

## Chapter 8

# Semi-automatic Microtransferator and Cell for the Bioluminescence Assay of ATP and Reduced NAD with Scintillation Counters

E. Schram and H. Roosens

*Vrije Universiteit Brussel, Belgium*

### INTRODUCTION

In previous articles<sup>1,2</sup> we have described the adaptation of the firefly-luciferase reaction to the bioluminescence assay of ATP with scintillation counters. It was concluded from our experiments that one is not bound to the measurement of the peak intensity of the luminescence curve (Fig. 1(a)), but that, under the proper conditions, the luminescence decreases exponentially over a rather long period of time while remaining proportional to the ATP concentration. This enables one to use the *integral mode* of counting (Fig. 1(b)), which is the only one compatible with the use of scintillation counters, and at the same time makes *flow-monitoring* possible. A lin-log plot of the luminescence versus time gives straight lines which run perfectly parallel over a wide concentration range and whose slopes are proportional to the concentration of the enzyme preparation. A similar technique was used for the bioluminescence assay of reduced NAD(P) and FMN by means of bacterial luciferase.

The main advantages of scintillation counters when used for the present purpose are their high sensitivity, the large concentration range which can be assayed, the digital display and/or output of the results, and their suitability for kinetic experiments or flow-monitoring (when used in the 'repeat' mode).

In view of the above experiments a special cell has been developed, suitable for flow as well as discrete measurements. A fixed cell, remaining in the counter, was preferred to vials in order, chiefly, to avoid phosphorescence phenomena. At the same time a device was built for the transfer of small volumes of liquid.

### CELL

The cell represented in Fig. 2 has been built to fit in the sample drawer of an Inter-technique SL 20 counter. This kind of counter offers the advantage that the counting chamber is easily accessible and that the connecting lines can be kept short. The body of the cell is made of quartz tubing in order to avoid long-lived phosphorescence after exposure to light (on installation or for maintenance). It occupies an eccentric position, closer to the photomultiplier that is used for the measurements; the dimensions of the holder, however, are similar to those of a regular counting vial so that it can be inserted without

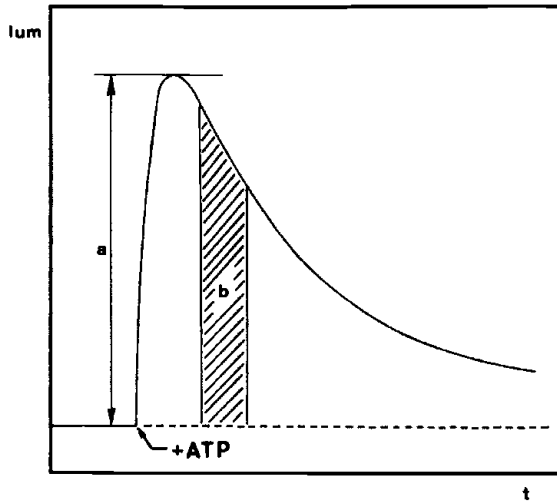


Fig. 1. Luminescence curve. a = peak intensity; b = integration of light intensity.

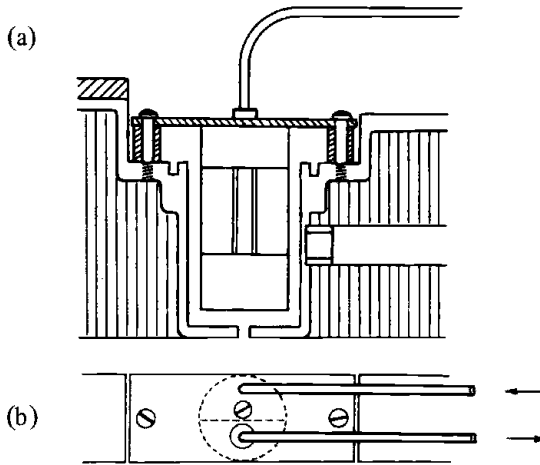


Fig. 2. Cell for discrete or flow measurement of bioluminescence; a) side view, b) top view.

any modification of the counter. The tendency has been towards a reduction of the cell volume, which may be as small as  $50 \mu\text{l}$ , thus reducing the enzyme consumption. On filling, the liquid level rises somewhat above the transparent segment, avoiding the need for a quantitative transfer. The effective volume and the geometry remaining constant, the reproducibility of the results is much increased above that attained with individual vials.

### TRANSFER DEVICE

Many techniques have been used for the transfer of samples, and are becoming in-

creasingly popular with the present expansion of automatic sample analysers. The sample solution is usually circulated by means of automatic pipettes (syringes), peristaltic pumps, compressed air or vacuum, or a combination of these systems. The flow of liquid is either unidirectional or bidirectional, intermittent or continuous. Most of these devices, unfortunately, are not adequate for the transfer of very small volumes and often require sample solution for rinsing. Our own efforts have tended towards the development of a reliable system which would not have these disadvantages.

Transfer of the sample solution into and out of the cell is achieved respectively by suction and pressure by means of a motor-driven microsyringe. When the cell is being emptied an excess of air helps to expell the last droplets of liquid from the outlet tubing, leaving less than a few tenths of a per cent of the sample solution in the cell and connecting lines. Hence no rinsing is necessary when samples of similar concentration are counted in succession. One rinse with distilled water by means of the transfer syringe is fully satisfactory in other cases. No trouble or contamination was encountered with the present device after several months of continuous operation.

Table 1. Reproducibility of results (measurements obtained on loading and unloading the same sample several times in succession).

Time (min)	Observed counts ( $10^3$ )	Theoretical <sup>a</sup> counts ( $10^3$ )	Deviation ( $10^3$ )
4	260.1	259.8	+ 0.3
6	246.0	245.9	+ 0.1
8	234.6	233.7	+ 0.9
10	222.0	221.7	+ 0.3
12	209.1	210.2	- 1.1
14	199.9	199.4	+ 0.5
16	188.6	189.1	- 0.5
18	179.5	179.4	+ 0.1

<sup>a</sup> As deduced from regression line.

Mean number of counts:  $217.4 \times 10^3$ .

Standard deviation: theoretical ( $\sqrt{\text{mean}}$ ): 467 or 0.22%  
observed: 584 or 0.27%

## RESULTS

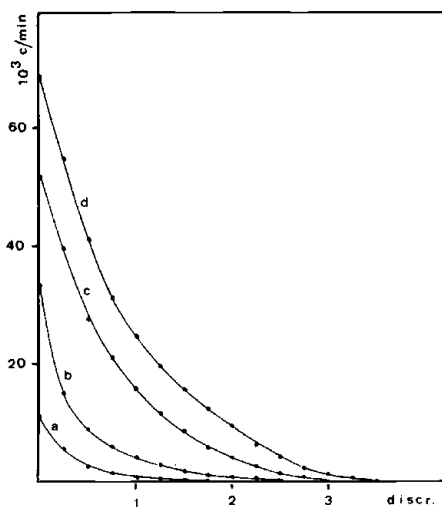
### Reproducibility

The reproducibility of the measurements was checked by loading and unloading the same sample several times in succession and observing the deviation with respect to the luminescence decay curve (calculated according to the method of least squares). These are shown in Table 1.

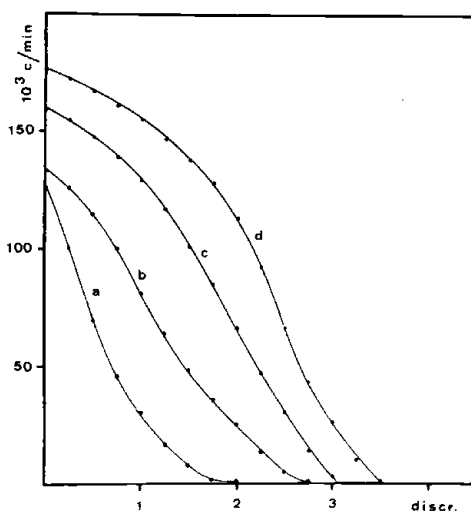
In other tests the luminescence decrease was calculated for different samples when recounted after the same time interval. The ratios found did not differ by more than 1% from the mean.

### High voltage and discriminator settings

A thorough study of the optimal counting conditions was performed, using the



*Fig. 3.* Energy distribution of background (SL 20 Intertechnique counter). a = 1000 V; b = 1100 V; c = 1200 V; d = 1300 V.



*Fig. 4.* Energy distribution of bioluminescence (SL 20 Intertechnique counter). a = 1000 V; b = 1100 V; c = 1200 V; d = 1300 V.

SL 20 Intertechnique scintillation counter. Overlapping of the photomultiplier noise and luminescence spectra is an important factor in this respect (measurements are performed with the coincidence circuit disabled). The statistical error was therefore determined for several luminescence to noise ratios as a function of the lower discriminator and high voltage settings. Increasing the high voltage above 1000 V did not improve the counting statistics. Moreover memory effects occurring at higher count rates could be eliminated at this voltage, and the dynamic range of the assay thus increased (see Figs. 3, 4 and 5).

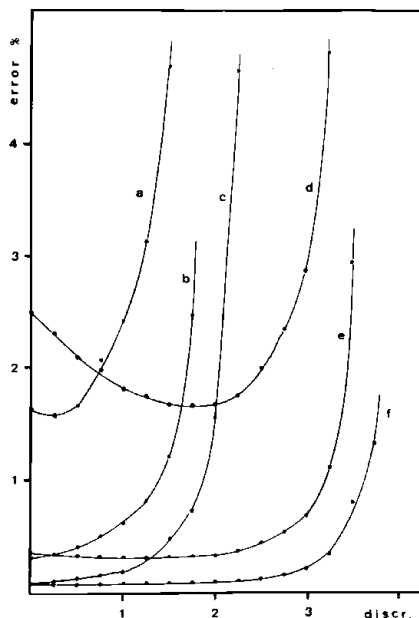


Fig. 5. Standard deviation at different ATP concentration levels and HV settings. a, b, c = 1000 V; d, e, f = 1300 V. a, d concentration so as to give a luminescence equal to the background at 1000 V; b, e =  $10 \times$  a; c, f =  $100 \times$  a.

### Sensitivity

The sensitivity of bioluminescence as an analytical tool depends not only on the proper use of the counting equipment, but also on the reagents. For our determinations endogenous ATP was separated from commercial firefly luciferase by gel filtration on BioGel P-10, with an appreciable reduction of background. Luciferin is eliminated as well (it has a similar molecular weight) but is readily replaced by synthetic luciferin at even higher concentration in order to reach maximum efficiency. The eluate from the filtration column does not need to be concentrated since diluted enzyme solutions correspond to a more adequate luminescence half-life.

When taking all necessary precautions, amounts of ATP of the order of  $10^{-15}$  mole can be measured. In many cases, however, such efforts will prove unnecessary since with regular commercial enzyme preparations,  $10^{-13}$  mole of ATP can be assayed with a statistical accuracy of better than 2%.

### APPLICATIONS

The present method for ATP determination has been used for the study of amino acid activation in *Bacillus stearothermophilus*, the periodicity of photophosphorylation in *Acetabularia mediterranea*, the development of amphibian eggs (*Xenopus laevis*); simultaneous determination of AMP and ADP after their enzymatic conversion to ATP has been achieved in blood and brain.

## REFERENCES

- 1 E. Schram, in *Liquid Scintillation Counting* (Ed. E. D. Bransome), Grune and Stratton, New York, 1970, p. 129.
- 2 E. Schram *et al.*, in *Organic Scintillators and Liquid Scintillation Counting* (Ed. D. L. Horrocks and C. T. Peng), Academic Press, New York, 1971, p. 125.

## DISCUSSION

**F. E. L. ten Haaf:** At what temperature are the measurements performed?

**E. Schram:** Luciferase shows an optimum activity around 23°C. All measurements are therefore performed at room temperature. Care should be taken to equilibrate all solutions at this temperature before mixing.

**F. E. L. ten Haaf:** When working with the very low pulse amplitudes resulting from the single photons from the sample, one is liable to have noise from the photomultiplier dynodes in addition to the thermal noise from the photocathode. Is this background sufficiently constant to enable correction for it?

**E. Schram:** No trouble was encountered as far as the stability of the background is concerned. As the photomultiplier noise is strongly temperature-dependent one should, of course, let the counter reach its equilibrium temperature after switching on. Dynode pulses are partly eliminated by discrimination (see text for background and luminescence spectra).

**J. P. Verhassel:** For these measurements do you use the output of just one photomultiplier or do you combine the output from both tubes?

**E. Schram:** Both methods are suitable but all counters cannot be used in both ways. With the Intertechnique SL 20 counter which we are using, only the tube with the best performance characteristics is used. In this case the counting cell is put closer to that tube in order to improve geometry.

**B. Scales:** You suggest that cyclic AMP can be measured making use of your system, since the sensitivity of the method is very high. Can you please briefly outline the enzyme system which you prefer for the conversion of cyclic AMP to adequate amounts of ATP?

**E. Schram:** Cyclic AMP has to be converted to 5'-AMP which can be assayed quantitatively with luciferase. However, the commercial enzyme used for this conversion still contains phosphatase activity and should therefore be further purified. Several authors have recently used their own enzyme preparations.

**E. D. Saggerson:** Is the firefly-luciferase reaction special for adenine nucleotides or will others, such as GTP, interfere?

**E. Schram:** The reaction is specific for ATP; although contamination of the enzyme with nucleoside diphosphokinase will lead to interference from other nucleoside triphosphates, these are generally present in small amounts compared with adenine nucleotides. Moreover, as the interconversion is not instantaneous the luminescence yield is only a small fraction of that obtained for an equivalent amount of ATP.