

THE USE OF THE LIQUID SCINTILLATION
SPECTROMETER IN BIOLUMINESCENCE ANALYSIS

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ABSTRACT

This review covers publications concerning analytical bioluminescence which in the main have appeared between mid-1973 and mid-1976. Outlines of some new assays and techniques are given together with modifications of existing procedures. Comments are presented on the use of the liquid scintillation spectrometer and other equipment for measuring bioluminescence. New applications are detailed and discussed.

INTRODUCTION

Light produced as a result of biological or enzymic reactions and which arises from electronically excited products is called bioluminescence. These reactions can be used to advantage for the analysis of certain compounds when they are present in rate limiting amounts. Thus a sensitive assay for adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) can be achieved using the luciferase enzyme-complex derived from the firefly *Photinus* and the bacterium genus *Photobacterium* respectively.

The present author reviewed extensively the literature on this topic three years ago (1) and since

then other reviews have been published (2-5). This review concerns in the main the literature appearing between mid-1973 and May 1976 and only limited attention will be paid to the basic principles since these have been covered elsewhere (1-5). For up-to-date accounts of the biochemistry of bioluminescence the reader is referred to recent reviews (6,7).

GENERAL ASPECTS

Introduction

Analytical bioluminescence is based on measuring the rate of the light emitting reaction. Thus it is necessary to determine the number of photons emitted per unit time at a fixed time or times after mixing the reactants. The actual rate will be the observed rate multiplied by an efficiency factor which must include not only the quantum efficiency of the photomultiplier at the wavelengths of the photons but also the optical efficiency of the detector assembly. The quantum efficiency is likely to be less than 10% for most photomultipliers (1) and the optical efficiency (percentage of emitted photons actually reaching the photocathode) is likely to be between 25 and 75%. Thus the observed rate is of the order 2 → 5% of the actual rate. The quantum yield of the reaction is the average number of photons produced per reacting molecule and for the firefly luciferase-ATP system this value approaches one. For other bioluminescence reactions it is frequently a good deal less and thus the worker usually only 'sees' a small fraction say around 1% of the number of molecules undergoing the bioluminescence reaction.

Temperature, pH, ionic strength etc. should be rigorously controlled since these will influence the rate of the reaction and consequently the final result of the assay.

It is important to have information about the kinetics of both the mixing of the reactants and the reaction itself since there are distinct advantages in being able to conduct an assay under conditions where the reaction kinetics are slow relative to those of mixing, that is when mixing is so fast that it has a negligible effect on

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the reaction rate. This has been accomplished by Rhee *et al* (8) who used a stopped flow spectrophotometer to measure ATP with the firefly luciferase.

Another approach to measuring these reactions is to integrate the luminescence-time curve over a fixed interval commencing at the time of mixing or at a fixed time thereafter (9,10). This latter approach is adopted by most workers using the liquid scintillation spectrometer since photons are counted from the time the vial is loaded until the selected preset time, usually 5 → 30 seconds, has elapsed.

Yet another approach is to mix the reactants in a continuous stream and to pass the mixture through the detector in a flow system (11). Effluents from chromatography columns can be monitored in this manner. However, considerably more reagents are consumed and less information about the kinetics of the reaction can be gleaned since generally, measurements are made of the steady state situation.

INSTRUMENTATION

Commercial

The rationale for using the liquid scintillation spectrometer to measure bioluminescence has been dealt with extensively elsewhere (1,2,3,5,9,10,12) but suffice to say that it still is the most sensitive unit which is available in most laboratories which can perform this task. Single photons can be counted by switching the photomultipliers out of coincidence and adjusting the pulse height analyzer to count low energy pulses. A tritium channel is suitable. Care should be taken so as not to overload the analyzers with high count rates ($>10^6$ cpm) since a non-linear response may be evident (1,10). Some workers prefer to use one rather than both photomultipliers and choose that with the best efficiency and/or the lowest background. Other instrumentation is now available commercially for bioluminescence assays and these have been assessed and used by various workers. The units include:

1. Lab-Line ATP-Photometer. Reagents are mixed in a scintillation vial externally to the photometer and then placed in it. Some 15 seconds after mixing, bioluminescence is integrated for one minute and the result is then displayed digitally.

2. DuPont Biometer. Reagents are mixed in a cuvette adjacent to the photomultiplier. The sample (10 μl) is introduced from a syringe through a septum and the total reaction volume is limited to 100 μl . Mixing is accomplished by the force of the injection and the efficacy and reproducibility of this procedure must be checked for the system under investigation. Bioluminescence is integrated for 3 seconds after mixing and the result is displayed digitally.

3. Aminco Chem-Glow Photometer. Reagents are mixed in a cuvette situated adjacent to the photomultiplier and mixing again depends on the force of the injection of the sample. The readout device is an analogue meter which can be coupled to a recorder so it is possible to obtain data over any required time interval. A flow cell is also available for use in automatic analyses.

4. Ortec - Brookdeal photon monitoring systems fitted with lock-in amplifiers would appear to warrant investigation for use in bioluminescence assays since they provide excellent signal to noise ratio, pulse pair resolution, maximum count capacity and are capable of very high counting rates. This unit has been used in the physical sciences but the author knows of no publications in which this system has been used for bioluminescence assays.

The silicon vidicon and vibrating mirror rapid scan spectrometers can be used for measuring bioluminescence spectra providing the light intensity is sufficient.

Recently two highly sensitive spectrofluorometers have been described (13,14) which no doubt could be used for measuring spectra from weak bioluminescence sources.

Some aspects of single photon counting

It has been shown (15) that the average count rate of anode pulses from a photomultiplier

$$\bar{n} = I_a/M_e = \gamma_a\Phi/M_e = \gamma_c\Phi/e$$

where I_a = average anode current, Φ = light flux on the cathode in watts, γ_a and γ_c are the sensitivities of the anode and cathode respectively (A watts⁻¹), M is the multiplication factor of the photomultiplier and e is the charge of the electron. Thus \bar{n} is proportional to Φ but not to the applied high voltage which determines M and this is a distinct advantage over the procedure involving analogue signals. Photoemission is a statistical process and given the signal to noise ratio is $\sqrt{\bar{n}t_c}$ (where t_c is the counting time interval) it means that a lower limit for a measurable rate can be established for a given system. The maximum count rate is also limited by this statistical process and it is recommended that the maximum count rate of the system (non-random pulses) exceed \bar{n} (random pulses) by at least a factor of twenty so as to avoid losing counts during times when the instantaneous rate exceeds this maximum. These workers also point out that the input time-constant (RC) of the photomultiplier will influence the height and width of the photoelectron pulses and ultimately of course limit its maximum counting rate.

The advent of very fast phase sensitive amplifiers will no doubt play an important part in single photon counting since in principle at least, a proportion of photomultiplier background pulses can be eliminated from true signal pulses just on the basis of their shape.

Special instruments

Beall and Haug (16) have designed an instrument to measure the very fast kinetics associated with the light emission of the photosynthetic alga *Scenedesmus* for a 20 μ sec period after a stimulus light was switched off. Such a system may be valuable for studying the kinetics of bioluminescence reactions although a recent report (17) suggests at least for the kinetics of firefly luciferase, such extreme measuring speeds are unnecessary since after mixing ATP, luciferin and luciferase there is a delay of some 25 msec before light is emitted and the maximum

intensity occurs after 300 msec.

Wettermark *et al* (18) have designed a photon counting device coupled to a multichannel analyzer operated in multiscale to measure ATP in single cells using the firefly luciferase system. A few femtomoles (10^{-15} moles) of ATP could be readily measured.

Rhee *et al* (8) have measured ATP in the picomole range using a stopped flow spectrophotometer and have concluded that the best index of ATP concentration is obtained by measuring the rate of the initial rise in bioluminescence following mixing. A similar conclusion has been reached by Lundin and Thore (19) who used a specially designed photometer fitted with an automatic dispenser/injector coupled to an electronic timer. These workers also studied the influence of injection velocity and hence the kinetics of mixing on the initial phase of bioluminescence and peak height reached.

Chappelle and co-workers have published details of the detection of microbial cells in urine and the automatic instruments they have employed (20-22) while Allen has described the development of a luminescence biometer for detecting microbial cells (23).

Automatic or semi-automatic instruments will no doubt be seen in increasing numbers where large sample numbers are involved such as for monitoring the microbiological disposal of sewage and in measuring biomass in soils and marine and fresh waters.

Automatic injection

To deal with large numbers of samples using a liquid scintillation spectrometer, some degree of automation is essential for mixing the reactants. Reproducible mixing is most important especially if the kinetics of the reaction is of the same order as that of mixing. While one kind of mixing may be satisfactory for one assay it may not be at all adequate for another especially if the densities of reactant solutions are substantially different or the solutions are viscous. Often workers and instruments rely on the forcible injection of a reactant to cause mixing. Stirring of reactants to cause mixing is an alternative but in the automatic LSC stirring is almost impossible but in

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the photometer this can be achieved, however, completely light tight seals around the stirrer are difficult to make. Magnetic stirring should be avoided since this may defocus the photomultipliers unless there is an adequate mu-metal shield.

Hammerstedt (24) has interfaced a Hamilton precision liquid dispenser to a liquid scintillation counter so that in the assay of ATP, the firefly luciferase is added to the next sample just as the data from the previous sample is being printed. Consistant and precision timing of the mixing process is thus accomplished. More recently a pneumatically operated manual dispenser has been described which mixes 10 μl ATP solution into 100 μl of the more viscous luciferase preparation (25). Samples were then counted in the spectrometer. Several types of dispenser were evaluated and the workers report that mixing which is too vigorous causes the enzyme to luminescence even in the absence of ATP. Reproducible injection of ATP solutions into firefly luciferase enzyme system has been described by Brunker (26). Here the injection of ATP is made from a modified syringe fitted into a scintillation vial. The syringe plunger is operated automatically by a hinge actuated by the light tight shutter of the detector of the liquid scintillation spectrometer.

ADVANTAGES

The advantages of bioluminescence assays or analytical bioluminescence include high sensitivity, specificity, speed and economy. Sensitivity will depend ultimately on the photomultiplier used; its thermal noise and quantum efficiency at the wavelength of interest being most important (1). The quantum efficiency of the reaction is of course very significant. Thus in the ATP-firefly luciferase system the value approaches unity. This is many orders of magnitude greater than the values recorded in chemiluminescence. It is of interest that ATP itself has been shown to be chemiluminescent with a quantum efficiency of 10^{-16} - 10^{-17} at pH 7 - 7.5 (27). Specificity for bioluminescence reactions is often high as is the case in the ATP-firefly luciferase system. No other naturally occurring nucleotide triphosphate is effective (however 3-iso-AMP and ϵ -AMP are active; see (1)). Bacterial luciferases can be used to measure NADH (10,12) but the

sensitivity of NADPH is twenty times less. Flavin mononucleotide is also required and thus can be measured in this reaction (10,12).

Specificity is high in the bioluminescent system of *Renilla* since only PAP (adenosine 3'-phosphate 5'-phosphate) is active. Thus the assay of PAP and PAPS (adenosine 3'-phosphate 5'-sulphatophosphate) using the LSC has been described (1,28,29,30). The occurrence of PAPS has been unequivocally established in plant materials using this method (30).

Bioluminescence reactions can often be performed quickly since they require no separation procedures such as are usually required for example with radioisotopic techniques. They are thus similar in many respects to spectrophotometric assays used for instance to follow the formation of NADH. The main difference to the spectrophotometric procedure is of course sensitivity, the bioluminescence assay being at least 20,000 times more sensitive. The assay of malate, oxalacetate, pyrophosphate, adenosine 5'-sulphatophosphate, PAPS, takes around seven or eight minutes to perform (1).

The cost of the assays is small since the reagents usually cost only a few cents per assay. Where large numbers of samples are concerned this becomes important as is the case in the field of clinical biochemistry for example in measuring various dehydrogenase enzymes or for screening the blood of newborn infants for creatine phosphokinase to detect muscular dystrophy (31).

NEW AND MODIFIED ASSAY PROCEDURES

Of the recent publications most are concerned in some way with ATP. Little about the assay of NADH and its conjugates has appeared.

Cheer *et al* (32) have described a procedure to measure ATP using a liquid scintillation spectrometer and it was similar to previously described methods except that counting was performed with the photomultipliers in coincidence. The potential problems associated with this approach have been discussed elsewhere (9,10,12). As would be expected the enzyme background was much reduced when

compared to that obtained with the out-of-coincidence technique. These authors provided good evidence that samples containing ATP were best kept at the temperature of liquid nitrogen or dry ice since at -20°C considerable losses of ATP were apparent. Kimmich *et al* (33) also used the photomultipliers in coincidence because of high and variable enzyme blanks. These workers have devised a method for measuring both AMP and ADP after converting them enzymically to ATP and in addition have achieved a five-fold activation of the crude extract of firefly luciferase by treating it with calcium phosphate. Further they employed a system buffered at a pH of 8.0 instead of the usual pH 7.4 and obtained an assay with an enhanced sensitivity.

Kimmich *et al* (33), Weiner *et al* (34) and Stanley (1,9) have recognized that ATP coprecipitates with potassium perchlorate formed when perchloric acid extracts are neutralized with KOH. If possible this neutralization step should be avoided and the extract assayed directly using an adequately buffered system (9). Davison and Fynn (35) however recommended that the sample be extracted into perchloric acid and then centrifuged to remove all protein since they have found in *Bacillus brevis* an ATPase which was not denatured by the acid. Without the centrifuging procedure the enzyme would be carried over at the subsequent neutralization and other steps and so hydrolyse ATP in the sample.

Manandhar and van Dyke have used the firefly luciferase system to measure adenosine tetraphosphate (36,37), guanosine triphosphate (37,38) and both purine and pyrimidine ribose and deoxyribose nucleoside triphosphates (39). The light output takes several minutes to reach a maximum and it seems likely that such kinetics are due to the various phosphates being converted to ATP by enzymes present in the crude firefly lantern extract. The authors give details of the thin layer chromatography necessary to separate the nucleotides prior to analysis (36,38).

The continued interest in the bioluminescence assay for cyclic nucleotides is shown by the publication of another sensitive assay for adenosine 3',5'-monophosphate (40) and another for the assay of guanosine 3',5'-monophosphate phosphodiesterase activity (41). Enzymic conversion to

ATP is the basis of both procedures.

A multichannel analyzer used in the multiscale mode is used by Quammen *et al* (42) to follow the kinetics of luminescence decay in the assay of ATP using a liquid scintillation spectrometer. In addition a flow type cell was employed so that the reactants could be mixed in the detector assembly rather than in the laboratory. The present author has followed similar kinetics using the same approach (1,28).

A recent publication (43) has highlighted a problem often not appreciated by workers new to the field and which tends to discourage them from exploiting analytical bioluminescence to its full extent. This is the problem of phosphorescence in glass and polythene vials. It is often caused by their exposure to fluorescent or direct sunlight. This spurious light is similar to bioluminescence in that it is composed of single photons and cannot be discriminated from it electronically. However it usually decays to negligible values within one to five minutes. Corredor *et al* (43) has shown the decay rate is different for various brands of vials presumably because different kinds or batches of glass and polythene were used in their manufacture. Further the light transmission (bioluminescence) can vary by a factor of more than 1.6 while the within vial variation of each type was 5 → 10%. Polythene vials gave the best transmission but also the highest phosphorescence, sometimes so high as to preclude their use.

A very sensitive assay for proteolytic enzymes has been described by Njus *et al* in which bacterial luciferase is used as the substrate (44). This luciferase enzyme has as its substrate FMN and not ATP. The luciferase was treated with the protease separately and the remaining active luciferase was measured at various times in a standard assay procedure. As little as 20 ng trypsin could be measured and no separation procedures were involved. The present author has shown that the assay can be followed in a dynamic fashion using the liquid scintillation spectrometer (set in the repeat count mode) when trypsin is mixed with the bacterial dehydrogenase/luciferase and with NADH and FMN (11) present in excess. Presumably trypsin acts on both the dehydrogenase as well as the luciferase.

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Nakamura (45) has standardized a light source containing luminol and has used it to measure quantum yields of bioluminescent reactions. Such a standard system should be considered by workers who quote the sensitivity of their procedures. Inter-laboratory and inter-instrument comparisons could be therefore readily made.

APPLICATIONS

Measurement of Biomass

In recent years ATP has shown great promise as an index of biomass and the pioneering work of Holm-Hansen and colleagues has been discussed previously (1). It is recognised that the efficient extraction of intact ATP from the microorganism in the water or soil is the most difficult and critical step. Perchloric, trichloroacetic and sulphuric acids, boiling water, boiling buffers including tris, tris-borate and glycine, dimethylsulphoxide, n-butanol, n-bromosuccinamide and boiling chloroform have been used (1,46). Soils and sediments are particularly difficult to deal with and it appears that procedures involving cold acid extraction are the methods of choice. Karl and LaRock (46) used sulphuric acid-EDTA and obtained extraction of 81-94% whereas with boiling tris the recovery was 3-6%. The selection of an appropriate internal standard is difficult since native ATP will be extracted much more readily for example than ATP derived from a microorganism adhering tenaciously to a particle of soil. Lyophilized bacteria may be employed or bacterial cells coated onto glass beads, similar in size to the soil particles, may be suitable. These of course should be added prior to the extraction process. Karl and LaRock (46) stress the importance of the effect of extracted cations and anions on the luciferase enzyme.

Moriarty (47) has recently shown that there is a correlation between muramic acid and biomass of bacteria in aquatic sediments. Since Gram-negative bacteria contain about 20 μg muramic acid/mg C and Gram-positives have 100 μg /mg C the relative numbers of each type must be estimated by other means. The method cannot be used in presence of blue green alga. Other publications on measuring biomass by the ATP index are to be found in the references (48-53).

Other applications of the firefly luciferase ATP assay

This procedure has been used extensively to study ATP in various components of blood. It is considered that the concentration of adenine nucleotides have critical influence on platelet aggregation *in vitro* and David and Herion (54) have devised and tested a technique for studying both ADP and ATP levels in such a system. The bioluminescence assay has also played its role in investigating the kinetics of thrombin-induced release of ATP by platelets (55). The level of ATP has been measured in red cells (56,57) and both groups of workers showed that the bioluminescence procedure gave falsely high readings due to luciferase being stimulated by a protein present in the extracts. The latter report (57) showed that denatured haemoglobin increases the light output.

The mechanism for the discocyte-echinocyte shape transformation in normal and ATP-enriched human erythrocytes has been studied using the firefly luciferase-ATP assay (58) and depletion or repletion of ATP in conjunction with intracellular or intramembrane factors has been suggested as being responsible for the equilibrium. Decreased levels of ATP in erythrocytes have been detected using the firefly luciferase-ATP procedure. Thus Wolf *et al* (59) have observed such a decrease in patients with either sickle cell anaemia, alcoholic cirrhosis, viral hepatitis or chronic renal disease.

The release or efflux of ATP from biological tissues (60) and from perfused heart during coronary vasodilation (61) have also been reported. The luciferase-ATP assay has also been used to study the effect on ATP levels of ultra high frequency radiation (2.45 GHz at 50 mwatts/cm²) on liver hepatocytes (62) and to measure ATP in Tarantula spider venoms. In the latter the concentration of ATP was surprisingly high (28-57 µg/µl venom) and it was shown to act synergistically with the venom toxin (63).

The luciferase-ATP method has also been used to effect in plant biochemistry to study the phytochrome mediated changes in ATP concentration in bean buds (64,65) and the light induced concentration changes of ATP in sporangio-phores of *Phycomyces* (66). In a study of ATP formation in the mitochondria of etiolated corn shoots treated with herbicide Rushness and Still (67) observed that the

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firefly luciferase assay was inhibited by the isopropyl-3-chlorocarbonilate and two of its hydroxylated metabolites. This observation indicates just how important it is to include the correct controls when bioluminescence assays are being used.

Considerable interest has centred on measuring ATP in bacterial cells (1) and this trend continues from both the research and applied side. Thus ATP pools in *Nitrobacter* have been shown to be as high as 8 picomoles per μg cell N (68) and Strange *et al* (69) in an older study investigated the effect of starvation on the ATP concentration in *Aerobacter* while Kao *et al* (70) have measured the pools in *E. coli* and *Pseudomonas*. The detection of foreign or contaminating microorganisms is pertinent here and Sharpe *et al* (71) have used the luciferase-ATP assay to detect microorganisms in various foodstuffs and found that during the incubation of these foodstuffs, the intrinsic ATP decreased while microbial ATP increased. In the clinical field the luciferase-ATP assay is being developed for the detection of bacteruria (72,73). ATP from mammalian cells which are present in the urine, was extracted by treatment with Triton X-100 and then hydrolysed with apyrase. The microbial ATP was then estimated after its extraction with boiling tris-buffer or perchloric acid. The lower limit of detection reported by both groups was around 10^5 cells. It has the advantage of speed and there is the potential for screening the sensitivity of organisms to various therapeutic agents.

The on-line measurement of the biosynthesis of ATP has been covered extensively elsewhere (1) and the procedure has been used recently to study the ATP produced in mitochondrial suspensions (74) and ATP release from platelets (55). The rate of ATP synthesis in bovine anterior pituitary slices has also been measured (75) and a study has been made of three iso-enzymes of human creatine phosphokinase and their levels following myocardial infarction (76). A sensitive enzyme assay for reverse reaction of the first histidine biosynthetic enzyme has been described recently (77). Thus low levels of ATP-phosphoribosyl transferase has been measured by estimating the ATP released from the enzyme's substrate, N'-(5'-phospho-D-ribosyl)-ATP.

Surprisingly little has appeared about the use of the NADH-bacterial luciferase assay although the present author has found it useful for measuring alcohol dehydrogenase and other dehydrogenases. Recently however, Hammar (78) has used bacterial luciferase to measure the epidermal activity of NAD-dependant iso-citrate dehydrogenase in skin biopsies during treatment with dithranol.

Izutsu *et al* (79) have studied a bioluminescence assay for ionic calcium which is based on its interaction with the protein aequorin derived from the jellyfish *Aequorea*. To obtain good reproducibility of results they found rapid mixing was necessary and concluded that the accuracy of the assay depended on the association constants of the calcium chelating agents in the test solution.

Kinetic studies have been made of the effect of various anaesthetics such as halothane and fluroxene on cell-free preparations of firefly lanterns (80,81) and the evidence for the observed inhibition of light output suggests that the anaesthetic acts at a hydrophobic site on the luciferase molecule causing a structural alteration and thus change in activity of the enzyme.

Luminescent bacteria continue to prove useful models for the study of certain anaesthetics since the levels inhibiting 50% bacterial luminescence and general anaesthesia in mammals is remarkably similar (82). Chloroform has been shown to be a competitive inhibitor of dodecanal in the light producing reaction (83). Two recent papers describe the effect of diethyl ether on the *in vivo* and *in vitro* light emission from *Vibrio fischeri* (84,85).

CONCLUSION

Analytical bioluminescence continues to be used by a gradually increasing number of workers and for a widening range of applications. Their low cost and high sensitivity make them particularly attractive for clinical biochemistry and also as enzyme labels or means of assay in the field of enzyme-immunoassay. The other area of potential is in the biochemistry of small numbers of cells as is the case for some tissue cultures and amniocenteses. The bacterial enzyme is particularly attractive for routine work since it can be obtained from readily grown cultures.

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