USE OF BIOLUMINESCENCE PROCEDURES AND LIQUID SCINTILLATION SPECTROMETERS FOR MEASURING VERY SMALL AMOUNTS OF ENZYMES AND METABOLITES

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ABSTRACT

The design and operation of a specially constructed analytical bioluminescence cell is described. The unit, which will fit into the detector chamber of a liquid scintillation spectrometer, is used to measure flash heights as well as to follow the production of light in bioluminescence assays.

The value of assaying metabolites and enzymes by a coupling to a bioluminescence reaction is illustrated by the measurement of picomole amounts of pyrophosphate and the enzyme ATP-sulphurylase using the firefly luciferin-luciferase system for determining adenosine triphosphate (ATP).

A new assay is described for determining adenosine 3'-phosphate 5'-phosphate (PAP) by the bioluminescence system of the sea pansy (Renilla reniformis). The sensitivity of the assay is around 1 picomole (10^{-12} mole). The procedure may also be used for measuring adenosine 3'-phosphate 5'-sulphatophosphate (PAPS, or "active sulphate") since this compound is readily hydrolysed to PAP.

INTRODUCTION

There is now a wide interest in the use of the liquid scintillation spectrometer as a quantum counter for measuring the single photons produced in analytical bio-

luminescence assays. The availability of photomultipliers with low-noise photocathodes enables the instrument to be used at room temperature, which is suitable for enzyme reactions. Thus, bioluminescence techniques are not only extremely sensitive but also very specific and easy to perform with equipment which is readily available.

In the field of analytical bioluminescence, two basic reactions have been developed: i) ATP, using the firefly luciferin-luciferase system (1,2,3,4) and ii) FMN* and NADH*, using the dehydrogenase-luciferase complex of the marine bacterium Photobacterium fischeri (2,3,5). They have been used mainly for measuring static levels of these compounds, but more recently the use of enzyme-coupled reactions in which ATP or NADH is either produced or utilized has enabled dynamic measurements to be made, e.g. ammonia (6) and APS (7). In this paper a new coupled system for measuring pyrophosphate and ATP-sulphurylase (ATP-sulphate adenylyl transferase E.C. 2.7.7.4) will be described.

A study of the metabolism of the sulphur nucleotides (APS* and PAPS) in biological systems has now been facilitated by bioluminescence procedures. Thus the one for APS has been described (7) and in the present paper a bioluminescence system from the sea pansy Renilla reniformis (8,9 10) is used for detecting PAP.

When assaying very small amounts of ATP or NADH (less than a picomole) it is better to measure "flash height" since light production occurs only for a few seconds (11). A unit specially designed to fit the detector chamber of a spectrometer is described which allows for the monitoring of samples from the time of mixing reactants.

MATERIALS AND METHODS

Analytical Bioluminescence Cell. The unit has been designed to fit a Model 3375 Packard Tri-Carb Liquid Scintillation Spectrometer but a simple modification will eanble the device to fit other units. The cell, which is illustrated in Fig. 1, consists of an opaque 5 ml polypropylene reaction/mixing vessel G (3.6 x 1.65 cm) fixed

*Abbreviations: APS, adenosine 5'-sulphatophosphate; FMN, flavin mononucleotide; NADH, reduced nicotinamide adenine dinucleotide.

at one end of a metal tube E (7.8 x 2.85 cm), which can be manually inserted into the detector chamber of the spectrometer so that G is positioned directly between the photocathodes of the photomultipliers. Extraneous light is prevented from reaching the photomultiplier tubes by three O-rings D and a large flange H (5.0 x 1.0 cm) which facilitates the removal of the unit from the detector chamber. A screw at the collar F (1.6 x 2.85 cm) attaches the reaction vessel G to the tube E in a gas-tight closure. The insert tubes A, made of 16 gauge stainless-steel tubing, conduct and mix the reactants, which are introduced into the tubes with syringes via the taps. Tube C, which extends to the bottom of the reaction vessel G, is used to extract the spent mixture, and B enables G to be kept at atmospheric pressure. The tube may also be used to introduce other gases.

The cell is introduced into the detector after switching off the high-voltage supply to the photomultipliers. The procedure is similar to that for fitting a flow-cell, as described by the manufacturer of the scintillation unit. Extraneous light reaching the photomultipliers will be evident as a high background in a tritium out-of-coincidence setting and this effect must be rectified immediately to avoid permanent damage to the photomultipliers.

The unit is used as follows: 1 ml of reactant is placed in each of two 2 ml glass syringes (fitted with Luer locks) together with an air bubble (0.25 ml). One syringe contains the purified luciferase and necessary cofactors and the other the energy source. The syringes are then attached at A, locked, and the pistons depressed simultaneously so that the reactants are mixed quickly. The air bubble in the syringes ensures that the tubes are emptied and that the reactants are thoroughly mixed. A small electrical switch is fitted to one syringe so that on depressing the piston, the switch closes, thus initiating a synchronising pulse to trigger multiscaling with the multichannel analyzer set at 100 msec per channel. The analyzer was connected to the ratemeter output of the RED channel set at 70-300, 100% amplification and out of coincidence. When multiscaling is complete, the counts in each channel are read and the flash height, occurring about 700-1000 msec after multiscaling had commenced, is measured.

Instruments. The bioluminescence assays reported here

were made in Packard 3375 and 3390-544 and Nuclear Chicago Mark II and Unilux III liquid scintillation spectrometers, all operating at 20° ± 0.5°. The Nuclear Chicago instruments were operated in the print-selected display mode, thus enabling counting to continue even during print-out. This is especially convenient during short counting periods, since very little information is lost when the counting rates are changing quickly.

A 200-channel Packard Spectrazoom Multichannel Analyzer, operated in multiscale, was used to follow bioluminescence reactions. It was operated at 100 or 1000 msec per channel. Alternatively, reactions were followed with a Packard ratemeter and chart recorder using a range setting of 3×10^5 or 10^6, a time constant of 1 second and a speed of 5 cm per minute.

Chemicals. Firefly lanterns and luciferin were obtained from Sigma Chemical Co., St. Louis, U.S.A. Preparation of crude firefly luciferin-luciferase, purified firefly luciferase and APS have been described elsewhere (1,12,13). The bioluminescence enzyme system from Renilla reniformis was prepared by extracting 10 g of an acetone powder of Renilla with 40 ml buffer (100 mM phosphate, 1 mM EDTA and 1 mM β-mercaptoethanol, pH 7.5) for 2 hr. The supernatant fraction remaining after centrifuging at 20,000*g* for 1 hr was passed through a Sephadex G-25 column to remove endogenous PAP and used without further purification (14).

Assay of Pyrophosphate. All solutions were filtered through a 0.22μ Millipore filter prior to use. Glassware was cleaned in hot detergent, washed four times in double-distilled water, dried at 100° and kept in an oven at 100° until required. Vials were not exposed to direct sunlight or fluorescent lighting, both of which result in phosphorescence of the glass. The room housing the spectrometers was lit only with tungsten lamps. The instrument was switched out of coincidence and a window set in the lower third of an unquenched tritium setting.

A scintillation vial containing 1 ml 50 mM Tris-HCl, 1 ml 15 mM sodium arsenate and 1 ml 5 mM $MgCl_2$ (pH 8.5) was equilibrated for 10 min at 20°. The vial was then counted for 2 min (6 x 0.1 min on the Packard units, 50 x 0.04 min on the Nuclear Chicago models). The appropriate amounts of ATP-sulphurylase and firefly extract and 2 nmoles APS were

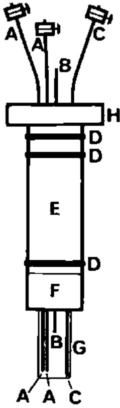


Fig. 1. A diagramatic representation of the analytical bioluminescence cell. See text for details.

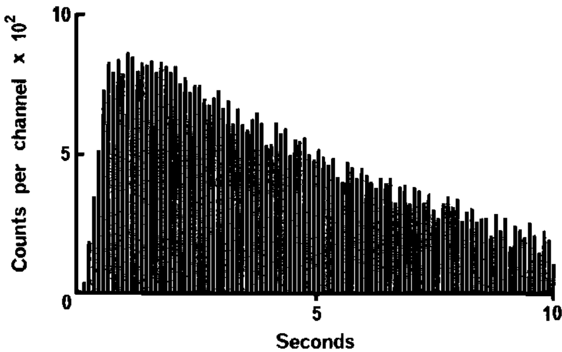


Fig. 2. Use of the analytical cell. A time course for the ATP-firefly luciferase-luciferin (5×10^{-14} moles ATP). Multichannel analyzer used in multiscale (100 msec per channel).

then added and a 2-min counting period initiated. Little difference in light output was observed during this time. The addition of pyrophosphate (1-100 pmoles) caused an immediate increase in light output which was monitored for a further 2 min. As well as a digital read-out, a continuous analogue display was available using the ratemeter and recorder or alternatively the multichannel analyzer operated in multiscale (1 sec/channel). The amounts of ATP-sulphurylase and firefly extract were varied according to the activity of the preparations. ATP-sulphurylase was readily assayed using a reaction mixture containing 2 nmoles APS and 20 nmoles pyrophosphate.

Assay of PAP (adenosine 3'-phosphate 5'-phosphate. A scintillation vial containing 2 ml of buffer (10 mM potassium phosphate, 1 mM Na-EDTA and 1 mM β-mercaptoethanol, adjusted to pH 7.5) was equilibrated at 20° for 10 min and then counted for 2 min (see pyrophosphate assay for spectrometer setting). The reaction was started by quickly adding PAP (1-100 pmoles) in a small vol (< 50 μl), 1 μl 0.3 mM luciferyl sulphate (in 0.01 M potassium phosphate pH 7.5 : ethanol, 1:1) and 50 μl Renilla enzyme system. The sample was then counted for a further 2 min, during which the light output increased linearly with time. The assay was calibrated internally by adding a standard of 10^{-10} moles PAP.

PAPS can be measured by the above procedure since it is readily hydrolysed to PAP in dilute acid (0.2 N HCl) at 37° for 30 min. This procedure has been used successfully for measuring the activity of APS-kinase (ATP:adenylyl-sulphate 3'-phosphotransferase E.C. 2.7.1.25).

RESULTS AND DISCUSSION

Some data used for measuring flash height in the analytical cell are presented in Fig. 2. Since the photons are produced randomly in the reaction, the counts recorded in each channel do not follow a smooth curve. To obtain an estimate of the flash height, the average was taken of the counts in the twenty channels immediately following the channel containing the maximum counts. For each batch of purified luciferase and luciferin it is necessary to make a calibration curve of ATP *vs* flash height over the desired range. The reproducibility of the assay depends on the level of ATP and the activity and blank of the enzyme.

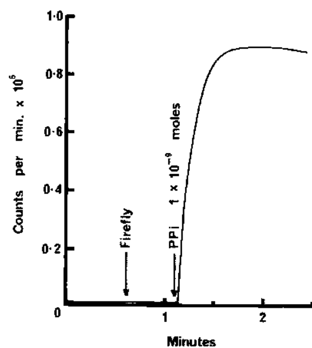


Fig. 3. Reaction sequence for measuring pyrophosphate

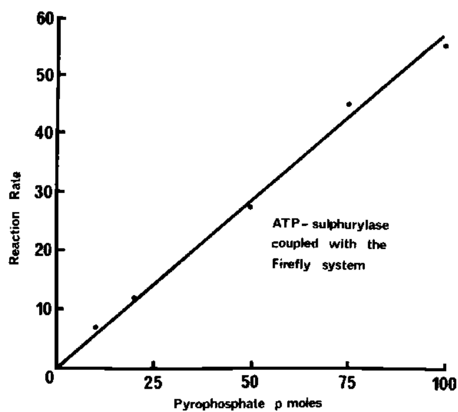
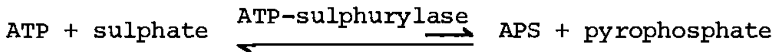


Fig. 4. A calibration curve for pyrophosphate (see text for details)

Thus, for a picomole (10^{-12}) the standard error from 25 measurements was 6.2%, while at a femtomole (10^{-15} mole) it was 14.8%. These errors might be reduced by using a mechanical or pneumatic means of depressing the piston, thus achieving a more consistent mixing. Even so the unit is valuable for measuring very small amounts of ATP (and NADH) or for working with reaction mixtures where considerable luciferase inhibition is apparent.

The basis of the reaction for measuring pyrophosphate is as follows:-

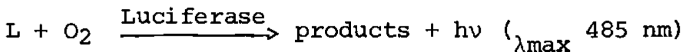


$$\text{ATP} + \text{sulphate} \underset{}{\overset{\text{ATP-sulphurylase}}{\rightleftarrows}} \text{APS} + \text{pyrophosphate}$$

The equilibrium constant is around 10^{-8} in favour of ATP production, so that it can be readily measured in a dynamic system by coupling to the firefly luciferin-luciferase bioluminescence system (1,7). The reaction can be followed in an analogue form using the multichannel analyzer in multiscale mode or the ratemeter and chart recorder, as shown in Fig. 3. A calibration curve for pyrophosphate against reaction rate or the rate of photon production is presented in Fig. 4. Thus, this procedure is several orders of magnitude more sensitive than the standard colorimetric methods. Because this system involves an enzyme reaction, it is affected by various inhibitory compounds. Internal standards are employed to assess this and also to correct for any coloured material which might absorb photons.

The reaction sequence resulting in light emission in the sea pansy *Renilla reniformis* is as follows (8,9):-

$$\text{Luciferyl sulphate} \xrightarrow{\text{L sulphokinase}} \text{L} + \text{sulphate}$$



$$\text{L} + O_2 \xrightarrow{\text{Luciferase}} \text{products} + h\nu \; (\lambda_{max}\ 485\ \text{nm})$$

L = luciferin

The structure of the luciferin has been reported recently (10). Since the K_m for PAP is 7.3×10^{-8} M (8), the system provides a very sensitive assay for not only this compound but also for PAPS, as illustrated in Fig. 5 where 1 to 100 picomoles of PAP are measured with the reaction sequence presented in Fig. 6. This assay has been used recently to

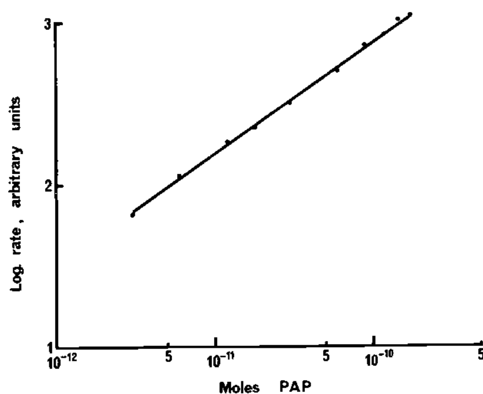


Fig. 5. Reaction sequence for measuring adenosine 3'-phosphate 5'-phosphate (PAP).

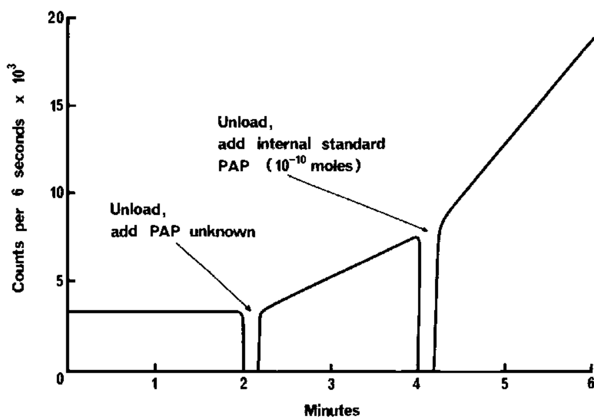


Fig. 6. A calibration curve for adenosine 3'-phosphate 5'-phosphate (PAP) using the bioluminescence system of *Renilla reniformis*.

show that higher plants synthesise PAPS from sulphate (15).

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REFERENCES

1. P.E. Stanley and S.G. Williams, Anal. Biochem. 29, 381 (1969)
2. P.E. Stanley in Organic Scintillators and Liquid Scintillation Counting, pp. 607-620 (D.L. Horrocks and C.-T. Peng, Eds.). New York and London : Academic Press (1971)
3. E. Schram, R. Cortenbosch, E. Gerlo and H. Roosens in Organic Scintillators and Liquid Scintillation Counting, pp. 125-135 (D.L. Horrocks and C.-T. Peng, Eds.). New York and London : Academic Press (1971)
4. E. Schram in The Current Status of Liquid Scintillation Counting, pp. 129-133 (Edwin D. Bransome Jr., Ed.). New York and London : Grune and Stratton (1970)
5. P.E. Stanley, Anal. Biochem. 39, 441 (1971).
6. D.J.D. Nicholas and G.R. Clarke, Anal. Biochem. 42, 560 (1971)
7. G.J.E. Balharry and D.J.D. Nicholas, Anal. Biochem. 40, 1 (1970)
8. M.J. Cormier, J. Biol. Chem. 237, 2032 (1962)
9. M.J. Cormier, K. Hori and Y.D. Karkhanis, Biochemistry 9, 1184 (1970)
10. K. Hori and M.J. Cormier, Proc. Natl. Acad. Sci. U.S. 70, 120 (1973)
11. S.E. Brolin, E. Borglund, L. Tegner and G. Wettermark, Anal. Biochem. 42, 124 (1971)
12. R. Nielson and H. Rasmussen, Acta Chem. Scand. 22, 1257 (1968)
13. C.A. Adams, G.M. Warnes and D.J.D. Nicholas, Anal. Biochem. 42, 207 (1971)
14. M.J. Cormier, Personal Communication
15. P.E. Stanley, B.C. Kelley and D.J.D. Nicholas, Manuscript submitted to Biochem. J.