

Analytical Bioluminescence Assays Using the Liquid Scintillation Spectrometer: A Review

P.E. Stanley ^a

Department of Agricultural Biochemistry, Waite Agricultural Research Institute,
The University of Adelaide, Glen Osmond, South Australia

INTRODUCTION

Bioluminescence is the enzyme- or protein-mediated production of light and is a form of chemiluminescence since it arises from the excited states of the products of exothermic reactions. The quantum yield, i.e. the total quanta emitted divided by the number of reacted molecules, is high for many bioluminescent species. Thus a value of close to one has been recorded for firefly luminescence,¹ whereas those of many chemiluminescent systems are frequently much less efficient^{2,3} with values as low as 10^{-15} . However, bioluminescent reactions differ thermodynamically from other biological reactions since the emission of an einstein (a mole of photons; 6.025×10^{23}) requires 63.5 kcal (2.75 eV) at 450 nm and 47.6 kcal (2.07 eV) at 600 nm. This is considerably more energy than is available in any single-step biological reaction. Thus the biochemistry and physical chemistry of bioluminescence has been of considerable interest. However, these aspects will not be discussed here and the reader is referred to recent books, reviews and symposia¹⁻¹⁷ for further information.

Since bioluminescence is generally a highly efficient process, it is extremely useful as an analytical tool, especially now that modern low-background photomultipliers are available to count photons with high quantum efficiency. An example will show the sensitivity that can ideally be attained for measuring adenosine triphosphate (ATP) using the luciferin-luciferase system of the firefly Photinus pyralis. When used to monitor such a reaction, a good photomultiplier with a background of 50 c.p.s. and an average quantum efficiency of 5% is capable of measuring approximately 10^{-19} moles ATP/s. Since light collection is not perfect and because the rate of the bioluminescent reaction diminishes with time, the estimate of sensitivity would have to be lowered by one or two orders of magnitude. In practice, the best sensitivities obtained are about 10^{-15} moles for ATP¹⁸⁻¹⁹ and 10^{-16} moles for reduced nicotinamide adenine dinucleotide (NADH).²⁰

^a Present address: Department of Clinical Pharmacology, The Queen Elizabeth Hospital, Woodville, Adelaide, South Australia.

GENERAL ASPECTS

In the early 1950's analytical bioluminescence assays were in use in few laboratories and most measurements were made using the photomultiplier as a photometer (current producing) rather than as a quantum or photon counter (pulse producing). Morton,²¹ Duquesne and co-workers,²²⁻²⁴ James²⁵ and Tohill²⁶ have considered various aspects of photon counting with special reference to low light levels. Seliger³ has compared the current and pulse procedures for detecting and measuring very low light levels and concludes that 'there is no a priori reason to use the more complex pulse counting techniques over the much simpler D.C. amplifier current measurements for optimum detection of very weak chemiluminescent reactions'. This is based on a photomultiplier with a figure of merit of one. Robben,²⁷ in a detailed review paper, has come to a similar conclusion when comparing the two techniques under conditions where the figure of merit approaches unity, but under the conditions employed by Duquesne et al.²² pulse counting gives the best sensitivity. However, the dearth of suitable photometers and the ready availability in many laboratories of liquid scintillation spectrometers, which can be readily adapted for quantum counting, has made this instrument the one of choice for work in the biological and medical fields. The modern unit, fitted with photomultipliers having low-noise bialkali photocathodes capable of excellent signal-to-noise operation at ambient temperatures, enables measurements of ATP and NADH to be made routinely in picomole (10^{-12}) amounts.²⁸⁻⁴¹

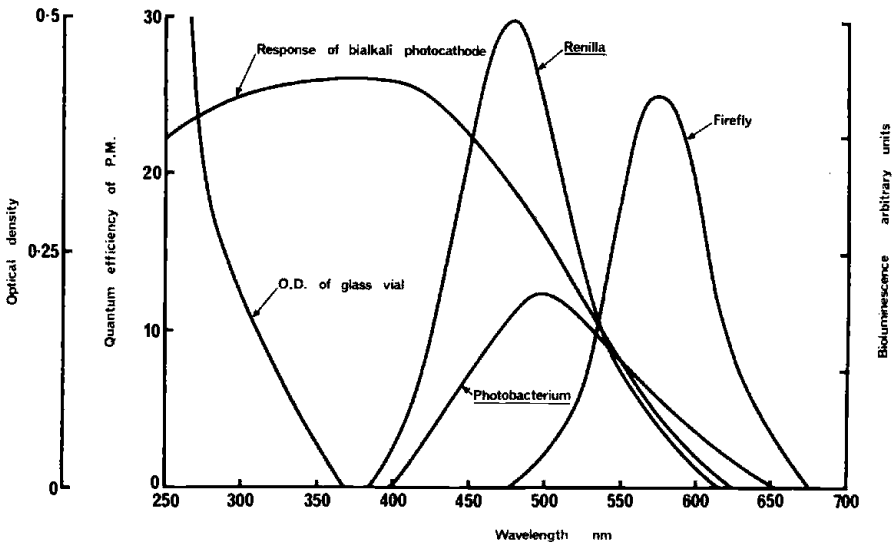


Fig.1. The quantum efficiency of a bialkali photomultiplier (EMI 9635QB 42a), the optical density of a glass scintillation vial together with emission spectra of the firefly, Photobacterium and Renilla. The emission spectra are not normalised.

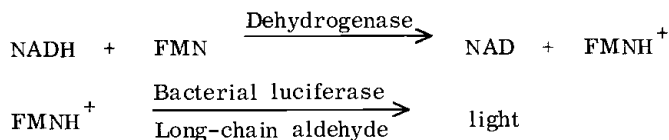
Figure 1 shows the quantum efficiency curve for a bialkali photocathode (SbKCs) fitted to a photomultiplier of a type commonly used in spectrometers, together with the emission spectra of the firefly (ATP), Photobacterium (NADH) and Renilla reniformis (adenosine 3'-phosphate 5'-phosphate, PAP). Thus good quantum efficiencies are attainable. Frequently, however, the instruments have features which are superfluous for analytical bioluminescence, e.g. refrigeration, coincidence

circuitry, external standardisation and several pulse height analysers, but until other less complex but equally reliable automatic pulse counting equipment becomes available commercially the conventional liquid scintillation spectrometers will continue to be used for bioluminescence and chemiluminescence assays.

Bioluminescence is composed of single discrete photons, whereas in liquid scintillation spectrometry the discrete scintillations consist of a burst of photons, some of which go to one photomultiplier and some to the other. Coincidence circuitry establishes the legitimacy of the pulses and electronically reduces the background derived from the thermal noise of the two photocathodes to negligible values. However, in order to count single photons efficiently in the spectrometer, the coincidence circuit must be switched off,^{32, 33, 37-40} this, of course, results in a concomitant increase in background which with good modern alkali photomultipliers is quite acceptable. Photons counted per unit time will thus be directly proportional to the ATP or NADH in the sample. In the coincidence mode a squared proportionality is observed^{32, 37} and the useful range is smaller.³⁷ In the out-of-coincidence mode the pulse height spectrum for these single photons is in the lowest sixth of the pulse height range for unquenched tritium³⁷ and the discriminators and amplifiers on the pulse height analysers should be set accordingly.

Schram³⁶ and others^{42a} have recently emphasised the importance of selecting optimal high-voltage and discriminator settings when maximum counting sensitivity is required. These settings are important in attaining the best signal-to-noise ratio and those used for the liquid scintillation counting of tritium are not adequate. Higher voltages do not necessarily give better separation of photomultiplier noise and signal, and these may give rise to memory effects at high counting frequencies.³⁶ On the other hand, Lin and Cohen⁴¹ advocate the use of higher voltages. The individual characteristics (gain and thermal noise) of the photomultipliers will no doubt dictate their best operating conditions.^{42a, b} The use of high counting rates causes another problem, which is associated with saturation of the pulse height analyser. The result is that individual pulses (from single photons) are not resolved and the apparent pulse height spectrum is distorted.³⁷ As a consequence, calibration curves deviate significantly from linearity at high counting frequencies. With modern equipment, about 2×10^6 random pulses/min is the upper limit for normal use. To employ closely set discriminators in order to diminish the observed counting rate does not overcome the problem since the analyser has to appraise all pulses entering it. Some equipment is fitted with live timing so that the real time clock is switched off during the processing of each pulse. The user must be aware that at high counting rates a considerable extra elapsed time will occur over and above the preset time and this will generally give false results because the bioluminescence rate usually changes with elapsed time.

All measurements of analytical bioluminescence are concerned with reaction rates. Thus on mixing ATP with firefly luciferin-luciferase (in the presence of O_2 and Mg^{2+}) there is a rapid increase in light output, reaching a maximum in about one second (the peak or flash height), and this is followed by a gradual exponential decrease in light production.⁶ The rate of decay depends on the amount of ATP, enzyme, pH and ionic strength,²⁸ as well as buffer type,²⁸ inhibitory ions^{30, 43} and temperature. Under proper conditions a decay period of 30 min or more can be observed and this rate remains directly proportional to the ATP concentration,³³⁻³⁵ so a log-lin plot of counts per unit time versus time gives a series of parallel straight lines over a wide concentration range of ATP. A somewhat similar, although not identical, situation is found in the case of in vitro bioluminescence of Photobacterium, which can be effectively employed to measure either FMN (flavin mononucleotide) or NADH^{34, 38, 39, 50} since the enzymes present catalyse the following reaction sequence :



When using the liquid scintillation spectrometer, conditions are usually so arranged as to minimise decay, e.g. use of arsenate buffer for ATP assays,²⁸ since quanta are counted over a fixed time interval starting at a preset time after mixing the reactants outside the instrument. This preset time is generally 5–10 s and the fixed time 5–30 s.²⁸⁻⁴¹ When a reaction is to be followed over many minutes, the repeat count mode is employed and the use of spectrometers fitted with buffered output (such as the Searle Analytic Unilux III and Mark II instruments) is particularly advantageous for this purpose since the next counting sequence is underway again as print-out commences. Since print-out may take from 5–15 s on some instruments, it follows that a considerable loss of data occurs in these when monitoring a reaction sequence taking, say, 2 min. Should a continuous analogue representation be required, this can be obtained by coupling a ratemeter (time constant 0.3 s or less) and a chart recorder (full-scale deflection in less than 1 s) to the output stage of one of the pulse height analysers.^{36,37,42} An alternative is to employ a multichannel analyser in multiscale. In this mode, channels are opened sequentially for a preset time, say 0.1 or 1 s, and the counts occurring in that time interval are stored in adjacent channels. This is especially useful for fast reactions.^{39,40,44} Analogue representation is usually available on an oscilloscope display screen and digital information can be retrieved by interfacing to a computer or teletype.

As mentioned earlier, photometers are also used for assaying bioluminescence but here the measurement is usually of the peak or flash height since some workers consider this value to be less susceptible to interference from other substances.⁴⁰ St. John,⁴⁶ however, recommends that light emission be integrated over a 30 s time interval commencing at the time of mixing the reactants. This gives high sensitivity, high reproducibility, convenience and speed. He has evidence that measurements of the peak intensity do not necessarily give the most reliable results for ATP in complex systems. A number of photometers designed for bioluminescence assays have been described^{18,19,47-54} and there are commercial units made by Du Pont (Biometer) and JRB Inc., San Diego, California (ATP photometer); the former is designed to measure the flash height and the latter integrates over a fixed time interval following mixing.³⁶ Prydz and Froholm⁵⁵ have described the use of Beckman DB spectrophotometer for the purpose and St. John⁴⁶ has used a bioluminescence sensing chamber (American Instrument Co.). Ausmus⁵⁶ has employed the highly sophisticated GeMSAEC Centrifugal Photometric Analyzer, designed to perform the analysis of ten samples, four standards and a blank simultaneously. The instrument is interfaced to a PDP8 computer for control of the unit and calculation of the results. Samples can be processed rapidly and accurately.

With the scintillation spectrometer, the use of a fixed cell within the counting vial gives more reproducible results when using small volumes than the vial produces alone.³³ A similar cell can also be employed to keep the reaction at a useful ambient temperature, using a cooled spectrometer,^{36,38,39} when the scintillation vial is filled with water at the required temperature. A cell fixed in the detector improves the reproducibility of results and obviates the problem of phosphorescence of glassware.³⁵ Transfer of reagents in this unit occurs by means of a motor-driven micro-syringe. Stanley⁴⁰ has described a cell which fits into the detector chamber and is filled manually with syringes; it may also be used to measure flash heights.

When large numbers of samples have to be processed, automation is an advantage. Hammerstedt⁵⁷ has interfaced to a commercial spectrometer a Hamilton

precision liquid dispenser, which adds a fixed amount of firefly luciferin-luciferase into the next sample at the time of printing data of the previous one; 1400 samples can be processed in a 7 hr session. Another approach has been to employ an auto-analyser on-line to a flow detector in a spectrometer.⁵⁸ The development of further automatic equipment is very important, especially for dynamic measurements when several reagents are to be added.^{38, 44, 59, 60}

A problem which is common to many bioluminescence assays is that of phosphorescence of vials. Thus, scintillation vials which have been exposed to sunlight or unfiltered fluorescent lighting will phosphoresce (single photons) for up to several hours, giving rise to both variable and high backgrounds ($10^3 - 10^4$ c.p.s.), and require 'dark' adaption for several hours prior to use. A room lit only with conventional tungsten lights is essential for this type of work. Some workers obviate the problem by employing special cells fixed in the detector chamber.^{33, 35, 40} Vial caps can also be a source of phosphorescence^{61, 62} but need not be used in analytical bioluminescence assays.

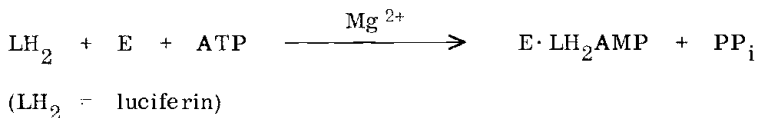
Not generally recognised is the problem of contamination of glassware and solutions with dust and micro-organisms. Thus the luciferase system of Photobacterium is readily absorbed on dust particles and gives erroneous data, while the presence of micro-organisms results in degradation of compounds such as NADH and ATP. Solutions are best prepared and then filtered through a bacterial filter. Even then, very dilute solutions of ATP ($< 10^{-9}$ molar) kept at 0°C at neutral pH appear to break down after a few hours.³⁷

SPECIFIC ASSAYS AND PROCEDURES

Beijerinck⁶³ in 1902 was the first worker to use bioluminescence as an analytical technique and assayed O_2 using luminous bacteria (Photobacterium). He detected oxygen produced during photosynthesis by crushed clover leaves covered with a suspension of luminous bacteria. McElroy⁶⁴ first used the firefly system to measure ATP in microgramme amounts. The development of sensitive photomultipliers in the early 1950's resulted in a rapid increase in the uses of bioluminescence assays in a few laboratories, and Strchler and Totter²⁸ published the first paper on the technique using quantum counting in 1952.

Assay of ATP (adenosine triphosphate)

The basis of the assay is the formation of an activated enzyme-substrate complex, specific for ATP:

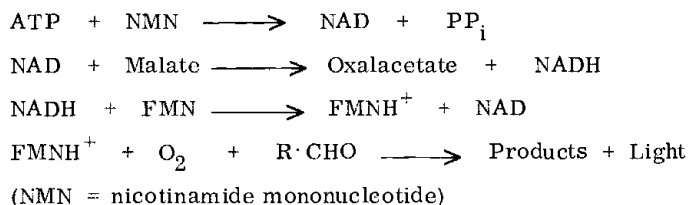


This complex then reacts with molecular oxygen to give an excited state enzyme-decarboxy keto luciferin product which, with a quantum yield of nearly unity, emits a yellow-green light ($\lambda_{\text{max}} = 562$ nm).¹ Thus with ATP the only reactant not in excess, the light emitted is proportional to the amount of this compound. The concentration of magnesium ions is important⁴³ under certain conditions but 5 mM is generally satisfactory. A cofactor requirement, D(-)luciferin, is apparent but this is present in crude firefly lantern extracts. About 1 mg of luciferin can be prepared from 2000 fireflies.¹⁷ Inactive extracts prepared from commercially available lanterns (perhaps old stock) can usually be traced to a deficiency of this compound. Fortunately synthetic luciferin is now available commercially (Sigma Chemical Co.) and may be synthesised by two different procedures.^{65, 66}

It is now standard practice to slow down the decay rate of luminescence by

conducting the reaction in phosphate or arsenate buffers, thus making measurements on spectrometers easier to perform.^{28, 37} A pH in the range 7.5 to 9.0 is usual for the reactions since the quantum yield exceeds 0.9 at these values. Below about pH 6.5 the yield is only 0.45 and the emission spectrum shifts further into the red (614 nm)¹ where the photomultipliers are considerably less sensitive (see Fig. 1). The use of internal standards is thus mandatory when measuring samples in acid solutions.³⁷

The specificity of the reaction for ATP is very high but recently two other nucleotides have been shown to be active in light production. Thus, 3-iso-AMP (ribose attached to N-3 of adenine)⁶⁷⁻⁷⁰ produces red light as does ϵ -AMP (but not ϵ -ATP).^{69, 70} However, these two compounds are unlikely to be present in biological materials and thus the reaction can be considered to be a specific assay for ATP. However, the use of crude luciferase preparations does lead to other problems of specificity associated with the presence of adenylate kinase and nucleoside diphosphate kinases. The enzymes mediate the in situ formation of ATP in the presence of ADP and other nucleoside triphosphates. This is readily detected since under these conditions light output increases steadily for at least half a minute rather than staying steady or decreasing slightly. Rasmussen and Nielsen⁴⁵ have purified the luciferase by filtration on high porosity gels and have considerably reduced the level of these two enzymes. While the purification and crystallisation of luciferase has been described,⁷¹ its use in pure form in these assays is not economical at the present time. Cormier and Totter¹⁴ have pointed out that ATP assays could be readily carried out without depleting the firefly population by using the readily available bacterial enzyme to assay NADH as follows:



The sensitivity attainable with a particular firefly preparation is of course dependent on the level of endogenous ATP. The latter can be reduced considerably by incubating the crude extract of 20°C for several hours. A better procedure to remove ATP is the use of Sephadex G-25 gel filtration.⁴⁵ Unfortunately this procedure also removes the luciferin cofactor, which must then be added back to the reaction mixture. About 10⁻¹⁵ moles ATP can then be detected by this method. Lyman and DeVincenzo¹⁹ recommend the addition of small amounts of apyrase to the luciferase to hydrolyse endogenous ATP. The present author finds this procedure difficult to control.

A good deal of attention has been paid to the proper extraction of ATP from biological material. Thus, it is important to assess whether its level has changed during sampling, extraction or storage.^{56, 72-75} Those extraction procedures tested have included: boiling water,²⁸ hot tris buffer,⁷³ perchloric acid,³⁷ trichoroacetic acid,⁷⁶ ethanol,^{46, 76} ethanol with Na-EDTA,⁷⁷ sulphuric acid for lake sediments,⁷⁸ dimethylsulphoxide at acid or neutral pH for spacecraft water supplies,^{79, 80} chloroform,¹⁹⁶ n-butanol in tris buffer,¹⁸ N-bromosuccinamide⁸¹ and butanol-octanol buffer⁷⁵ for soils. Each extraction procedure has usually been developed specially for the material under investigation. Dilute perchloric acid is a highly efficient extractant for many biological materials and ATP is quite stable in it at 0°C.³⁷ However, the

removal of perchlorate as the insoluble potassium salt should be avoided since ATP co-precipitates and is consequently lost.⁸²⁻⁸⁴ A number of anions and cations are inhibitory, including perchlorate and chloride, and which reduce luminescence.^{28, 30, 45, 46, 85} This is remedied by using more luciferase. The importance of using an internal standard in such cases cannot be overemphasised. Phenolic material is frequently a problem since it inhibits luciferase⁷⁶ and must therefore be removed from plant extracts before assaying for ATP. Stenlid⁸⁶ has reported that iso-flavones also inhibit. The use of internal standards is mandatory for coloured samples, since absorption quenching occurs under these conditions.

The presence of ATPases in tissues, especially plants, makes the estimation of endogenous ATP very difficult and it is usually important to denature them before assay. Thus, in micro-organisms, the injection of the sample into boiling water, boiling buffer or cold acid is often possible, but for multicellular organisms another procedure is required. In our laboratory, freezing small portions of the sample in isopentane (-79°C), then in liquid nitrogen, followed by grinding (-196°C) in a top-drive homogeniser, has proved successful. The powder is then mixed with ice-cold 0.4N perchloric acid and as the powder thaws, perchloric acid rapidly enters the cells, denatures the enzyme and extracts the ATP.⁸⁷

Assay of FMN and NADH

Light emission from the bacterial system appears to come from a protonated flavin, FMNH^+ , since its spectral emission corresponds very closely with the bioluminescence spectrum of the species.^{20, 88, 89} Since reduced FMN has a half-life of around 0.1 s in air, it follows that the analytical assay for the reduced form is unlikely to be of value. However, with the aid of NADH-linked dehydrogenase, the system can be coupled to the bacterial luciferase and thus makes possible the assay of both FMN and NADH when these are in turn rate limiting.^{34, 38-40, 50} The system may also be used to measure NADPH but the sensitivity is only 5% of that for NADH.^{34, 38-40}

The requirement of a long-chain aldehyde is a peculiar feature of the luciferase,⁹⁰ and decanal and tetradecanal are frequently employed because they give near optimum light output,^{91, 92} although optimal chain length varies somewhat with bacterial type. The reaction is very temperature dependent and has an optimum of about 28°C .^{38, 39} The amount of light produced is also dependent on the ionic strength of the buffer as well as on its pH.³⁹ The reaction is insensitive to cyanide, azide and fluoride at 1 mM and is inhibited by 10^{-5}M Zn^{2+} and Ca^{2+} .³⁹ The sensitivity of the procedure is in the picomole range,³⁹ but this depends very much on the luciferase preparation. However, Lee *et al.*,²⁰ using a luciferase prepared from *Photobacterium fischeri*, have obtained a maximum sensitivity of 10^{-16} mole and were limited only by the thermal noise of the photomultiplier. The proportionality between substrate concentration and photon output holds over a wide range, is far superior in sensitivity to fluorescence methods and is not subject to interference.

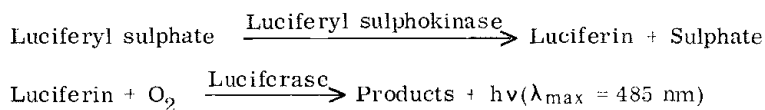
As mentioned previously, the assay procedure may be used for FMN.³⁹ However, the number of photons counted is not directly proportional to the amount of FMN present, since on increasing its concentration its yellow colour absorbs increasing amounts of emitted light³⁹ and there is some evidence that FMN may be a substrate inhibitor for the dehydrogenase.⁹³ Neither FAD nor riboflavin can replace FMN in the reaction; however, it is claimed that the reduced forms of FAD, riboflavin and other flavins are weakly active in bacterial luminescence but give different emission spectra.^{94, 95}

Extraction of NADH⁹⁶ and NADPH⁹⁷ must be made in alcoholic alkali or with 0.1M NaOH⁵⁴ since they are acid labile. NAD⁹⁸ and NADP⁹⁹ may be extracted using dilute perchloric acid. When NAD/NADH ratios are to be determined, the NADH is measured first and then the NAD is reduced using malic dehydrogenase and a further measurement is made. Mixtures of NADH and NADPH can be measured selectively and specifically by oxidising NADPH with oxidised glutathione and glutathione reductase or specifically by oxidising NADH with acetoacetate and 3-hydroxybutyrate dehydrogenase. The use of internal standards to monitor quenching is of course mandatory.

FMN is usually structurally bound to tissue proteins and can be released by perchloric acid,¹⁰⁰ boiling water or aqueous phenol for yeast.¹⁰¹ Total phosphoflavin (FMN plus FAD) may be estimated by boiling perchloric acid extracts to convert FAD to FMN. FAD alone can be measured by performing the assay before and after the hydrolysis.¹⁰⁰

Assay of PAP (adenosine 3'-phosphate 5'-phosphate)

The bioluminescent system of the coelenterate *Renilla reniformis* has been studied extensively by Cormier and his associates^{8, 14-17, 102-106} and it has been shown that light production in cell-free extracts of this organism is absolutely dependent on PAP. The bioluminescent system involves two stages, thus:^{102, 103}



Since PAP is readily formed by the mild hydrolysis of PAPS (adenosine 3'-phosphate 5'-sulphatophosphate), a sensitive method becomes available for these compounds, which are of considerable interest in sulphate metabolism in micro-organisms, plants and animals. Since the K_m for PAP in the reaction is 7.3×10^{-8} M,¹⁰² such a system is likely to be very sensitive. A simple method using the liquid scintillation counter and the *Renilla* system has now been developed by the author⁴⁰ and a fuller account will be published later. The sensitivity attained thus far is a few picomoles of PAP. The procedure depends on adding PAP to the *Renilla* enzyme complex together with luciferyl sulphate,^{107, 108} after which the photon output increases linearly with time and the slope is proportional to the amount of PAP up to about 150 picomoles in the assay procedure.⁴⁰ An internal standard is, of course, required and this can be added to the reaction mixture after the unknown sample has been assessed. This method has been of value in showing that PAPS is produced in higher green plants, but depends on the presence of AMP to protect the PAPS from hydrolysis by various nucleotidases.⁴⁰

COUPLED REACTIONS

Strehler and Totter²⁸ were the first to apply bioluminescence reactions *in vitro* to determine important intermediates in biological systems. They used firefly extracts to assay not only ATP but also a number of substrates and enzymes by measuring the light produced with a quantum counter cooled in liquid nitrogen.^{109, 110} More recently, Strehler has published several reviews and articles based on coupled reactions with the firefly system.^{51, 52, 111, 112}

In the coupled system, whether it be for enzyme or substrate, the procedure is dependent on the material to be assayed being present in rate-limiting amounts. Thus to obtain a linear response, the concentration should be less than the K_m value.

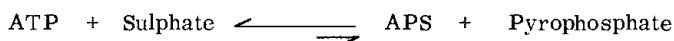
The sensitivity achieved is several orders of magnitude greater than for spectrophotometry. Thus NADH measured in a spectrophotometer at 340 nm with an o.d.

of 0.06 in a 1 cm path 2.5 ml cuvette would be about 2.5×10^{-8} moles. Since a picomole is readily measured^{38, 39} with the Photobacterium system (similar volume), it follows that the bioluminescence system is some 25,000 times more sensitive.

The applications for coupled reactions fall into three classes :

1. The assay of the substrate (or closely related substrate, such as NADH), e.g. ATP, NADH, NADPH, FMN, PAP.
2. The assay of enzymes. Strehler⁵¹ mentions adenylate kinase and creatine kinase as examples.
3. The assay of related compounds which can be used to mediate a reaction in which ATP or NADH/NADPH is formed or utilised. Strehler⁵¹ lists ADP, phospho-creatine, phosphoenolpyruvate and glucose in this category.

In classes 2 and 3 there have been recent reports concerning the assay of other compounds. Balharry and Nicholas⁵⁹ have described the measurement of ATP-sulphurylase and adenosine phosphosulphate (APS); both the enzyme and APS are particularly difficult to assay by other means since they frequently occur at low levels and APS is very labile. Stanley^{40, 44} has used the reaction mediated by ATP-sulphurylase to measure pyrophosphate :



The enzyme is readily isolated and purified from baker's yeast and the reaction carried out at 20°C. The incubation mixture consists of 1 ml 50 mM Tris-HCl, 1 ml 15 mM sodium arsenate and 1 ml 10 mM MgCl₂, all at pH 8.5. This is counted in a vial for 2 min (0.1 or 0.04 min counts); ATP-sulphurylase (amount depends on its activity) and 20 µlitres firefly extract³⁷ are then added together with 2 nmoles APS¹¹³ and the mixture counted again for 2 min. Addition of 1-100 picomoles of pyrophosphate cause an immediate rise in the count rate as monitored by the liquid scintillation spectrometer, or by a ratemeter or multichannel analyser operated in multi-scale. The initial slope of this third segment of the reaction is proportional to the amount of pyrophosphate. A calibration curve and internal standards are of course essential. The use of phosphate buffers should be avoided since pyrophosphate is a frequent contaminant.

Malate and oxaloacetate can be measured in picomole amounts by coupling to the luminous system of Photobacterium.⁴⁴ The reaction is mediated by malic dehydrogenase, thus :



The equilibrium constant at neutral pH is such that malate formation is heavily favoured but conditions for the reverse reaction, to measure malate, can be made more favourable by increasing the pH to 9.5 and using a large excess of NAD or acetyl pyridine NAD. The initial rate at which NADH is utilised or produced is a direct measure of oxaloacetate or malate respectively.

The reaction mixtures used are as follows :

Malate assay	Oxaloacetate assay
2 mlitre 0.05M Tris-HCl, pH 9.5	2 mlitre 0.05M phosphate, pH 7.0
2 µlitres β-mercaptoethanol	2 µlitres β-mercaptoethanol
20 µlitres tetradecanal in ethanol (saturated)	20 µlitres tetradecanal in ethanol (saturated)
2 nmoles FMN	2 nmoles FMN

20 μ litres 1 mg/ml dehydrogenase/ luciferase from <u>Photobacterium</u>	20 μ litres 1 mg/ml dehydrogenase/ luciferase from <u>Photobacterium</u>
5 μ litres 0.5 mg/ml malate dehydro- genase (pig heart; sp. act. 1100 units/mg)	5 μ litres 0.5 mg/ml malate dehydro- genase (pig heart; sp. act. 1100 units/mg)
500 nmoles NAD	10-1000 picomoles NADH (depends on level of oxaloacetate to be assayed)
10-200 picomoles malate	10-1000 picomoles oxaloacetate

Preparation of the tetradecanal solution requires special attention.^{39,40} Pig-heart malate dehydrogenase was obtained from Boehringer (Mannheim, Germany), Photobacterium luciferase from Sigma (St. Louis, Missouri, U.S.A.) and tetradecanal from Fluka (Switzerland).

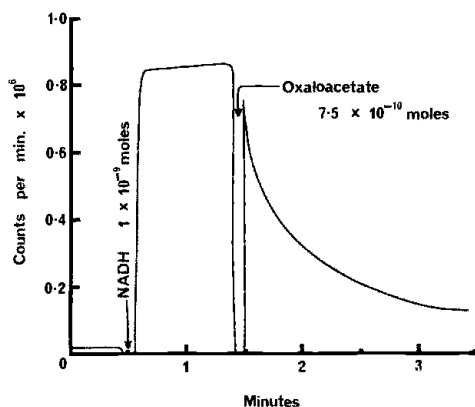


Fig. 2. Reaction sequence for the assay of oxaloacetate (see text for details).

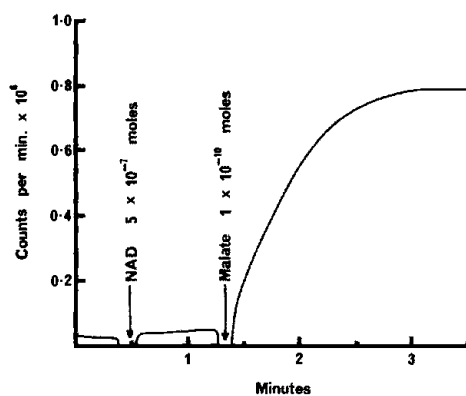


Fig. 3. Reaction sequence for the assay of malate (see text for details).

Figures 2 and 3 show the reaction scheme for oxaloacetate and malate, and calibration curves for these substrates are presented in Figs. 4 and 5. It should be noted that the apparent equilibrium level of NADH after the reaction is less than expected due to the presence of inhibitory or coloured material in the oxaloacetate sample. This illustrates well the necessity for always including a second assay with an internal standard. The non-linearity of the calibration curve for oxaloacetate is due to the level of NADH employed, only 120 picomoles. Thus at higher concentrations of oxaloacetate (>50 picomoles), a significant proportion of the NADH is utilised very quickly and despite the very favourable equilibrium constant the reaction rate slows.

Another technique utilising the Photobacterium complex is that described by Nicholas and Clarke⁶⁰ for measuring ammonia. The coupled reaction involved the oxidation of NADH in the presence of glutamic acid dehydrogenase and α -oxoglutaric acid. The sensitivity is around 10^{-8} moles.

Another enzyme which has been measured by a bioluminescence procedure is phosphodiesterase.¹¹⁴ The assay involves making the enzyme rate-limiting in a reaction sequence in which adenosine 3', 5'-monophosphate (cyclic-AMP) reacts via adenosine 5'-phosphate to give ATP. This enzyme can be readily detected in as little as 0.1 μ g of brain tissue.¹¹⁴

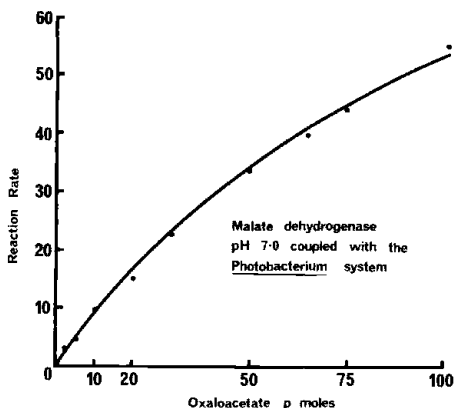


Fig.4. Calibration curve for oxaloacetate.

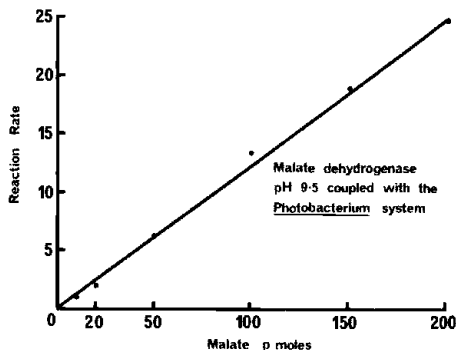
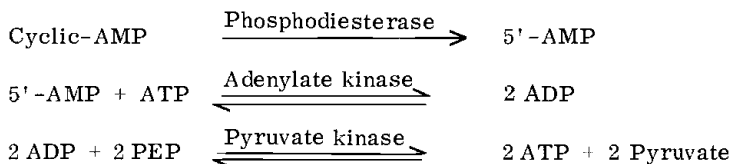


Fig.5. Calibration curve for malate.

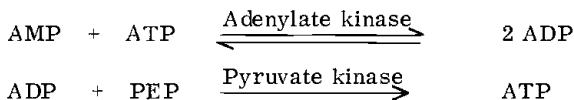
Johnson *et al.*¹¹⁵ have used phosphodiesterase to assay adenosine 3', 5'-monophosphate (cyclic-AMP) using the following reaction sequence and then measuring the ATP produced:



PEP = Phosphoenolpyruvate

The sensitivity is around 1 picomole cyclic-AMP. Johnson¹¹⁶ has published further on the measurement of cyclic-AMP by this procedure and Ebadi¹¹⁷ has reviewed the method for measuring cyclic-AMP in urine and various tissues.

The balance of AMP, ADP and ATP is especially important in the control of cellular processes and a number of workers have used the firefly luciferase technique to measure mixtures of these compounds. Parkinson and Medley¹¹⁸ measured these compounds in human plasma using the following enzyme transformations:



Thus a number of measurements of ATP are required and the amounts of nucleotides are obtained by difference. Pradet¹¹⁹ has used a similar technique for estimating these compounds in plant tissues. Brooks¹²⁰⁻¹²² has measured ATP and ADP in spermatazoa and blastocysts and Holmsen *et al.* their levels in plasma¹²³ and blood platelets,⁷⁷ again employing the standard pyruvate kinase transformation.

The use of a standard light source

In the preceding sections mention has been made of the importance of calibration curves from which results can be obtained. However, to check an instrument from day to day or to work on an absolute basis, a standard light source is required. Hastings and Weber¹²⁴ have described a source prepared from hexadecane-1-¹⁴C in a scintillator of PPO, POPOP in either toluene or monoisopropyl biphenyl.

This of course does not give rise to single discrete photons but to scintillations and is thus suitable for photometers rather than for quantum counters. Lee *et al.*¹²⁵ recommend a luminol standard which can be the source of controlled chemiluminescence (single photons). However, a more convenient single-photon source may be a light-emitting diode (LED) of good long-term stability, preferably doped with gallium arsenide and coated with a phosphor to give an emission between 500 and 600 nm. A small mercury battery to drive the LED and the LED itself could fit readily into a scintillation vial.

GENERAL APPLICATIONS

During the last few years bioluminescence has been used to determine cell numbers and biomass in water and soil. An interesting example is a method used in the NASA programme to detect extraterrestrial life by the presence of either ATP^{18, 81} or FMN,⁵⁰ which are products of living organisms. The measurement of low levels of bacterial contamination of potable water in spacecraft^{79, 80} is also possible by this procedure. Chappelle and Levin,¹⁸ in a survey of nineteen species of bacteria, found a mean of 2×10^{-16} g ATP/bacterium with a coefficient of variation of 100% due to abnormally high levels in three of the nineteen species, while Hamilton and Holm-Hansen¹²⁶ report a mean of 2.3×10^{-15} g ATP/bacterium (with a coefficient of variation of 100%) for seven species grown in a chemostat. This ten-fold difference may well be associated with the physiological state of the bacteria,¹²⁶ and other work supports this suggestion.¹²⁷ However, D'Eustachio *et al.*^{128, 129} find that the ATP level is relatively constant at different growth stages and have also reported levels of ATP for a number of bacterial species.

An important aspect of detecting low concentrations of micro-organisms by these methods is that it provides a very sensitive technique for checking the sterility of solutions. This is especially important in the pharmaceutical and beverage industries since the assay is quick in operation, e. g. a result can be obtained within 10 min. Ames¹³⁰ has used this technique to test urine samples for bacteruria and to check the effectiveness of various antibiotic treatments. NASA has also used ATP as a measure of urinary tract infection in humans¹³¹ and units have now been specially designed to automatically determine ATP in such samples.^{132, 133}

A particularly important aspect of measuring micro-organisms is in the assessment of biomass (bacteria, fungi, algae, protozoa) in fresh and marine waters, and more recently in soils. Holm-Hansen and his co-workers have pioneered this type of work in the aquatic environment^{73, 74, 126, 134-140} and it has now been established that ATP is present only in living cells. The nucleotide diffuses rapidly out of dead cells and probably does not adsorb on non-living matter since it is rapidly broken down. A number of workers have established that the ratio of cellular organic carbon to ATP is around 286³ for marine and freshwater algae,^{74, 134, 135, 141-143} bacteria,^{72, 126, 127, 144, 196} fungi¹⁴⁵ and tumour cells.¹⁴⁶ However, the ratio of carbon to chlorophyll *a* is around 100^{135, 147} and so the ATP assay provides extra evidence for the validity of biomass estimates. Lee *et al.*^{78, 148} have used the ATP procedure for measuring microbial biomass in lake sediments, and Patterson *et al.*¹⁴⁹ used it for activated sludge. A number of workers have used the method for measuring biomass in soils^{56, 75, 81, 150, 151} but absorption of ATP to clay particles appears to be a problem, as well as poor recoveries with certain soil types.

Numerous applications of the technique for measuring ATP in various tissues have been reported including blood,¹⁵¹ crayfish stretch receptor cells,⁴¹ human, bovine and octopus spermatozoa,¹⁵²⁻¹⁵⁴ houseflies,¹⁵⁵ adrenal chromaffin cells,¹⁵⁶ preimplantation mouse embryos,¹⁵⁷ cerebral mitochondria,¹⁵⁸ *Chlorella*,⁴⁶ erythrocytes⁵⁸ and plants.^{76, 87}

Most bioluminescence systems require oxygen and their sensitivity to it varies widely. Very low levels of oxygen are sufficient to cause light emission in the luminous bacteria and thus they make convenient detectors of this gas. Harvey^{4, 159} provides evidence that as little as 0.007 mm Hg can be sufficient for bioluminescence, whereas Meyer¹⁶⁰ states that 1 part O₂ in 10¹⁰ parts N₂ at atmospheric pressure is enough. The partial pressure needed for maximum light emission is also small, being only 1-2 mm Hg.^{161, 162} More recently Schindler¹⁶³⁻¹⁶⁵ has measured O₂ down to 0.0004 μM and states that luminescence is linearly related to O₂ concentration below 0.1 μM.

The luminous bacteria have been used to monitor the effects of ionising radiation,^{166, 167} anaesthetics,¹⁶⁸⁻¹⁷¹ air pollutants¹⁷²⁻¹⁷⁶ and rocket fuels in space capsules.¹⁷⁷ The effects of temperature, pressure and narcotics on bioluminescence in intact cells have been thoroughly investigated.¹⁷⁸ The bacteria have also been employed to screen antibiotics since it has been shown that there is a correlation between the therapeutic effectiveness of the drugs and the brightness of luminescence.¹⁷⁹⁻¹⁸³ Harvey⁵ has listed a number of papers since 1889 on the effects on the luminous bacteria of such diverse materials as tobacco smoke, alcohols, metal salts, ethers and alkaloids. The bacteria may also be useful in the future to screen algacides for their effectiveness in inhibiting photosynthesis (and hence O₂ evolution) in those algae which cause pollution in reservoirs.

Bioluminescence in the hydromedusid Aequorea does not involve the usual luciferin-luciferase system. Instead a photoprotein, aequorin, luminesces in the presence of calcium ions.^{184, 185} The process does not have an oxygen requirement. Shimomura *et al.*¹⁸⁶ proposed its use as a microtest for Ca²⁺ and it has been used to examine intracellular Ca²⁺ ion transients in electrically stimulated single muscle fibres of the barnacle Balanus nubilis.¹⁸⁷⁻¹⁹⁰ They conclude that there is no relationship between Ca²⁺ concentration and tension. This procedure has also been applied to mitochondria¹⁹¹ and single nerve fibres in Loligo forbesi.¹⁹²

Shimomura *et al.* have suggested the use of Odontosyllis extracts as a microtest for cyanide^{193, 194} and Chaetopterus extracts to assay ferrous ions.¹⁹⁵

CONCLUSION

The light emission from bioluminescence reactions has been shown to have a wide range of analytical applications. While some of those discussed have to date been measured only in a photometer or even with the naked eye, the liquid scintillation spectrometer could, in most cases, have been used and with increased sensitivity. The areas in which advances can be expected are those for the assay of specific enzymes and substrates and their uses will no doubt expand rapidly when an instrument becomes available commercially which can automatically process samples. This will then make procedures useful not only in the research field but also in the clinical field of micro-biochemistry, e. g. in testing for genetic enzyme deletions in amniocentesis samples, where currently routine tests are not available. Thus far more emphasis has been placed on ATP-based assays, but because of the extremely wide range of dehydrogenase enzymes it is likely that the NADH assay system will become more important in the assay of a wide range of compounds with a high degree of specificity and sensitivity, e. g. prostaglandins.

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DISCUSSION

B.E. Gordon: Has this technique been in fact used for O₂ determination in the low ppm (even 0.01 ppm) region? Also for a flowing system?

P.E. Stanley: My references will quote Meyer¹⁶⁰ who states that 1 part of O₂ in 10¹⁰ parts of N₂ at atmospheric pressure can be detected! Schindler¹⁶³⁻¹⁶⁵ has shown that luminescence is linearly related to O₂ down to 0.0004 μM. These are of course for the bioluminescent bacteria. Higher concentrations above around 1-2 mm Hg need a different organism since this level of O₂ is saturating for the bacteria. I have no knowledge of such a system being used for flow measurements. Perhaps the bacteria could be metered in with the gas and the light measured continuously as the mixture passes a photomultiplier placed close to the mixing point.

J. de Bersaques: What are your experiences in the determination of enzyme activities?

P.E. Stanley: The system is very good for dehydrogenases and ATP-sulphurylase. The appearance of NADH or ATP can be followed continuously. We have used it for measuring malate dehydrogenase and adenylate kinase and as long as the correct controls are used (plus internal standards) these assays are perfectly satisfactory and at least a thousand times more sensitive than conventional spectrophotometric techniques.