation

(1) Extract Rhozyme concentrate in H_2O (8 g powder concentrate in 92 ml H_2O) overnight at 10°C.

(2) Centrifuge at 12,000 RCF $\times$ G, and filter supernate (0.2μ).

(3) Store supernate at 10°C, and use within 48 hours.

Sodium Sulfate (Na_2SO_4)

Sodium sulfate has recently been substituted for H_2O as the diluent in the nitric acid extraction procedures. It has been determined that Na_2SO_4 lowers the blank reading by approximately 70 percent with a concurrent 50 percent loss in luciferase activity, thereby effecting a net increase in sensitivity. The Na_2SO_4 is used to adjust the ionic strength of the injected sample to equal or exceed the ionic strength of the luciferase preparation. The sample pH after Na_2SO_4 addition should be 1.8 to 2.1.

Sodium Sulfate: Certified ACS (Anhydrous)

MW 142.04

Fisher Scientific Company

Store at room temperature

TRIS Buffer

The lyophilized luciferase powder is rehydrated in TRIS buffer, containing magnesium, a cofactor in the reaction. One vial of powder is rehydrated in 1.5 ml TRIS-Mg buffer. The pH and concentration of the TRIS are critical. For optimum light response, the final pH after injection of the sample should be 7.75 ± 0.1. If the sample contains HNO_3, the concentration and pH of the TRIS must be adjusted to maintain the reaction pH optimum. Use of the lowest concentration and pH of TRIS that will maintain pH 7.75 will minimize luciferase activity loss. TRIS is also used to buffer samples requiring rhozyme to approximately pH 8.0.

Trizma Base (Tris [hydroxymethyl] aminomethane: Reagent Grade)

MW 121.1

Sigma Chemical Company

Store at room temperature

Preparation of Stock (100 ml) 2 M TRIS

(1) Add 24.2 g of Trizma Base to 80 ml of H_2O. Mix occasionally to dissolve; do not heat.

(2) Bring solution to final volume of 100 ml.

(3) Filter through 0.22 μ membrane.

(4) Store at $-20^\circ C$.

Preparation of Rehydrating Buffer

(1) 0.05 M TRIS: 2.5 ml 2 M stock/100 ml
     0.25 M TRIS: 12.5 ml 2 M stock/100 ml

(2) If TRIS is to be used to reconstitute luciferase, 0.01 M $MgSO_4$ and 1×10^{-3} M Cleland's reagent are required. For every 100 ml of TRIS to be made, decrease volume of H_2O by 1.0 ml and add 1.0 ml 1 M stock $MgSO_4$ and 15.43 mg Cleland's reagent. (EDTA may also be added at 1×10^{-3} M for increased luciferase stability, but since rehydrated enzyme is seldom stored for more than a few hours, EDTA is not routinely placed in the rehydrating buffer.)

(3) The pH of the solution is adjusted before bringing it up to final volume: pH 7.75 for 0.05 M TRIS and pH 8.20 for 0.25 M TRIS. To allow for the addition of pH reagents (HCl and NaOH), the solution should be brought to about 80 percent of final volume before pH adjustment. It should then be brought to full volume and the pH rechecked.

Triton X-100

Frequently, a specimen to be analyzed for bacteria contains contaminating non-bacterial cells such as mammlian cells: red blood cells, white blood cells, or epithelial cells. To obtain true bacterial values, these other sources of ATP must be removed prior to bacteria rupture. The nonbacterial ATP is released

by Triton X-100 (TX), a nonionic detergent, making the ATP accessible to hydrolysis by potato apyrase. Triton X-100 is added at 0.001 percent to all reagents used in filtration procedures to facilitate rapid sample and reagent flow.

Triton X-100 (Octyl Phenoxy Polyethoxyethanol)

Sigma Chemical Company

(Triton is a trademark of the Rohm and Haas Company)

Store at room temperature

Preparation of 100 ml of 6-Percent TX-100 Stock Solution:

(1)   Fill pipette with 6.0 ml of TX, do not overfill (use a pipette filler). Wipe off outside of pipette.

(2)   Rinse pipette in 94 ml of warm H_2O until all TX is dissolved. Swirl gently to mix.

(3)   Heat TX solution just to boiling. It will become cloudy, but will clarify on cooling.

Preparation of 100.0 ml of 10-Percent TX-100 Stock Solution:

(1)   Heat 90.0 ml of H_2O as above.

(2)   Add 10.0 ml of TX-100 as above.

-CAUTION-

Care should be taken not to swallow any TX-100, as ingestion of TX in rats has been shown to cause subcapsular cysts on the ovaries. If TX should come in contact with skin, wash immediately with volumes of tap water.

Typical Listing of Reagent Supplies

E. I. Du Pont de Nemours and Company, Inc.
Instrument Products Division
Wilmington, Delaware 19898
(302) 774-1000

Fisher Scientific Company
711 Forbes Avenue
Pittsburgh, Pennsylvania 15219
(412) 781-3400

Mallinckrodt Laboratory Chemicals
223 West Side Avenue
P. O. Box 384
Jersey City, New Jersey 07303
(201) 432-2500

Rohm and Haas Company
Independence Mall West
Philadephia, Pennsylvania 19105
(215) 592-3143

Sigma Chemical Company
P. O. Box 14508
St. Louis, Missouri 63178
(314) 771-5765

ALTERNATIVE EXTRACTION PROCEDURES

In addition to HNO_3, several extractants were tested in conjunction with our procedures development including ionizing organic solvents (90-percent dimethyl sulfoxide (DMSO), 10-percent formamide), nonionizing organic solvents (90-percent acetone, butanol, chloroform, ethanol, methanol, methylene chloride), boiling TRIS buffer, and other inorganic acids (H_2SO_4, H_3PO_4, and perchloric). Of these extractants, only a few proved efficient in the extraction of bacterial ATP: 90-percent acetone, boiling TRIS, 90-percent methylene chloride, 90-percent DMSO, H_2SO_4, perchloric acid, and HNO_3.

When acids are used as extracting agents, the molarity of TRIS used for luciferase rehydration must be high enough to maintain optimal reaction pH. Nonionizing organic solvents (removed in extraction procedure by heating) and boiling TRIS do not alter the reaction pH on injection. This allows the use of low molarity TRIS at pH 7.75 which increases enzyme activity. Acid extraction, however, is instantaneous and has the added advantage of inactivating ATPases which may be released on bacterial ATP extraction or added for removal of soluble ATP.

The injection of DMSO (at about 70-percent concentration) results in a 90-percent loss in luciferase activity. Dilution of the DMSO extracted sample before assay reduces this loss. Potential sensitivity losses are negated by low blank levels (compared to acid extractants).

-CAUTION-

It should be remembered that methylene chloride and DMSO
are hazardous compounds. Do not mouth pipette or allow
skin contact.

Inorganic Acids: HNO_3 H_2SO_4, PCA (perchloric acid)

Extraction Procedure—

- Add 0.2 ml of 0.1 N acid to bacterial (sample) pellet. Wait 5 minutes. Dilute with 0.2 ml of 0.15 M Na_2SO_4. Vortex.

  Or add 0.1 ml of 1.5 N acid to 0.5 ml sample fluid. Vortex. Wait 5 minutes. Dilute with 4.4 ml of 0.085 M Na_2SO_4. Vortex.

- Assay: luciferase prepared in 0.25 M TRIS, pH 8.2, 1×10^{-2} M $MgSO_4$, 1×10^{-3} M Cleland's reagent.

Nonionizing Organic Solvents: Acetone, Methylene Chloride

Extraction Procedure—

- Add 5 ml of 90-percent organic solvent to bacterial pellet or 4.5 ml of 100-percent organic solvent to 0.5 ml of sample fluid. Vortex. Heat at 90°C for 40 minutes to evaporate solvent. Add 0.4 ml of 0.085 M Na_2SO_4. Vortex.

- Assay: luciferase prepared in 0.05 M TRIS, pH 7.75, 1×10^{-2} M $MgSO_4$, 1×10^{-3} M Cleland's Reagents.

Boiling Tris

Procedure—

- Add 5 ml of boiling 0.02 M TRIS, pH 7.75, to pellet or 4.5 ml of boiling 0.02 M TRIS, pH 7.75, to 0.5 ml of sample fluid. Mix. Heat 5 minutes at 100°C. Cool in ice bath (or dilute with TRIS).

- Assay: luciferase prepared in 0.05 M TRIS, pH 7.75, 1×10^{-2} M $MgSO_4$, 1×10^{-3} M Cleland's reagnet.

DMSO

Filter Procedure using 90-percent DMSO—

- Filter sample on 0.22 or 0.45 μ Gelman Acropore membrane.

- Wash with 10 ml saline.

- Add 2 ml of 90-percent DMSO. Wait 3 minutes. Collect extract.

- Add additional 2 ml of 90-percent DMSO. Wait 3 minutes. Collect extract.

- Add 1 ml of H_2O. Collect extract.

- Assay: luciferase prepared in 0.05 M TRIS, pH 7.75, 1×10^{-2} M $MgSO_4$, 1×10^{-3} M Cleland's reagent.

MEASUREMENT OF SOLUBLE ATP IN BODY FLUIDS

Measurable quantities of soluble ATP pervade most body fluids in addition to the ATP that may be extracted or freed from bacteria. Cellular debris or membrane fragments of mammalian cells found in urine, for example, are also sources of soluble ATP. Because ATP is an important energy-rich compound required in the proper functioning of all cells, fluctuations in soluble ATP levels may be indicative of bodily changes occurring in response to a particular environment, malfunction, or disease. Such fluctuations can be monitored by selectively measuring free ATP using the firefly luciferase assay. The amount of light produced from the reaction between the body fluid and the luciferase is proportional to the amount of ATP present in solution.

Reagents

Apyrase

0.03 M $CaCl_2$

1.5 N HNO_3

Sterile deionized water

Luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2.

Standard ATP

Procedure

<u>Removal of all ATP for Blank Measurement</u>—Filter urine through a 0.45 μ Millipore cellulose filter to remove all particulate sources of ATP. To a 0.5-ml sample of prefiltered urine, add 0.1 ml of 10 mg apyrase per ml 0.03 M $CaCl_2$. Vortex, and wait 15 minutes for enzyme to hydrolyze all soluble ATP. Add 0.1 ml 1.5 N HNO_3, and dilute with 4.3 ml sterile, deionized water.

Assay by injecting 0.1 ml of the sample into 0.1 ml of luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2. Record light units.

<u>Selective Measurement of Soluble ATP</u>—To a second 0.5-ml sample of prefiltered urine add 0.1 ml 1.5 N HNO_3 and 0.1 ml of 10-mg apyrase per ml 0.03 M $CaCl_2$. In the presence of the nitric acid, the apyrase is inactive and serves only to simulate the chemical environment of the blank. Dilute with 4.3 ml H_2O and assay by injecting 0.1 ml of the sample into 0.1 ml luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2. Record light units.

Discussion

The difference between the light units generated by the sample and the blank light units represents the light signal due to soluble ATP. For greater accuracy in the measurement of small amounts of soluble ATP, the blank and sample light unit values must emanate from a common chemical environment. Nitric acid is added to the blank after apyrase has hydrolyzed all free ATP in order to create the same background solution as in the sample which contains nitric-acid-inactivated apyrase.

To translate light units into micrograms of ATP per ml, an ATP standard must be processed in the same manner as the blank, adding a known concentration of ATP diluted in the nitric acid. The signal due to soluble ATP will then be directly proportional to the ATP standard signal times the known concentration of ATP in the standard.

Example of Values

The soluble ATP detection procedure demonstrates a range of sensitivity from 10^{-5} micrograms to 10^{-1} micrograms ATP per ml sample. Soluble ATP values found in random clinical urine samples are given in the following list*:

Specimen #	μg Soluble ATP/ml
1	1.67×10^{-2}
2	2.53×10^{-1}
3	4.62×10^{-3}
4	4.80×10^{-2}
5	7.48×10^{-2}
6	5.29×10^{-2}

Conditions for Use

The usefulness of soluble ATP measurements in predicting or evaluating the course of an infection or metabolic disease remains to be determined by hospital studies. Dynamic changes in ATP levels may prove meaningful in following nutritional programs, diagnostic treatments, or the recovery of health.

MEASUREMENTS OF MAMMALIAN CELL ATP IN BODY FLUIDS

Flexibility in the use of reagents that hydrolyze ATP and rupture cells allows for selective measurement not only of soluble or bacterial ATP, but also of ATP associated with mammalian cells such as leukocytes, erythrocytes, platelets, epithelial, muscle, and germinal cells as they occur in body fluids. Measurements of erythrocyte ATP levels have been used as a means of monitoring the freshness of blood for transfusion.** Decreases in ATP levels of stored blood appear to be associated with loss of freshness due to age and unfavorable storage conditions.

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*Jaffee, B. D., Hahnemann Medical College and Hospital, Department of Microbiology, 230 N. Broad St., Philadelphia, Pennsylvania 19102, unpublished data

**Nakasako, Harber General Hospital, Department of Microbiology, 1124 W. Carson St., Torrance, California 90502, personal communication

The procedure for measuring ATP in blood cells involves initial hydrolysis of soluble ATP by an ATPase, inactivation of that ATPase, rupture of blood cells, and reaction of the freed ATP with luciferase. Since blood cells contain about one thousand times as much ATP as bacterial cells, the presence of any bacterial ATP in the sample will be insignificant, unless the sample has been concentrated for bacteria. Following is a list of concentrations of ATP per cell type common to biological fluids.

Cell Type	μg ATP
Average Bacterium	2.5×10^{-10}
Platelet	3.2×10^{-9}
White Blood Cell	2.1×10^{-7}
Red Blood Cell	1.2×10^{-7}
Total ATP per ml Whole Blood	6×10^{2}

QUANTITATION OF BACTERIA IN BODY FLUIDS BY MEASURING ATP

Introduction

Although the ATP content of a bacterium may vary somewhat through a growth cycle (References 26 and 27) and with species (Reference 28) an average of 2.5×10^{-10} μg ATP per organism has proved useful in relating bacterial ATP levels and bacterial cell numbers. In order to quantitate bacteria in clinical urine or blood samples, however, any soluble ATP or ATP from nonbacterial cells such as erythrocytes and leukocytes must be removed first (References 29, 30 and 31). The combined use of reagents that selectively rupture mammalian cells and hydrolyze free ATP, prepares the sample for subsequent deactivation of hydrolysis, lysis of bacteria and measurement of bacterial ATP by the firefly luciferase assay.

Several procedures for quantitating bacteria in pure cultures, in clinical urine and in blood samples have been developed (Reference 21). Pure cultures or samples containing high levels of bacterial ATP and low levels of competing sources of ATP may be assayed without concentration of the sample. For

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greater sensitivity and more efficient removal of interfering ATP, the organisms in the sample may be concentrated by either centrifugation or filtration, both of which permit washing of the bacteria. In detecting the presence of bacterial ATP in blood cultures, most of the mammalian cells are removed physically rather than chemically by selective centrifugation. Once the bacteria are detected and quantitated, rapid antibiograms can be obtained by subjecting an inoculum of the concentrated organisms to antibiotics and measuring differences in ATP levels over a 2.5 hour incubation period.

Controls and Calculations

In order to calculate micrograms of ATP per ml from recorded light unit readings for the sample being assayed, blank and ATP-standard values must be obtained. The blank sample contains the background fluid, such as sterile urine, sterile broth or sterile saline, and is treated with all reagents in the same manner as the test sample. The purpose of the blank is to determine the amount of light which is produced by the chemical environment of the sample and which is not a result of bacteria. Generally, the blank level is low enough to be insignificant; however, when the sample light unit reading is very low, it becomes important to subtract the blank value.

The basic conversion of sample light units to micrograms of ATP per ml involves a comparison of sample light units with light units obtained from a known amoutn of ATP standard solution. If the proportion of the original sample assayed and that of the ATP standard solution assayed are the same, then the concentration of ATP in the unknown sample is directly proportional to the ratio of the light units from the sample to the light units from the standard ATP times the concentration of ATP in the standard solution. This relationship may be expressed as

$$\frac{\text{Sample L.U. (Minus Blank)}}{\text{Standard L.U. (Minus Blank)}} \times \begin{array}{c}\text{Concentration}\\ \text{of ATP in the}\\ \text{Standard}\end{array} = \begin{array}{c}\text{Concentration of}\\ \text{ATP in the sample}\end{array} \quad (5)$$

where L.U. = light units

If the proportions of sample and standard assayed are not the same, a simple methematical correction for volume differences is necessary.

An ATP standard can be prepared in two ways. If a small volume (0.05 ml) of an ATP dilution is added to the sample tube, the control is referred to as a recovery standard. The sample light units obtained before the addition of ATP must be subtracted to obtain a light-unit reading due to the added ATP alone, as opposed to the ATP of the organism. The volumes must be noted in order to correct proportions for the above calculations.

Rather than adding ATP to a sample tube from which aliquots have been removed for injection, the preferred method involves preparation of a duplicate blank or sample to which a solution of ATP, diluted in the required volume of extractant, is added. In this type of standard, referred to as a processed standard, the added ATP is subjected to the same extracting environment as the organism ATP. In addition, the sample and standard volumes are identical, making calculations straightforward.

Nonconcentrated Procedure for Measuring Bacterial ATP

Reagents—

Apyrase

0.03 M $CaCl_2$ (0.6 percent TX-100)

1.5 N HNO_3

Sterile, deionized H_2O or 0.085 M Na_2SO_4

Luciferase rehydrated in 0.25 M TRIS,
    0.01 M $MgSO_4$, 10^{-3} M Cleland's, pH 8.2

Procedure—

- Removal of Interfering ATP—To a 0.5-ml sample in a sterile, polypropylene test tube, add 0.1 ml of 10 mg apyrase per ml 0.03 M $CaCl_2$ (with 0.6 percent TX-100 if contaminating mammalian cells are present). Vortex and wait 15 minutes for apyrase to hydrolyze free ATP.

- Release of Bacterial ATP—Add 0.1 ml of 1.5 N HNO_3 to deactivate the apyrase and rupture the bacteria. Vortex and wait 5 minutes for complete extraction. Dilute extracted ATP with 4.3 ml of sterile, deionized H_2O or 0.085 M Na_2SO_4. Vortex.

- Assay Bacterial ATP—Inject 0.1 ml of processed sample into 0.1 ml of luciferase, rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2. Record light units.

Conditions for Use—Since this procedure does not allow for concentration of bacterial ATP, it is recommended for the analysis of pure cultures or of those samples which contain high levels of bacterial ATP and low levels of such competing sources of ATP as blood cells or tissue cells. By forfeiting the sensitivity that can be achieved with concentration, the procedure remains rapid and simple;

a sample of minimal volume can be assayed in 30 minutes with few manipulations.

Sensitivity—This nonconcentrated procedure actually effects a ten-fold dilution of the original sample. Nevertheless, the lower limit of sensitivity approaches 1×10^5 bacteria per ml.

Concentration Procedures

For greater sensitivity in the detection of low numbers of bacteria in biological fluids, the organisms can be concentrated by centrifugation or filtration. The concentration procedures that follow also allow soluble factors that might otherwise inhibit the luciferase-ATP reaction to be physically removed in the supernate or filtrate, and the bacteria to be washed free of contaminating sources of ATP. The concentration procedures can effectively prevent mammalian cells (at levels as high as 3×10^6 white blood cells per ml urine sample, or a 10-percent blood contamination) from interfering with the final bacteria ATP measurement (Reference 32).

Centrifugation Procedure for Urine—

- Reagents—

    6-percent TX-100

    Apyrase

    0.03 M $CaCl_2$

    0.9-percent saline

    0.5 M malic acid, pH 4.25

    0.1 N HNO_3

    Sterile, deionized H_2O or 0.15 M Na_2SO_4

    Luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2.

- Procedure—

    (1) Removal of Interfering ATP—To a 10-ml sample in a sterile 12-ml polypropylene centrifuge tube add 0.2 ml of 6-percent TX-100 to selectively lyse contaminating nonbacterial cells

such as erythrocytes and leukocytes. Centrifuge at 10,400 x G for 15 minutes at 20°C to concentrate bacteria in a pellet; decant supernate to remove soluble inhibitors; invert tube and drain 5 minutes.

Add 1.0 ml of 10-mg apyrase per ml of 0.03 M $CaCl_2$ to hydrolyze remaining soluble ATP. Vortex well. Add 5.0 ml of 0.9-percent saline as a wash, vortex, and wait 15 minutes to allow apyrase to work.

Add 1.0 ml of 0.5 M malic acid, pH 4.25. At this pH, the membrane-bound nonbacterial ATP is released and then hydrolyzed by the apyrase. Centrifuge at 10,400 RCF x G for 15 minutes at 20°C, decant supernate to remove apyrase and malic acid, invert tube and drain for 5 minutes.

(2) Release of Bacterial ATP—To the pellet add 0.2 ml of 0.1 N HNO_3, vortex well and wait 5 minutes. The low pH of the nitric acid deactivates any remaining apyrases and extracts ATP from the bacterial cells. Add 0.2 ml of sterile, deionized water or 0.15 M Na_2SO_4 to dilute the nitric acid, and limit its effect on the extracted ATP and the luciferase-ATP reaction.

(3) ATP Assay—Inject 0.1-ml of final processed sample into 0.1 ml of luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2. Record light units.

● <u>Controls and Calculations</u>—To determine the micrograms of ATP per ml and the number of bacteria per ml of urine sample from the recorded light unit reading requires blank and ATP standard values. A quick "processed" standard can be obtained by assaying 0.1 ml of a mixture of 0.2 ml of 0.1 N HNO_3 and 0.2 ml of a known ATP dilution in water. A more accurate standard for a clinical urine sample, which reflects the total chemical environment ot which the bacterial ATP is exposed, requires treating a 10-ml sample of sterile urine with all of the reagents enumerated in the foregoing procedure, adding a given concentration of ATP with the extractant. A reagent blank is obtained in the same fashion, omitting the addition of ATP.

● <u>Sensitivity</u>— The centrifugation procedure effects a 25-fold concentration of the bacteria from a 10-ml sample of urine. Because interfering particles and nonbacterial cells are also concentrated in the pellet, ⸱ malic acid wash is included to allow apyrase hydrolysis of membrane-bound ATP as well as hydrolysis of soluble ATP which is released by

the selective rupturing agent TX-100. Concentration of the bacteria and more effective removal of nonbacterial ATP result in a detection sensitivity as low as 1×10^4 bacteria per ml of the original 10 ml sample.

- Practical Applications—Bacterial counts as determined by the luciferase assay of bacterial ATP in urine, using the centrifugation procedure, have been correlated with standard hospital plate counts. The ATP assay successfully separated uninfected urine samples from those with medically significant bacterial infections of 3×10^4 bacteria per ml or greater with a false negative rate of only 1.3 percent (Reference 32). Such correlation indicates that the procedure could be a useful, accurate, and rapid tool for screening clinical urine samples. At this false negative level, however, 25 percent of the urines are reported as false positives. The causes of this disagreement with routine plate count (a method which itself has a certain percentage of error) are under investigation.

Although this procedure involves a number of manipulations, many of the steps lend themselves to automation. The filtration procedure yet to be described holds the most promise for development of a completely automated rapid bacterial detection system for clinical urine samples.

Either concentration procedure, centrifugation or filtration, can be integrated with a rapid antibiotic susceptibility test that is based on measurements of bacterial ATP before and after incubation of the organisms in the presence of antibiotics. The initial inoculum of bacteria required for the antibiotic test can be prepared using the quantitative information provided by the detection assay.

The processing time of the centrifugation procedure can be shortened to less than one hour by eliminating one centrifugation step and the use of malic acid. Because this shortened procedure is not guaranteed to remove bound ATP from nonbacterial sources in the sample, it is useful for analysis of pure bacterial cultures in known media rather than clinical urine or blood samples which contain other sources of nonbacterial ATP. Practical applications include pure culture work for evaluating new antibiotics and quality control studies of a standard bacterial strain. The procedure is as follow:

(1) Removal of Interfering ATP—To a 5.0-ml sample of the bacterial culture in a 12-ml centrifuge tube add 1.0 ml of 10-mg apyrase per ml of 0.03 M $CaCl_2$ (0.6 percent TX if mammalian cells are present). Vortex and allow 15 minutes for hydrolysis of soluble ATP by the apyrase. Centrifuge at 10,400 RCF x G at 20°C

for 15 minutes, discard supernate and invert tube to drain for 5 minutes.

(2) Release of Bacterial ATP—Add 0.2 ml of 0.1 N HNO_3 to the pellet, vortex well, and wait 5 minutes for extraction of ATP. Dilute with 0.2 ml sterile, deionized H_2O or 0.15 M Na_2SO_4.

(3) ATP Assay—Assay by injecting 0.1 ml of sample into 0.1 ml of luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2. Include an assay of a processed blank and ATP standard for calculations.

Centrifugation Procedure for Blood—Standard laboratory methods for the detection of bacteremia depend on growth, with subsequent gross and microscopic examination of blood culture media, which requires an incubation period of at least 18 to 24 hours to produce a visibly "positive" result. The presence of multiplying bacteria can be detected chemically by measuring increases in the ATP content of a blood culture sample. Depending on the species of organism and the initial concentration of bacteria in the blood sample, the presence of bacteria in blood can be detected by the firefly luciferase ATP assay after an incubation period of only 5 to 12 hours (Reference 33). Although many factors contribute to mortality from blood infections, the development of techniques that provide earlier detection and treatment of bacteremia might serve to reduce the overall fatality rate which is presently 20 to 75 percent (References 34 and 35).

- Reagents—

  Dextrose Phosphate Blood Culture Media (Scott Laboratories, Cat. No. 3013-2331)

  6.0 percent Triton X-100

  Apyrase in 0.03 M $CaCl_2$

  0.9 percent NaCl

  0.5 M Malic acid (pH 3.75)

  0.1 N HNO_3

  Sterile, deionized water

  Luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2.

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● <u>Procedure—</u>

Standard Preparation and Incubation of Blood Culture Sample—Using
sterile technique, withdraw 5.0 ml of blood from patient, using a sy-
ringe containing 100 units of heparin. Transfer blood by syringe into
a Dextrose Phosphate Blood Culture bottle containing 90 ml of broth,
mix well, and incubate at $37^{\circ}C$.

Measurement of Bacterial ATP at t_1 (a time interval for growth, usually
5 hours after inoculation)—The physical removal of erythrocytes and
leukocytes is accomplished as follows:

Shake culture bottle to ensure even dispersal of contents before remov-
ing portion for processing. Aspirate 12 ml of fluid through a sterile
needle into a 20-ml, sterile, plastic syringe (modified by cutting off
all but 4 cm of the plunger). With a hemostat, pull plunger until it is
flush with the end of syringe. Occlude the needle-mount of the syringe
with a plastic cap or parafilm. Place syringe in centrifuge head, needle-
mount up, and secure with cotton packing. Return culture bottle to in-
cubator for continued growth. Centrifuge the withdrawn sample at 160
RCF × G for 5 minutes at $20^{\circ}C$ to settle most of the cellular elements
of the blood into a pellet. Bacteria are not precipitated at this speed,
and most will remain in the supernate. Attach a sterile needle, bent
at a 90 angle, to the syringe mount, and carefully transfer 10 ml of the
supernatant fluid to a 17 × 100-mm polypropylene tube without disturbing
the pellet.

Removal of nonbacterial ATP is accomplished as follows:

Add 0.2 ml of 6.0 percent Triton X-100 to the 10-ml sample to lyse any
remaining nonbacterial cells. Concentrate bacteria by centrifuging at
10,400 RCF × G for 15 minutes at $20^{\circ}C$. Discard supernate and invert
tube to drain for 5 minutes. To the pellet add 1.0 ml of 10-mg apyrase
per ml 0.03 M $CaCl_2$ and 5.0 ml of 0.9 percent NaCl. Vortex and allow
15 minutes for hydrolysis of soluble ATP. Add 1.0 ml of 0.5 M malic
acid, pH 3.75, to free any membrane-bound ATP for hydrolysis by the
still-active apyrase. Reconcentrate bacteria by centrifuging at 10,400
RCF × G for 15 minutes at $20^{\circ}C$. Discard inhibitory soluble factors
with the supernate and invert tube to drain for 5 minutes.

The release of bacterial ATP is accomplished as follows:

To the pellet add 0.2 ml of 0.1 N HNO_3 in order to deactivate any remaining apyrase and to lyse bacteria. Vortex and wait 5 minutes for complete extraction of the bacterial ATP. Dilute with 0.2 ml sterile, deionized H_2O and vortex again.

Assay ATP by injecting 0.1 ml of the final processed sample into 0.1 ml of luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2. Record light units.

Measurement of Bacterial ATP at t_2 (a time interval for growth, usually 8 hours after inoculation). Remove, process and assay second portion from the blood culture bottle as described above. An increase in light units from t_1 to t_2 indicates the presence of multiplying bacteria and, therefore, the detection of bacteremia.

Controls—To ensure sterility of the system, incubate, process, and assay a sample of sterile blood or broth as a blank. If quantitation of the infecting bacteria present after t_2 is desired, include a processed standard, adding a known amount of ATP at the point where bacterial ATP otherwise would be released. Use the blank, sample, and ATP standard values for calculating bacteria per ml. (See "Controls and Calculations" section.)

Practical Considerations—Although this procedure involves meticulous techniques, many of the steps lend themselves to automation, and with experience it may be possible to simplify some of the procedures. Recent experiments indicate that filtration may be a promising alternative to centrifugation as a means of concentrating and washing the bacteria from blood in preparation for an ATP assay.

The biggest potential source of error in using this procedure to detect bacteremia is maintaining the sterility of the system. Five or more hours of incubation under optimal growth conditions might give any contaminating bacteria ample opportunity to multiply and cause a false indication of the presence of bacteremia. Contaminating bacteria introduced during withdrawal of the t_1 sample might also have enough growth time to falsely elevate the t_2 sample value. To ensure sterility, the blood sample is removed from the culture bottle with a sterile needle and syringe. In no case, in studies by Schrock (Reference 93) has a control sample revealed any bacterial contamination due to manipulations, even after incubation for 10 days and periodic subcultures on agar.

After careful withdrawal from the blood culture bottle and low-speed centrifugation to allow separation of the bacteria from most of the mammalian blood cells, the sample is assayed for bacterial ATP by the same concentration procedure previously described for urine samples. With semiquantitation of the bacteria present in the blood culture medium, an inoculum of about 10^6 organisms per ml of trypticase-soy broth can be developed for subsequent antibiotic susceptibility testing, using ATP assays as described for urine samples in the section, "Integrated Procedure for Detection and Antibiotic Susceptibilities of Bacteria Infecting Clinical Urine Samples."

● Example of Values—Detection of bacteremia by the firefly luciferase ATP assay has been tested using healthy blood samples inoculated with a known concentration of bacteria per ml. An example of values obtained using various inoculum sizes of an E. coli strain American Type Culture Collection (ATCC #25922) is given in Table 2.

Table 2
Values Obtained from Various Inoculum Sizes

Original Inoculum	t_1 Light Units (5 hours)	t_2 Light Units (8 hours)
0 bacteria per ml (control)	0.91×10^5	0.53×10^5
5 bacteria per ml	4.46×10^5	6.32×10^7
25 bacteria per ml	4.76×10^5	9.69×10^7
50 bacteria per ml	1.70×10^6	2.50×10^8

The low control values indicate that nonbacterial sources of ATP such as erythrocytes and leukocytes have been effectively removed by the slow centrifugation step and the use of Triton X-100 detergent. After 5 hours of incubation, a significant difference is noted between the control and all three inoculated samples, indicating bacterial growth. Since light unit readings on the Du Pont Biometer in the range of 1×10^5 to 1×10^6 are often within blank range (depending on particular reagent and luciferase lots), it is recommended that the culture be allowed to incubate at least 8 hours (12 hours for gram-positive organisms) in order to see an unquestionable change in ATP levels as demonstrated by the example above. Negative cultures should be resampled at intervals up to 12 hours to preclude the possibility of a slow-responding organism.

- <u>Sensitivity</u>—In preliminary studies of 5 gram-negative and 5 gram-positive organisms, the presence of multiplying gram-negative organisms from an initial inoculum of about 50 colony-forming units per ml was readily detected within 6 hours. Lower inocula of 1 to 7 colony-forming units per ml could usually be detected by 8 hours. Although gram-positive strains exhibited slower growth or production of ATP, positive results were obtained after 12 hours. False-positive readings, where ATP analysis indicated bacteremia and agar plating resulted in no growth, occurred in only 6 percent of instances. In no case was any growth observed in the control samples (Reference 36).

- <u>Conditions for Use</u>—The successful detection of bacterial growth in blood cultures by ATP analysis has been established using blood samples which have been drawn from healthy volunteers and inoculated with a known number of bacteria. The technique has not been tested extensively on blood samples from patients with symptoms of bacteremia. Continued study using clinical as opposed to laboratory cultures is required before the method can be recommended for hospital use.

<u>Filtration Procedure for Urine</u>—The length of time needed for each processing step requiring concentration or washing of the sample can be reduced by filtration. As a result, more steps can be included to resolve the bacterial ATP signal from interfering biological and chemical background—soluble or membrane-bound nonbacterial ATP, and luciferase-inhibitory insoluble components of the sample, such as urine crystals (salts), cell casts, and blood-cell fragments. Particulates and macro-molecular elements that cannot be selectively washed away prior to bacterial extraction will be retained in the filter matrix rather than collected in the extract to be assayed. Filtration procedures may be adapted for concentration in completely automated systems.

Unfortunately, while all samples are readily centrifuged, not all fluids of biologic origin lend themselves to rapid filtration in the volumes required for detection of low-level bacterial contamination by the ATP assay. When a specimen contains a large number of particulates (such as urine crystals), high mammalian cell levels (such white blood cells in infected urine), or mucoid material, special treatments are required to facilitate sample concentration and subsequent processing (Reference 37). Varying the temperature or pH of a specimen prior to filtration may alter the solubility of components and enhance filtration, while changing the pH after sample concentration (as a wash step) may remove otherwise insoluble inhibitory compounds. Enzymatic degradation of selected sample fractions (protein complexes, mucopolysaccharides, and lipids) may minimize filter clogging and remove or inactivate inhibitory components. Proper membrane selection may be critical with more complex fluid specimens. In some

cases, two filters must be combined for optimum filtration-one prefilter (a cellulose ester membrane which tolerates more debris without clogging but also retains more fluid in its matrix), and one bacteriologic filter (a polycarbonate membrane which clogs easily but has minimal fluid retention).

When processing individual samples, any filtration apparatus allowing both filtrate disposal and collection of extracted sample under pressure or vacuum will suffice. In dealing with most samples, however, it is advantageous to employ a vacuum device designed for multiple sample processing with minimal manipulation, such as the Millipore Sampling Manifold. This unit consists of a top plate with 12 sample ports, a lower filter support plate, and a reservoir base with removable tray for the collection of filtrate in standard test tubes. The unit accommodates about 15 ml of sample per port but provides only about 2.5 square cm of filter surface (18-mm diameter), a limited filtration area which increases the difficulty of concentrating complex fluids.

Two basic filtration procedures have been developed—a simple procedure for fluids requiring no pretreatment, and a complex procedure designed to deal with infected urines which might contain, for example, 5 percent whole human blood and approximately 10^8 bacteria, thereby representing a considerable challenge to the filtration system. Assay sensitivity varies with the procedure used. When low levels of mammalian cells are present, a range of 700 to 5000 bacteria per ml can be detected, depending on the urinary pathogens involved. In 5-percent bloody urine, using the complex filtration procedure, 5000 organisms per ml of a 10-ml sample are routinely detected. The sample is concentrated 12.5 or 20-fold, depending on the procedure used.

- Simple Filtration Procedure—This procedure is suitable for enumeration of bacteria in a sample containing low levels of mammalian cell contamination, where filtration difficulties are not anticipated. When the fluid can not readily be filtered (due to high membrane, particulate, or mucoid content), the complex filtration procedure should be utilized. In either case, a reagent blank and ATP standard should be included to relate sample light units to micrograms of ATP per ml or number of bacteria per ml.

    Reagents:

    6 percent Triton X-100 (TX)

    10 mg/ml Apyrase in 0.03 M $CaCl_2$

    0.1 N HNO_3 (in 0.001 percent TX)

0.15 M Na_2SO_4 (in 0.001 percent TX)

0.9 percent saline (with 0.001 percent TX)

1.0 M Malic acid, pH 4.25

(prefiltration of reagents is advisable)

Procedure—The removal of soluble ATP is accomplished as follows:

To a 10-ml sample in a test tube, add 0.2 ml of 6 percent TX and 1.0 ml of 10 mg/ml apyrase in $CaCl_2$, vortex, and wait 15 minutes for lysis of mammalian cells by TX and hydrolysis of soluble ATP by apyrase.

The removal of membrane-bound ATP is accomplished as follows:

This optional step is required only for detection of low bacterial levels in the presence of mammalian cells. Add 1.0 ml of malic acid buffer to lower the pH and release bound ATP. Vortex and wait 5 minutes for further hydrolysis by apyrase.

The sample is concentrated as follows:

Filter sample through 0.22 μ membrane and wash with 10 ml of saline. Filter to dryness to prevent dilution of extractant.

Extraction is accomplished as follows:

Apply 0.25 ml (this volume suitable for up to a 25-mm diameter filter) of 0.1 N HNO_3 to filter surface to inactivate ATPases, lyse bacterial cells, and free bacterial ATP. Wait 5 minutes for complete extraction, and dilute by adding 0.25 ml Na_2SO_4 to filter. Collect extract by filtration.

Assay by injecting 0.1 ml of vortexed extract into 0.1 ml of luciferase rehydrated in 0.25 M TRIS, pH 8.2, 1×10^{-2} M $MgSO_4$, and 1×10^{-3} M Cleland's reagent. Record light units.

● Complex Filtration Procedure—This procedure is designed to minimize mucoid and particulate clogging of the filter and remove high levels of nonbacterial ATP. It is particularly adapted for the elimination of physical and chemical interferences associated with mammalian tissue specimen contamination (that is, infected urines).

Reagents:

6 percent Triton X-100 (TX)

10 mg/ml Apyrase in 0.03 M $CaCl_2$ (with 0.001 percent TX)

8 percent Rhozyme-41

1.5 M TRIS pH 8.3

1.0 M Malic acid pH 4.0 (in 0.001 percent TX)

0.15 M Na_2SO_4 (in 0.001 percent TX)

0.1 N HNO_3 (in 0.001 percent TX)

(prefiltration of reagents in advisable)

Procedure—Pretreatment of sample to minimize filter clogging is accomplished as follows:

To a 10-ml sample in test tube add 0.2 ml of 6-percent TX, 1.0 ml of 1.5 M TRIS, and 1.0 ml of 8-percent rhozyme-41. Vortex and wait 5 minutes for lysis of mammalian cells and degradation of proteins (glycoproteins, blood membranes, histones, etc.) in the buffered sample.

Concentration of sample is accomplished as follows:

Filter the pretreated sample on the Millipore Sampling Manifold using an 8μ cellulose ester prefilter membrane overlaying a 0.4μ bacteriologic polycarbonate membrane.

The release and hydrolysis of residual nonbacterial ATP is accomplished as follows:

Wash the filter with a minimum of 10 ml of malate buffer to lower pH and release bound ATP. Apply 1.0 ml of 10 mg/ml Apyrase in 0.03 M $CaCl_2$ and wait 15 minutes for hydrolysis of ATP. Remove apyrase by filtration. Wash filter with 10 ml of Na_2SO_4 to prevent buffering of the extractant by residual malic acid and apyrase. Filter to dryness.

Extraction is accomplished as follows:

Apply 0.4 ml 0.1 N HNO_3 to filter, wait 5 minutes for inactivation of ATPases and release of bacterial ATP. Dilute the extract by adding 0.4 ml of Na_2SO_4. Collect extract by filtration.

Assay by injecting 0.1 ml of vortexed extract into 0.1 ml of luciferase rehydrated is 0.25 M TRIS, pH 8.2, 1×10^{-2} M $MgSO_4$, and 1×10^{-3} M Cleland's reagent. Record light units.

Filtration Procedure for Whole Blood—It is possible to filter 5 ml of whole blood on the 18-mm diameter Millipore Sampling Manifold filter surface, using the double membrane system (8.0μ and 0.4μ) previously described. The blood must be pretreated for lysis and membrane digestion as follows:

To 5 ml of whole blood, add 1.0 ml of 10 percent TX, 2.0 ml 1.5 M TRIS, pH 8.3, and 2.0 ml 8 percent Rhozyme-41. Vortex, wait 5 minutes, and filter. Ten ml of whole blood is readily filtered on one 47-mm diameter, 0.45μ filter by doubling the volumes listed above. The concentration of TX required to lyse whole blood is apparently 1-percent (acting concentration), while only 0.1 percent is necessary to lyse 10-percent blood. TX at these high concentrations may affect the viability of organisms extracted from blood, therefore, modification of this filtration procedure is advisable (that is, dilution to allow use of less TX or use of another blood cell lysing agent).

The 5-percent blood-dextrose-phosphate broth described in the section "Centrifugation Procedure for Blood" can be filtered using the Millipore Sampling Manifold. Pretreatment of the blood culture (TX-Rhozyme-buffer) allows concentration of at least 20 ml, enabling bacterial enumeration and antibiotic susceptibility testing following the filtration procedures previously outlined.

INTEGRATED PROCEDURE FOR DETECTION AND ANTIBIOTIC SUSCEPTIBILITIES OF BACTERIA INFECTING CLINICAL URINE SAMPLES

The present laboratory techniques for microbial sensitivity testing, namely, agar diffusion (Bauer-Kirby), broth dilution, and agar dilution, require overnight incubation after primary isolation of the infecting organism. Accurate susceptibility data are available to the physician a minimum of 48 hours after the specimen is received in the laboratory. This delay often results in the initiation of empirical and/or broad-spectrum antimicrobial therapy in urgent clinical situations. The availability of a rapid bacterial susceptibility test would allow

prompt selection of the most effective agent and would avoid inclusion of inappropriate, unnecessary, or toxic agents in the initial therapeutic regimen. The procedure described below, using the firefly luciferase ATP assay, detects and quantitates bacteria present in the specimen and provides accurate, reproducible antibiograms in approximately 4 hours (References 38, 39, and 40).

Reagents

Trypticase Soy Broth

Apyrase

0.03 M $CaCl_2$ – 0.18 percent TX-100

0.9 percent Saline

0.25 M Malic acid

0.1 N HNO_3

Sterile deionized H_2O

Luciferase in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2

Antibiotic solutions (see following section)

0.03 M $CaCl_2$ – 0.6 percent TX-100

1.5 N HNO_3

Antibiotics

The antimicrobial agents that have been tested extensively for use with the ATP assay are listed below with the appropriate final concentration to be used for results comparable to Bauer-Kirby test (Reference 40). Concentrations were determined empirically by measuring the effect of the mean inhibitory concentration (MIC) breakpoints generally quoted for the agar diffusion method on a

panel of prototype organisms. The concentrations below fall somewhere between these MIC breakpoints.

Antibiotic	mg/ml
Sodium penicillin	8
Sodium ampicillin	8
Sodium nafcillin	0.6
Disodium carbenicillin	128
Sodium Cephalothin	16
Tetracycline hydrochloride	6
Chloramphenicol	12.5
Clindamycin phosphate	2
Erythromycin	4
Gentamicin sulfate	6
Sodium colistimethate	8
Kanamycin	15
Streptomycin	10
Nitrofurantoin	50

Most of the listed antibiotic powders are water soluble and prepared by addition of an appropriate volume of sterile, deionized water. Erythromycin and chloramphenicol are initially dissolved in about 2 ml of 95 percent ethanol, then further diluted with water. Nitrofurantoin is dissolved in Trypticase Soy Broth after heating and requires separate A_0 and $A_{2.5}$ controls that include an equivalent volume of broth instead of water. Rapid sensitivity testing to sulfonamides and to nalidixic acid appears impossible by the ATP assay method because consistently false resistant indices result.

Procedure for a Urine Sample Tested Against 12 Antibiotics

<u>Preparation of Concentrated Test Portions</u>—Centrifuge a 35-ml clinical urine sample at 10,400 RCF × G for 15 minutes at 4 C. Discard supernate and reconstitute pellet with 1.75 ml of Trypticase Soy Broth to achieve a 20-fold concentration of the original sample. Vortex well.

Transfer by pipette 0.5 ml of concentrated sample (representing 10 ml original sample) to a 17 × 100-mm polypropylene tube for subsequent detection assay. Store the remaining 1.25 ml (representing 25 ml of original sample) at 4°C to be used later for antibiotic susceptibility tests. At this temperature, no change in the bacterial concentration should occur.

<u>Detection Assay</u>—

- <u>Removal of Interfering ATP</u>—To a 0.5 ml sample add 1.0 ml of 10 mg apyrase per ml 0.03 M $CaCl_2$ - 0.18 percent TX-100 and vortex. Add 4.5 ml of 0.9-percent saline as a wash, vortex again, and allow apyrase to hydrolyze soluble ATP for 15 minutes. Add 1.0 ml of 0.25 M malic acid, vortex, and centrifuge at 10,400 RCF × G for 15 minutes at 4°C. The apyrase continues to work during centrifugation on membrane-bound ATP released by the malic acid. Discard supernate and drain for 5 minutes in the inverted position.

- <u>Release of Bacterial ATP</u>—To the pellet add 0.2 ml of 0.1 N HNO_3, vortex and wait 5 minutes for extraction of ATP. Dilute with 0.2 ml of sterile deionized water and vortex.

- <u>ATP Assay</u>—Inject 0.1 ml of sample into 0.1 ml of luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2. Record light units and calculate bacteria per ml using blank and ATP standard values. If a medically significant concentration of bacteria exists, proceed with antibiotic testing.

<u>Antibiotic Susceptibility Test</u>—

- <u>Preparation and Incubation of Test Portions</u>—Retrieve 1.25 ml of concentrated sample from refrigeration and dilute further with Trypticase Soy Broth to achieve a concentration of about 10^6 bacteria per ml. Preincubate sample for 30 minutes at 37°C to allow organisms to reach log growth phase.

  Aliquot two 0.45-ml portions to serve as growth controls before and after a 2.5-hour incubation period. These controls are referred to

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as A_0 and $A_{2.5}$. Aliquot one 0.45-ml portion for each antibiotic to be tested. These samples are referred to as B_1, B_2, B_3, etc. Add 0.05 ml of antibiotic solution (10 times the final desired concentration) to each of these test protions and vortex. To the controls, A_0 and $A_{2.5}$, add 0.05 ml of sterile, deionized water to simulate antibiotic test portions. Incubate each test portion and the $A_{2.5}$ control at $37°C$ for 2.5 hours.

- Extraction of Bacterial ATP in the A_0 Control—Add 0.1 ml of apyrase (10 mg/ml 0.03 M $CaCl_2$ – 0.6 percent TX) to hydrolyze soluble ATP. Vortex and wait 15 minutes. Add 0.1 ml of 1.5 N HNO_3, which simultaneously deactivates the apyrase and releases bacterial ATP. Vortex and wait 5 minutes. Dilute with 4.3 ml of sterile, deionized water, and vortex. The sample may be stored at room temperature for later assay with the $A_{2.5}$ control and all antibiotic test portions.

- Extraction of Bacterial ATP in Incubated Samples—At the end of the 2.5-hour incubation period, remove all samples from the incubator and to each tube add 0.1 ml of apyrase (10 mg/ml 0.03 M $CaCl_2$ – 0.6 percent TX). Vortex and wait 15 minutes for hydrolysis of soluble ATP. Add 0.1 ml of 1.5 N HNO_3 for apyrase deactivation and ATP extraction. Vortex and wait 15 minutes. Dilute with 4.3 ml sterile, deionized water.

- ATP Assays—Assay all samples (A_0, $A_{2.5}$, B_1, B_2, B_3, etc.) by injecting 0.1 ml of the final processed sample into 0.1 ml of luciferase rehydrated in 0.25 M TRIS, 0.01 M $MgSO_4$, 10^{-3} M Cleland's reagent, pH 8.2. Record light units.

Practical Considerations

Blanks and standards are not absolutely required for the antibiotic susceptibility test since the final index of inhibition is determined not by an absolute calculation of micrograms of ATP per ml, or number of bacteria per ml, but rather by an examination of relative differences between light signals before and after incubation with the antibiotic. If the infecting bacteria reconstituted in broth after centrifugation are resistant to a particular antibiotic, growth will occur during the incubation period. The increase in ATP that occurs with growth will be reflected in a significant difference between light units recorded for the A_0 control and the B sample. If the bacteria are sensitive, the increase in light units will be negligible, or even negative. Uninhibited growth is represented by the $A_{2.5}$ control. The ratio of growth in the presence of antibiotic to growth in the antibiotic-free control provides a direct measurement of the drug effect.

Since the ATP susceptibility test relies on an increase in bacterial ATP over time, any nonbacterial sources that might falsely elevate the A_0 determination would elevate the $A_{2.5}$ by the same increment. This constant contribution becomes mathematically insignificant in the final ratio calculation. For this reason, the increased specificity for bacterial ATP afforded by the use of malic acid and a second centrifugation step in the original detection procedure can be sacrificed in the antibiotic test to achieve greater simplicity and rapidity.

The original detection value for bacteria per ml of urine sample provides the basis for dilution of the stored portion to achieve a working inoculum of 10^6 organisms per ml for the antibiotic tests. Although the standard Bauer-Kirby Antibiotic Disc Susceptibility Method requires a standard inoculum size, the ATP method requires only a range of 10^6 to 10^7 bacteria per ml. Beyond these range limits, accuracy of ATP results does decrease slightly as compared to Bauer-Kirby results.

A laboratory experienced with the ATP antibiotic susceptibility test could determine a dilution schedule directly from the detection light signal, bypassing the need to process a blank and ATP standard for calculations. Light signals are dependent, however, on the luciferase lot and daily conditions in the lab; therefore, inclusion of an ATP standard for quality control is recommended.

It is possible that growth of some bacterial strains may be inhibited by exposure to TX. To avoid this problem, the detergent required in the detection assay is added after the initial centrifugation step has taken place and after an aliquot for later growth in antibiotic testing has been removed.

The procedure outlined in this section represents an integration of detection and antibiotic susceptibility testing, designed to limit requirements for clinical sample volume, reagent volumes, length of time, and manipulations. The procedures may be performed separately, of course, without sacrificing accuracy or sensitivity, particularly if large numbers of urine samples are to be screened for significant bacterial infections before antibiotic testing is considered.

Calculations

The ATP index of inhibition for a given antibiotic is determined according to

$$\text{ATP INDEX} = \frac{B_t - A_0}{A_t - A_0} \quad , \text{ where}$$

B_t = sample light units for organisms incubated with antibiotic

A_t = control light units for organisms incubated without antibiotic

t = time of incubation period

A_0 = control light units for organisms without antibiotic at time zero.

An ATP index ≤ 0.05 indicates sensitivity (S) to the antibiotic and index > 0.05 indicates resistance (R).

Example of Values

Antibiotic susceptibility testing on a clinical urine sample with greater than 10^5 E. coli per ml by plate count resulted in the values indicated in Table 4.

Table 3
Examples of Antibiotic Susceptibility Test Values

Sample	Light Units	ATP Index	Interpretation
A_0 Control	9.14×10^5	–	–
$A_{2.5}$ Control	3.61×10^7	–	–
B penicillin	3.59×10^7	+.99	R
B ampicillin	1.58×10^5	–.02	S
B nafcillin	4.13×10^7	+1.15	R
B cephalothin	2.46×10^5	–.02	S
B tetracycline	1.43×10^6	+.01	S
B chloramphenicol	1.33×10^6	+.01	S
B clindamycin	3.41×10^7	+.94	R
B erythromycin	2.53×10^7	+.69	R
B gentamcin	1.67×10^5	–.02	S
B colistin	1.86×10^5	–.02	S
B kanamycin	2.09×10^5	–.02	S
B streptomycin	2.24×10^6	+.04	S
B nitrofurantoin	2.88×10^5	–.02	S

Proposed Filtration Procedure for Antibiotic Susceptibility Testing

Determination of antibiotic susceptibility utilizing a filtration technique has been adequately tested to date (July 1976) only for seeded prefiltered urines and broth cultures, i.e., samples requiring no pretreatment that might affect the viability of bacterial cells in a specimen (Reference 41). Although it has been shown that the use of TX-Rhozyme-buffer as a pretreatment does not decrease the level of bacterial ATP in a specimen, it does not necessarily follow that these bacterial cells would replicate normally under the requisite incubation conditions, or that a typical antibiogram pattern would be produced. These questions are presently under investigation.

The filtration susceptibility test is preceeded by any of the detection methods (centrifugation or filtration) previously outlined. An inoculum representing approximately 10^6 organisms is prepared from the specimen, as determined from the detection data. The specimen is either diluted (in saline) to allow the application of a 1.0-ml inoculum, or if necessary, up to 10 ml of specimen may be filter-concentrated for processing low-level infections. If more than 5 ml of undiluted specimen are to be processed, it may be necessary to pretreat the sample with TX-Rhozyme-buffer to prevent clogging of membrane pores. Familiarity with the filtration procedure and various specimen conditions will allow the technician to determine the advisability of pretreatment.

Development of the filtration procedure for susceptibility tests has involved use of a Millipore Sampling Manifold. As shown in figures 15 and 16 a minimum of two plate sets is required for processing, each set being composed of a top sample port plate and lower filter support plate. To prevent leakage of the sample and filter movement, the two plates are joined by "C" clamps, creating one unit or "set".

One plate set is required to process A_0's and determine the initial light response of the inoculum (without incubation or antibiotic treatment). The A_0 samples must be extracted separately, while the $A_{2.5}$ (normal growth in the absence of anti-biotics) and B (antibiotic-treated) samples are incubated on the second plate set(s). It is recommended that both a standard and blank be processed on each plate set (that is, with and without incubation) for reagent and sterility control. As any contamination of the culture medium would result in a false elevation of inoculum ATP level, it is particularly prudent to obtain the blank light response for use in calculating the net change in sample light response after incubation. (For further clarification refer to figures 15 and 16.)

PLATE SET I

AO(1)
12
1
11
AO(2)
2
10
BLK
9
3
STD
8
4
7
6
5

INITIAL LIGHT RESPONSE
(NO INCUBATION,
NO ANTIBIOTICS)

PLUGGED (UNUSED)
SAMPLE PORT

PLATE SET (SETS) II

STD
BLK
12
A2.5
(1)
11
1
B4(2)
B1(1)
10
2
B3(2) 9
3 B2(1)
8
4
B2(2)
B3(1)
7
6
5
B1(2)
A2.5
(2)
B4(1)

INCUBATION W/O
ANTIBIOTICS
SAMPLE 1

ANTIBIOTICS 1-4
SAMPLE 1

ANTIBIOTICS 1-4
SAMPLE 2

INCUBATION W/O
ANTIBIOTICS
SAMPLE 2
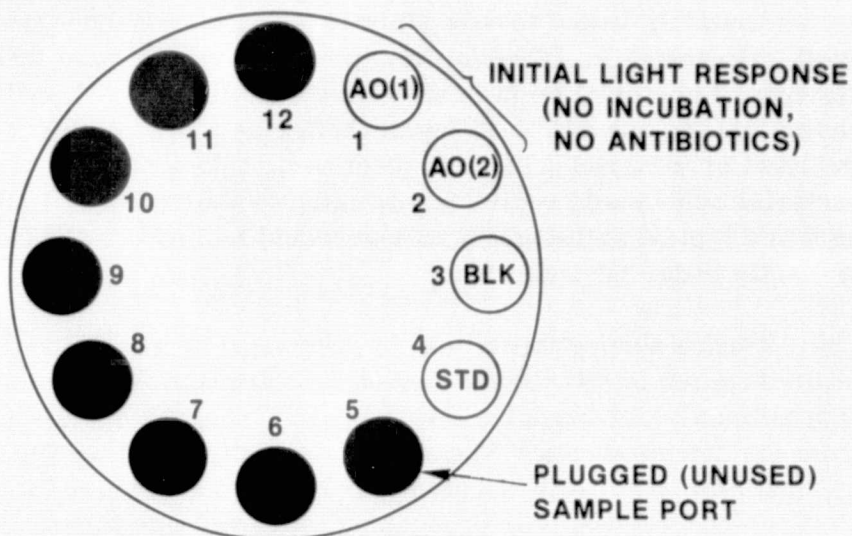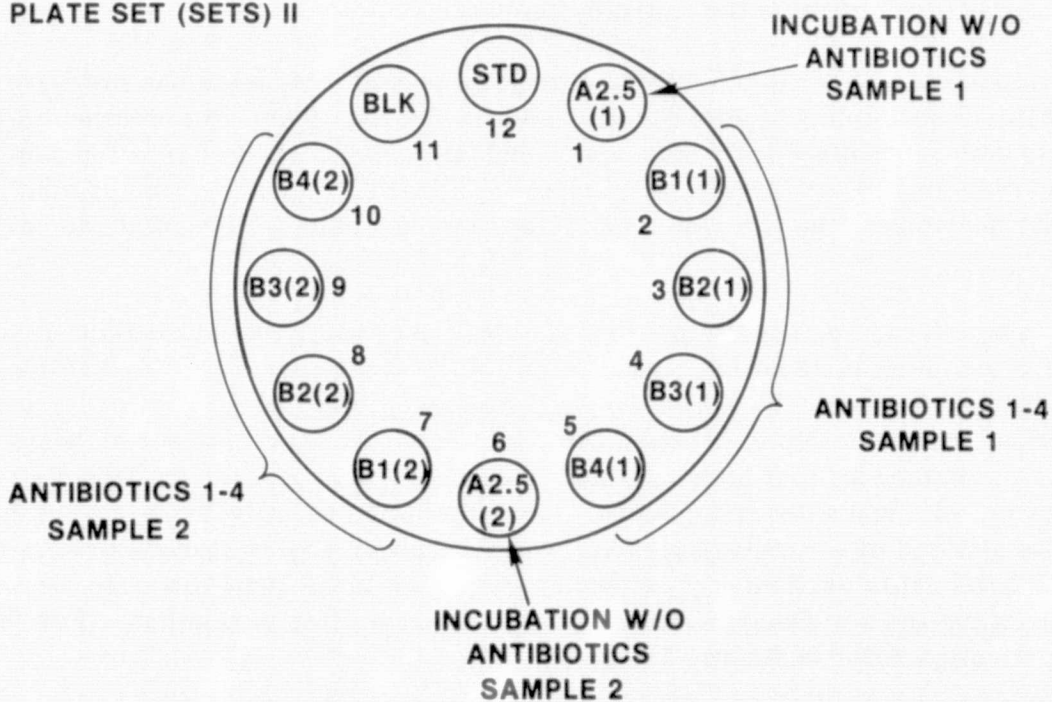
Figure 15.   Antibiotic Susceptibility by Filtration

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HANDWHEEL NUT

SAMPLE PORT PLATE

FILTER SUPPORT PLATE

TEST TUBE RACK

RUBBER GASKET

LUCITE PLATE

RESERVOIR BASE
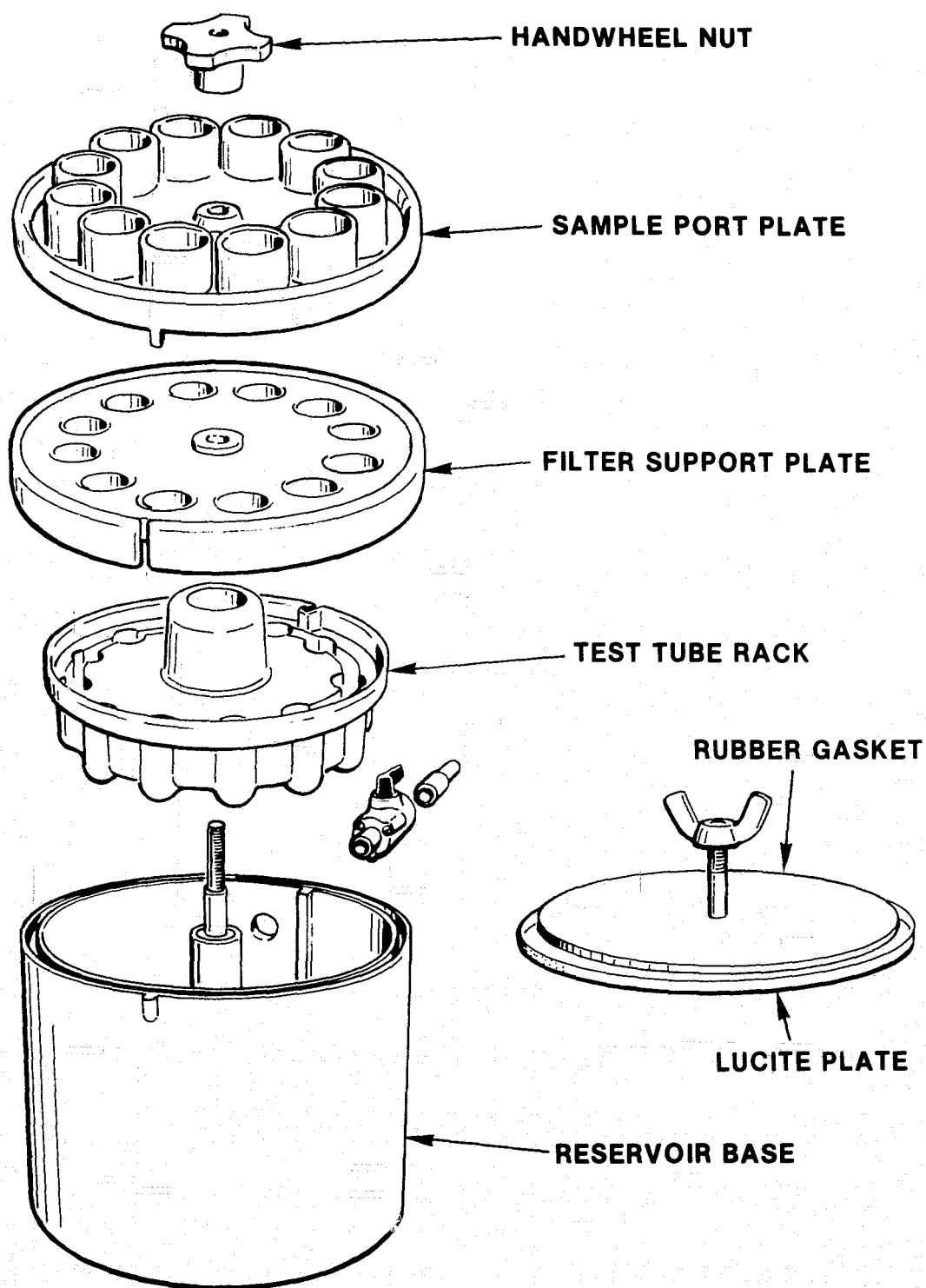
Figure 16.   Millipore Sampling Manifold

Reagents—

6 percent Triton X-100 (TX)

8 percent Rhozyme-41

1.5 M TRIS pH 8.3

Saline (0.9 percent)

0.9 percent Saline (with 0.0001 percent TX)

0.1 N HNO_3 (in 0.001 percent TX)

0.15 M Na_2SO_4 (in 0.001 percent TX)

10 mg/ml apyrase in 0.03 M $CaCl_2$

Antibiotics (listed previously in this section)

Trypticase Soy Broth (TSB)

(pre-filtration of reagents is recommended)

Procedure—

- Inoculation of Filters, Concentration of Organisms—One inoculum portion (10^6 organisms) is required for A_0 (plate set I), $A_{2.5}$, and each antibiotic tested (plate set II). A sample requiring pretreatment is brought to 10 ml (with saline) in a test tube, then treated with 0.2 ml of TX, 1.0 ml of Rhozyme-41, and 1.0 ml of TRIS buffer. Vortex and wait 5 minutes for mammalian cell rupture and degradation of protein complexes by Rhozyme.

  The Sampling Manifold is prepared with 8.0 μ cellulose ester membrane over a 0.4 μ polycarbonate membrane.

  Pipette inoculum (1.0 ml diluted specimen or 1-10 ml undiluted specimen or total volume of pretreated specimen) into sample ports.

  Vacuum filter to impinge bacteria on filter membranes.

  Wash membranes by filtering 10 ml of saline.

- **Preincubation of All Samples to Reach Log Growth Phase**—Remove both plate sets from manifold chamber and secure on plastic rubber bases with wing nuts.

  Add 0.9 ml of TSB to all sample ports. Incubate all plate sets 30 minutes at 37°C.

- **Antibiotic Testing**—Remove plate set(s) II from incubation for introduction of antibiotics.

  Add 0.1 ml of each antibiotic to appropriate B sample ports.

  Add 0.1 ml of H_2O to each $A_{2.5}$ port.

  Swirl plate unit for proper mixing. Return plates to 37°C incubation for a 2.5-hour incubation period.

- **Determination of A_0 Level**—Remove plate set I from incubation after a 30-minute preincubation. Extract A_0 ports (as indicated in the following step).

  Refrigerate collected extract until antibiotic testing is completed and all extracts can be assayed. (Be sure to allow approximately 10 minutes for room temperature equilibration prior to assay of refrigerated A_0 extracts.)

- **Extraction Procedure**—Secure plate unit on manifold reservoir base. Vacuum filter to remove broth.

  Add to filter 0.1 ml 6 percent TX and 1.0 ml of 10 mg/ml Apyrase in $CaCl_2$. Swirl to mix reagents and wait 15 minutes for lysis of mammalian cells and ATP hydrolysis.

  Vacuum filter to remove apyrase solution. Wash by filtering 10 ml saline-TX to remove any remaining reagent or sample interference. Filter to dryness.

  Remove plate unit, drain off filtrate, and insert collection test tubes in rack provided. Replace plate unit on manifold base.

  Add 0.4 ml of HNO_3 to inactivate ATPases and lyse bacterial cells releasing ATP. Wait 5 minutes. Dilute extract by adding 0.4 ml Na_2SO_4. Collect extract by vacuum filtration to dryness.

- Assay of All Extracts (A_0, $A_{2.5}$, B's)—Vortex all tubes.

   Assay by injecting 0.1 ml of extract into 0.1 ml of luciferase prepared in 0.25 M TRIS, pH 8.2, 1×10^{-2} M $MgSO_4$ and 1×10^{-3} M Cleland's reagent.

- Calculate ATP index according to equation given on page 53.

BACTERIAL LUCIFERASE FLAVIN MONONUCLEOTIDE (FMN) ASSAY

INTRODUCTION

Flavin compounds are ubiquitous in all organisms. The detection of such compounds can give quantitative information on the number of bacteria present due to pathological disorders in body fluids such as urine or blood. In addition, by using selective methods, abnormalities of flavin concentrations due to metabolic disorders may also be evaluated (Reference 8). The bioluminescent reaction for the detection of flavins is more sensitive than fluorometric techniques and does not suffer from interferences which would affect other techniques. The sensitivity of methods for FMN assay are listed as follows:

Sensitivity of Methods for FMN Assay (Reference 8)

Method	Lower limit of detection (micrograms)
Paper chromatography	0.01
Cytochrome c reductase	0.01
Lactic oxidase	1
Fluorometry	0.0001
Bacterial bioluminescence	0.00001

The proposed applications of the bacterial luciferase FMN assay include enumeration of bacteria in sewage effluent, water, blood, urine, ambient air, (Reference 42), industrial wastes, and clean-room air studies. The assay has been used in the determination of riboflavin, FMN, FAD (flavin adenine dinucleotide) flavin enzymes, flavin vitamins, flavoproteins, and a variety of inorganic and organic chemicals in air samples.

Luminous bacteria have been used to study the mechanism of the action of drugs (Reference 43). Johnson et al., (References 44 and 45) using dimming time as a measure of respiration of luminous bacteria, were able to measure the effects of various narcotic agents on the luciferase enzyme.

An in vitro system using Photobacterium phosphoreum has been developed which can determine the concentration of an aqueous solution of gentamicin in concentrations as low as 1.25 μg/ml. This is done by measuring the light intensity of a control culture and the subsequent decrease in light intensity of the bacterial culture when exposed to gentamicin. This test system is being developed for measuring antibiotic levels in blood serum (Reference 46).

LUMINESCENT BACTERIA

Luminescent bacteria have been found in fresh water, sea water, in insects, fresh and salt water shrimp, amphipods, and other marine organisms. They have also been found living saprophytically on dead fish or meat and in mutualistic associations in the luminous organs of fish or squid. Incubation of dead fish or squid at 15° to 20°C overnight will reveal small luminous colonies developing on the animals' surfaces. When some of the glowing material is transferred to an agar plate, the colonies that develop may reveal motile or sessile luminous bacteria of long or short rods, cocci or vibrios. The characteristic of light emission is not a constant feature and the ability to emit light may be lost during maintenance on artificial laboratory culture media.

Some of these bacteria, which can be obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, are discussed in the Appendix.

CULTURE MEDIA

Various complex media have been used to isolate and culture these microorganisms. The media generally contain sea water or artificial sea water. Some strains have been grown on synthetic media, also.

The complex media may contain a natural carbon and energy source such as fish muscle extract, peptone or tryptone. Glycerol has also been used as a carbon and energy source. In one species, P. phosphoreum, methionine appeared to be an essential growth factor (Reference 47).

A commercially available medium is Photobacterium Broth (Difco) which is suitable for many marine luminescent bacteria.

Another medium is artificial sea water consisting of 0.4 M NaCl, 0.1 M $MgSO_4 \cdot$ 7 H_2O, 0.02 M KCl, and 0.02 M $CaCl_2 \cdot$ 2 H_2O (Reference 48). Basal medium broth (BM) contains 50 mM TRIS HCl (pH 7.5), 19 mM NH_4Cl, 0.33 mM K_2HPO_4. 3 H_2O), 0.01 mM $FeSO_4$ and half-strength artifical sea water (Reference 48). Basal medium agar (BMA) is prepared by sterilizing separately and then mixing equal volumes of double-strength BM and 20 g of Difco Noble Agar per liter. Compounds serving as sole sources of carbon and energy are filter-sterilized (0.2μ Nucleopore) and added to the already autoclaved medium. For a complex broth or agar add 5 g Bacto-Peptone, 3 g Difco Yeast Extract, and 3 ml glycerol to the recipe from BM.

BACTERIAL LUCIFERASE

Luminescent bacterial luciferases are self-induced enzymes, induced by an active compound (autoinducer) which accumulates in the culture medium. When the concentration of autoinducer attains a critical level, synthesis of luciferase occurs (Reference 49). The autoinduction mechanism has been described by Eberhard (Reference 49), Nealson, and (Reference 50 and 51). Autoinducer can be extracted from the culture medium by 2 successive two-volume extractions with ethyl acetate. When a strain of V. fischeri (ATCC 7744) was grown in a culture medium, the growth of the cells was continuous from the time of inoculation, while luminescence lagged for nearly 3 hours (Reference 51). The explanation for this delay consists of two factors. The first is an inhibitor which is present in complete medium and must be removed (References 47 and 49). Various marine bacteria can remove this inhibitor* leaving what Nealson refers to as a conditioned medium. The second factor is the time needed for production of autoinducer which stimulates synthesis of the luminous system.

pH Range of Bacterial Luciferase

At ordinary temperatures, activity of the bacterial luciferase appears to be stable in the acid range down to pH 4.0. Tests performed in our laboratory indicate that the luciferase system from V. fischeri, strain A-13[†] is active from a pH of 5.8 to 7.2 with optimal luminescence between 6.8 and 7.0. Stability of the system from V. fischeri, strain MJ-1[††] is between pH 6.0 and 7.3 with

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*Nealson, Scripps Institute of Oceanography, LaJolla, California 92093, unpublished data.

†Obtained from Dr. John Lee, Dept. of Biochemistry, University of Georgia, Athens, Georgia 30601.

††Obtained from K. H. Nealson, Scripps Institute of Oceanograph, LaJolla California 92093.

an optimum between pH 6.3 and 6.8. The differences between the enzymes may be slight as far as pH optima are concerned. However, in transferring cultures for maintenance of the MJ-1 strain of V. fischeri, light intensity is more consistently reproduced than in the A-13 strain, where appearance of dark mutants is common.

Preparation and Partial Purification of Luciferase

Culture and Harvest of Vibrio fischeri (Reference 52)—Incubate organisms in a shaking flask of Photobacterium Broth (Difco) at 25°C for a minimum of 5 hours. When brightly glowing, spread culture on a large shallow tray containing Photobacterium broth and 0.8 percent agar at a depth of 1/4 to 3/8 inches. Cover trays to prevent agar from drying out and incubate at 25°C overnight. Harvest the luminescent colonies by scraping the surface of the agar with a rubber spatula.

Extraction of Proteins—Add 500 ml of cold acetone to the cells and blend for one minute at 10°C in a Waring blender. Collect extracted proteins on a Whatman #41 filter using a Buchner funnel and air dry (Reference 53).

Removal of Proteins Other than Luciferase—Add 150 ml of 0.05 M TRIS, 10^{-3} M Cleland's pH 7.1, to 3 grams of the acetone powder. Shake this suspension for 15 minutes to allow for extraction of the soluble protein fraction containing luciferase. Centrifuge at 8000 RCF x G for 20 minutes at 5°C, collect the supernate (130 ml) and discard the pellet.

Add 22.88 grams of $(NH_4)_2SO_4$ to the supernate (30 percent saturation) and shake for 15 minutes at room temperature to precipitate proteins other than luciferase. Centrifuge at 30,000 RCF x G for 20 minutes at 20°C, collect the supernate (140 ml), and discard the pellet.

Precipitation of Luciferase—To the supernate add 22.88 grams of $(NH_4)_2SO_4$ (70 percent saturation) and shake the suspension for 15 minutes at room temperature. This concentration of ammonium sulfate allows for precipitation of the luciferase enzyme. Centrifuge at 30,000 RCF x G for 20 minutes at 20°C, discard the supernate, and wash the residue once in 100 ml of 70 percent $(NH_4)_2SO_4$, pH 7.1, at room temperature. Let stand for 10 minutes.

Elution of the Enzyme—Apply the precipitate to the top of a Sephadex G 200 column and elute off the column in 0.05 M TRIS, 10^{-3} M Cleland's reagent, pH 7.1. Collect samples in a fraction collector.

<u>Assay for Active Fractions of Luciferase (Reference 52)</u>—To 1.0 ml standard
FMN solution (100 μg/ml) in a polypropylene test tube, add 1.0 ml PdCl$_2$ solution
(0.17 mg/ml in 0.1 M TRIS, pH 7.0) and 1.0 ml NaBH$_4$ (14.8 mg/ml in same
TRIS). Wait 10 minutes for reduction of FMN to FMNH$_2$.

Inject 0.1 ml of sample into a cuvette containing 0.2 ml enzyme fraction plus 0.1
ml dodecyl aldehyde. Pool the most active fractions, those producing the most
light when reacted with the reduced FMN standard, for subsequent use in cellular
and/or soluble FMN assays.

<u>Known Sources of Prepared Bacterial Luciferase</u>

Sigma Chemical Company
P. O. Box 14508
Saint Louis, Missouri 63178

Luciferase Type I, Bacterial; Purified from <u>V. fischeri</u> ATCC No. 7744
   (prepared for Sigma)

Luciferase Type II, Bacterial; Purified from <u>V. fischeri</u> ATCC No. 7744

ICN Pharmaceuticals, Inc.
26201 Miles Road
Cleveland, Ohio 44128

Luciferase, Partially purified powder from <u>V. fischeri</u>

Worthington Biochemical Corporation
Freehold, N. J. 07728

Luciferase, Partially purified dried powder from <u>V. fischeri</u>

DETERMINATION OF FMN IN FLUIDS

<u>Reagents</u>

<u>Extractants</u>—Original work with the FMN assay involved the use of perchloric
acid as an extractant (Reference 53). However, a 0.2 N extraction concen-
tration of nitric acid (HNO$_3$), or a solution of 0.1 M boiling TRIS, may also be
used as an effective extractant of FMN and FAD. The following procedures
incorporate the use of nitric acid as the extractant.

Lumiflavin, a photolysis product of riboflavin, may be added to the acid extracting solution to retard photodegradation of FMN and FAD without inhibiting the luciferase reaction. Immediately before use, dissolve 10 mg lumiflavin in 250 ml of distilled H_2O and add 1.0 ml of this solution of 100 ml of the acid extracting solution.

Palladium Chloride ($PdCl_2$)—A 0.002 M solution of $PdCl_2$ in 0.1 M TRIS buffer at pH 7.0 or in 0.4 N potassium hydroxide (KOH) serves as a catalyst for the reduction of FMN to $FMNH_2$. KOH neutralizes the acid extractant. Purified palladium chloride is available from Fisher Scientific Company.

TRIS (Hydroxymethyl Amino Methane)—A 0.1 M solution of TRIS at pH 7.0 is used as a buffer.

Sodium Borohydride ($NaBH_4$)—Sodium borohydride reduces FMN. Prepare a 0.4 M solution in 0.4 N KOH immediately before use. A 98 percent pure powder is available from Fisher Scientific Company.

Dodecyl aldehyde—Dodecyl aldehyde is a necessary cofactor for the bacterial luminescent reaction. To prepare immediately before assay, suspend 0.1 ml of concentrated aldehyde in 5.0 ml of luciferase solution and disperse with shaking. The aldehyde can also be added before lyophilizing the preparation of luciferase to give the same final concentration when the enzyme is hydrated with distilled water or buffer.

Luciferase—The bacterial luciferase solution used in the FMN assay is the result of pooling active fractions from Sephadex G-100 columns in 0.05 M TRIS, pH 7.0. The subsequently lyophilized enzyme is rehydrated by adding 4.5 ml of 0.4 M TRIS buffer, pH 7.0. (Refer to section on preparation and partial purification of bacterial luciferase.)

Flavin Mononucleotide (FMN)—Serial dilutions of FMN in distilled water are used for preparation of standard curves from which unknown concentrations of FMN can be determine:.

Procedure for Measuring Soluble FMN (Reference 8)

Reduction of FMN—To a 1.0 ml sample add 1.0 ml $PdCl_2$ solution (0.7 mg/ml in 0.1 TRIS, pH 7.0) to serve as a catalyst for FMN reduction. Add 1.0 ml $NaBH_4$ solution (14.8 mg/ml in same TRIS, prepared immediately before use) and allow for an FMN reduction time of 10 minutes. Sample must be assayed within 20 minutes after reduction by borohydride.

Assay for FMN—Assay by injecting 0.1 ml sample into 0.3 ml luciferase solution (0.2 ml luciferase, 0.1 ml dodecyl aldehyde) in a 6 × 50-mm test tube mounted in front of a photomultiplier tube. Record light units.

Procedure for Measuring Cellular FMN

Extraction of Flavins from Cellular Material—To one ml of an aqueous cellular suspension add 1.0 ml of PdCl$_2$ solution made up in nitiric acid (0.17 mg/ml in 0.4 N HNO$_3$). Allow acid to react for 5 minutes to rupture cells and free FMN and FAD from protein.

Reduction of FMN—Add 1.0 ml of the NaBH$_4$ solution (14.8 mg/ml in 0.4 N KOH, prepared immediately before use) to reduce FMN in the sample. Add 1.0 ml 0.4 M TRIS, pH 6.4, to stabilize pH for optimal enzyme activity. Allow for a reaction time of 10 minutes (not more than 20 minutes).

Assay for FMN—Assay by injecting 0.1 ml of sample into 0.3 ml of the luciferase solution (0.2 ml luciferase, 0.1 ml dodecyl aldehyde) in a 6 × 50-mm test tube mounted in front of a photomultiplier tube. Record light units.

Practical Considerations

The extraction of flavins from cellular material and subsequent assay steps should be carried out with minimal exposure to light. In order to minimize surface adsorption and degradation of the flavins, use polypropylene containers, pipettes, and syringes as much as possible.

The addition of TRIS buffer to the sodium borohydride cuases the evolution of hydrogen gas bubbles. The solution should be pipetted with a safety pipetter or a rubber bulb attached to the pipette. When adding to the cells in a calibrated test tube, the borohydride solution is added until the liquid reaches the 3 ml mark. The addition of borohydride causes the FMN solution to darken and many small bubbles to appear. While still dark and bubbly, the solution should be assayed.

The temperature of the extractant used is critical for determining FMN content versus the total phosphoflavin (FMN plus FAD) content of a sample. Acid extractants at cold temperatures (near 4°C) allow for specific measurement of FMN only; complete conversion of extracted FAD to FMN for a total phosphoflavin measurement can be ensured by boiling the sample before adding PdCl$_2$. The difference between the total phosphoflavin and FMN values represents the concentration of FAD in the sample.

When extracting and measuring FMN from pure bacterial cultures, the percentage of extracellular FMN that might falsely elevate the bacterial FMN value is so low as to be insignificant. To detect bacteria in fluid samples containing more substantial levels of background flavins, the cells can be concentrated and washed to remove extracellular flavins and to increase sensitivity. At present, no proven method exists for simple hydrolysis of interfering flavins before extraction of bacterial FMN, although a selective enzymatic hydrolysis system is under investigation at Goddard Space Flight Center (Reference 54).

Calculations

The FMN content of a sample is calculated by reference to a standard curve prepared from measurements of light intensity obtained with known concentrations of FMN subjected to the same conditions as the unknown sample. The concentrations prepared should be such that the injected 0.1-ml volumes contain quantities of FMN ranging from $10^{-1} \mu g$ to $10^{-4} \mu g$. Values from blank assays using water in place of the FMN solution should be subtracted both from the standard curve and from the unknown values. Since the reaction is linear, a single point of standard FMN may be used for calculating the unknown values.

Examples of Values

One ml of 0.1 M boiling TRIS buffer at pH 6.7 extracts $7 \times 10^{-4} \mu g$ FMN per ml of Pseudomonas aeruginosa, corresponding to approximately $1 \times 10^{-10} \mu g$ FMN per cell. Following is a list of the total amount of FMN and FAD extracted by boiling in perchloric acid from various bacteria grown to early maximum stationary phase*.

Organism	μg FLAVIN/Cell ($\times 10^{-10}$)
Bacillus globigii	61.0
Brevibacterium helvolum	9.0
Escherichia coli	1.9
Flavobacterium arboresceus	65.4
Kelbsiella pneumoniae	1.8

*Picciolo, Chappelle, Goddard Space Flight Center, Greenbelt, Md., (unpublished data).

Organism	μg FLAVIN/Cell ($\times 10^{-10}$)
Micrococcus lysodeikticus	21. 0
Pseudomonas aeruginosa	1. 8
Pseudomonas fluorescens	2. 6
Proteus vulgaris	1. 4
Serratia marcescens	1. 6
Spirillum serpens	0. 2
Staphylococcus aureus	0. 7

The flavin content of several biological fluids has been determined using a 6 percent butanol solution in 0.01 M TRIS buffer as an extractant (Reference 8):

Fluid	μg FMN/ml
Urine	1×10^{-11}
Whole blood (5.5×10^6 rbc/mm^3)	5. 0
Serum	1. 1

Sensitivity

Under the conditions cited in the description of procedure, approximately 10^5 bacteria per ml are required for reliable results. A sample containing fewer bacteria will have to undergo concentration by centrifugation or filtration.

Conditions for Use

The FMN bacterial detection system remains to be applied to clinical specimens. Predictable advantages over the firefly luciferase ATP assay would include low cost of enzyme and the measurement of a metabolically less variable cellular constituent. The determination of an ATP/FMN relationship may prove to be a useful description of the health or energy potential of cells in a sample.

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LUMINOL CHEMILUMINESCENT SYSTEM

INTRODUCTION

The luminol chemiluminescence method for detecting bacteria in fluid samples may provide an alternative to the more expensive assays already described. Like the other luminescent reactions, the luminol chemiluminescence method is a sensitive, simple, and rapid method for bacterial detection. Unlike the luciferase enzymes, however, luminol is readily available in large quantities at low cost and remains stable indefinitely. Although previously applied to the analysis and detection of iron II (Reference 55), hydrogen peroxide as a result of glucose oxidation (Reference 56), ferricyanide (Reference 57), and hematin compounds (Reference 58), the luminol chemiluminescent reaction has been modified for detecting bacteria in fluid samples by incorporating interference removal techniques. (References 59 through 63.)

Luminol (5-amino -2, 3-dihydro -1, 4-phthalazinedione) chemiluminescence is produced by the reaction of an aqueous alkaline luminol solution with hydrogen peroxide and an activating agent consisting of an oxidizing agent such as ferricyanide, hypochlorite or a chelated transition metal such as iron or copper. In bacterial detection systems, it is assumed that iron porphyrins (peroxidase, cytochrome, catalase), contained in microbial cells serve as the activators for luminol chemiluminescence.

REAGENTS

Luminol Stock

Combine 0.8860 g of luminol (Sigma Chemical Co., St. Louis, Mo.)* and 60 g sodium hydroxide per liter deionized water to obtain a stock luminol solution that remains stable for several months.

Hydrogen Peroxide (H_2O_2)

A 3 percent solution of hydrogen peroxide is added to the sample as a pretreatment for elimination of extra-bacterial porphyrins and is also required for the luminol reaction.

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*Also available from J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg N.J. 08865; Eastman Kodak Co., Eastman Organic Chemicals, 343 State St., Rochester, N.Y. 14650; and other chemical companies.

Sodium Hydroxide (NaOH)

A final concentration of 0.75 N sodium hydroxide extracts porphyrins from bacteria and provides the pH necessary for the luminol reaction.

Luminol Reagent

Combine 10 ml of luminol stock, 6.6 ml of 3 percent hydrogen peroxide, 90 ml of 1.5 N sodium hydroxide, and 93.4 ml of deionized water to achieve a 200-ml solution of 2.5×10^{-4} M luminol, 0.1 percent hydrogen peroxide, and 0.75 N sodium hydroxide that remains stable for at least 24 hours.

APPARATUS

The luminol reaction can be performed as an injection system using the instruments described previously. However, a flow system has been developed that lends itself to automation. This luminol flow system requires a light-tight chamber containing a coiled glass flow cell (available from the American Instrument Company for use with the Chem-Glow), and a four-channeled Buchler peristaltic pump.

PROCEDURE (FLOW SYSTEM)

Sample Pretreatment

The sample is introduced into the flow system (see figure 17) by the action of the peristaltic pumps and mixes with 3 percent hydrogen peroxide for a period of 2 minutes. Hydrogen peroxide pretreatment causes rapid degradation of free porphyrins and as a result removes 90 percent of any soluble extracellular iron porphyrins which may be contaminating the sample.

Luminol Reaction

The next step allows the sample to react with the luminol reagent for a period of 10 seconds before reaching the flow cell and photomultiplier. This step, reaction rate resolution, eliminates the short-lived luminol responses due to inorganic interferences (see figure 18). Since the peroxide pretreatment eliminates soluble porphyrins, the peak light response after 10 seconds reflects only the bacterial porphyrin concentration.
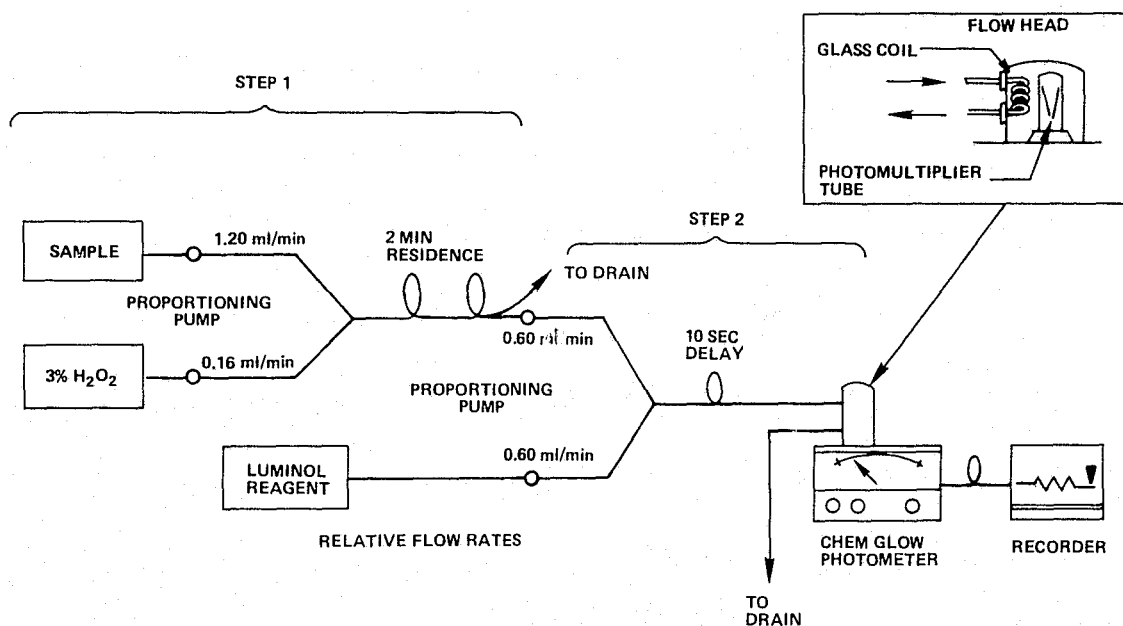
Figure 17. Automated Luminol Flow System, Incorporating Hydrogen Peroxide Pre-
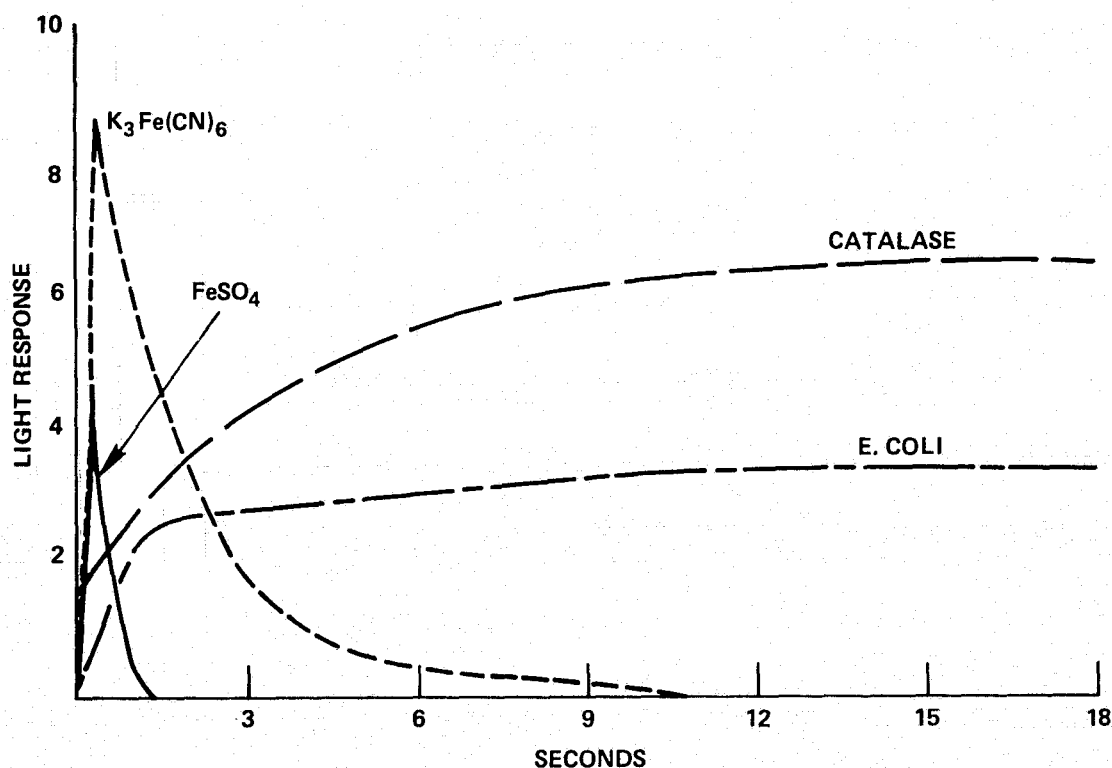treatment and Reaction Rate Resolution for Eliminating Interference

Figure 18. Reaction Rate Curves for Various Luminol Oxidizing Agents

ALTERNATIVE PROCEDURE (INJECTION SYSTEM)

Sample Pretreatment

To a 7. 5-ml sample in a test tube add 1. 0 ml of 3-percent hydrogen peroxide and let stand 2 minutes for degradation of soluble porphyrins.

Luminol Reaction

Inject 0. 15 ml of the peroxide-treated sample into a cuvette containing 0. 15 ml of luminol reagent. Record light units after ten seconds to preclude measurement of short-lived light responses from inorganic interferences.

CALCULATIONS

Luminol light units are converted to bacteria per ml by referring to a standard bacterial concentration curve obtained by measuring luminol responses of serial dilutions of washed bacterial cells, E. coli for example. Concentrations are determined by standard plate-count methods. With the luminol flow system one light unit has been shown to be equivalent to about 1.5×10^6 bacteria per ml sample.

SENSITIVITY

The luminol flow or injection system can be used to detect bacterial levels as low as 1×10^4 E. coli per ml. If concentrations of bacteria are expected to be less than 10^4 per ml, a method of concentration is necessary. Filter an appropriate volume of sample through a 0.45μ, 22-mm or 47-mm Gelman acropore filter and holder. Add 5. 0 ml of 0. 5 percent hydrogen peroxide to the filter and wait 2 minutes before vacuum-washing with about 10 ml of deionized water. Extract bacterial porphyrins with 0. 1 N sodium hydroxide – 50 percent ethanol for 30 seconds and collect by vacuum. Assay with the luminol reaction system without using the 3 percent hydrogen peroxide step.

MEDICAL APPLICATIONS

Another luminol procedure has been used by Ewetz to detect bacteriuria, "combining a high rate of positively identified bacteriurias (>99 per cent) due to its high sensitivity, with an acceptably low rate (7 per cent) of false positives in an unselected clinical material" (Reference 61). Since hemoglobin is a luminol activator, a problem does exist when blood is present in the sample, however this method may still be a valuable tool in screening large numbers of urine samples for bacterial infections.

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The determination of iron porphyrins by luminol in clinical samples, either as an assay for bacterial levels or perhaps as a measure of blood or hemoglobin, may prove useful as long as interference from other materials that activate the response is removed. A ratio of values (luminol/ATP/FMN) may be shown in the future to characterize various body fluids.

SUMMARY

The characterization of a clinical sample by a simple, fast, accurate, automatable analytical measurement is important in the management of infectious disease. Luminescence assays offer methods rich with options for these measurements. The instrumentation is common to each assay, and the investment is reasonable.

Three general procedures have been developed to varying degrees of completeness which measure bacterial levels by measuring their ATP, FMN and iron porphyrins. Bacteriuria detection and antibiograms can be determined within half a day. The characterization of the sample for its soluble ATP, FMN or prophyrins can also be performed.

The utility of these assays for clinical samples as well as their versatility will be further demonstrated as their clinical use expands (Reference 65).

APPENDIX
LUMINESCENT BACTERIA

1. <u>Vibrio fischeri</u> (<u>Photobacterium fischeri, Achromobacter fischeri</u>) (Reference 64). (nomenclature from Bergey's Manual of Determinative Bacteriology 8th Ed)

<u>Vibrio fischeri</u> are found in sea water and marine animals. The cell, a motile rod, (mainly lophotrichous although some strains are monotrichous) is gram-negative and usually requires 2.8 percent NaCl for growth. It grows at $5°C$ but not at $37°C$. The temperature for optimum luminescence is $25°C$. The organism can be grown only aerobically.

2. <u>Photobacterium phosphoreum</u>
The cells are coccobacillary or rods, usually motile with polar flagella, and are gram-negative. Some strains do not grow well on nutrient media, unless the NaCl concentration is 2.0 to 3.0 percent.

They are chemorganotrophs with a carbohydrate metabolism that is fermentative. The organism can grow at $5°C$ but not at $37°C$. The optimum temperature for luminescence is approximately $15°C$.

3. Genus <u>Beneckea</u>.
The genus <u>Beneckea</u>, as described by Baumann et al., 1971, (Reference 66) is regarded as a genus of uncertain taxonomic position by the authors of Bergey's Manual, 8th Ed. (Reference 64).

Baumann describes these organisms as straight or slightly curved rods, gram-negative, facultatively anaerobic marine organisms. Some of the strains have single polar flagella when grown in either liquid or solid culture media; other have single polar flagella in liquid culture, but when transferred to solid culture media, they develop peritichous flagella. They decompose chitin and ferment glucose with the production of acid but no gas. Some strains grow at $4°C$; some at $35°C$, and some at as high as $40°C$. Their luminescence has been studied by Hastings (Reference 67).

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