Chapter 7

# The Estimation of ATP, ADP and AMP in Human Plasma using Luciferin/Luciferase and a Scintillation Counter

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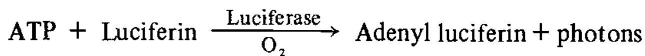
## INTRODUCTION

The measurement of adenosine triphosphate (ATP) using luciferin and the enzyme luciferase results in an emission of photons and therefore lends itself to scintillation counting.



$$\text{ATP} + \text{Luciferin} \xrightarrow[O_2]{\text{Luciferase}} \text{Adenyl luciferin} + \text{photons}$$

We are measuring ATP in amounts of $10^{-12}$ to $10^{-9}$ mole in aqueous solution using a method adapted from that described by Stanley and Williams.[1]

The technique of treating whole blood to obtain deproteinized plasma is crucial as destruction of the cellular elements of blood and degradation of ATP occur readily and distort results.

## TECHNIQUE

Deproteinized plasma is obtained in the following way: 7 ml of blood is drawn into a syringe containing 1 ml of normal saline and 21 mg of EDTA (pH 7.4) at a rate of 1 ml/ 5 s. Air is drawn into the syringe and the syringe is inverted eight times. EDTA binds calcium ions and thus inhibits both coagulation and ATPase activity (see Table 1). The contents of the syringe are placed in a siliconized glass tube and spun at 5000 G for 30 min. Table 2 illustrates the effect of spinning six sequential samples from the same patient in siliconized glass or plastic tubes. The increase in counts in plastic tubes we attribute to release of ATP from platelets. 3 ml of the supernatant is measured into a plastic tube to which 5 ml of 0.5 m perchloric acid is added. The effect on the ATP counts of leaving pooled plasma to stand at room temperature is shown in Table 3. The tube is shaken and spun at 5000 G for 10 min. This supernatant is decanted and neutralized to pH 7.0 with potassium hydroxide (see Table 4). The precipitate of potassium perchlorate is removed by cooling and spinning at 5000 G for 10 min and the supernatant is again decanted. The volume is made up to 8.5 ml with distilled water. To this is added 2 ml of 0.55 m triethanolamine buffer and 1 ml of a solution containing 4 mmole magnesium sulphate, 1 mmole potassium chloride and 0.01 mmole phosphoenol pyruvate(PEP)/10 ml.

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Table 1. Effect of varying the mixing of EDTA with blood.

| | Counts |
|---|---|
| Syringe not inverted | 22 824 |
| Syringe inverted twice | 544 720 |
| Syringe inverted four times | 717 747 |
| Syringe inverted eight times | 832 539 |

Table 2. Effect of spinning samples in siliconized glass or plastic tubes.

| | Counts |
|---|---|
| Siliconized glass tubes | 883 585 |
| | 847 070 |
| | 892 162 |
| Plastic tubes | 2 332 645 |
| | 1 885 000 |
| | 1 615 185 |

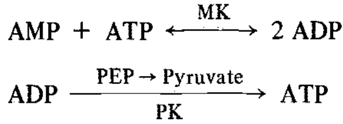
Table 3. The effect on ATP counts when plasma is left standing at room temperature (pooled plasma).

| Time standing | Counts |
|---|---|
| 13 min | 50 120 |
| 25 min | 51 747 |
| 29 min | 38 808 |
| 53 min | 37 989 |
| 62 min | 31 943 |
| 75 min | 27 840 |

Table 4. The inhibiting effect of potassium perchlorate on the luciferase reaction.

| Dialysis fluid + $10^{-10}$ mole ATP + $HClO_4$ + KOH + spin | Dialysis fluid + $10^{-10}$ mole |
|---|---|
| Counts | Counts |
| 11 074 | 75 119 |
| 9 842 | 85 646 |
| 10 806 | 1100 466 |
| 10 697 | 58 850 |

Three 1 ml aliquots of this solution are taken for triplicate estimations of ATP, 0.02 mg of pyruvate kinase (PK) is then added to convert adenosine diphosphate (ADP) to ATP and the samples are left to incubate at room temperature for 15 min before counting again in triplicate. 0.2 mg of myokinase (MK) is then added and the samples are left to incubate for 1 h to convert adenosine monophosphate (AMP) to ATP:



$$\mathrm{AMP} + \mathrm{ATP} \xleftrightarrow{\mathrm{MK}} 2\,\mathrm{ADP}$$

$$\mathrm{ADP} \xrightarrow[\mathrm{PK}]{\mathrm{PEP} \rightarrow \mathrm{Pyruvate}} \mathrm{ATP}$$

and a further three aliquots are taken for counting. Subtraction of the figures obtained gives an estimate of ADP and AMP.

The remaining 2.5 ml of deproteinized plasma solution in one tube is mixed with the remnants from other samples from which four 1 ml aliquots are estimated for their ATP content. To another four aliquots of the same plasma solution a known amount of ATP is added and they too are estimated for their ATP content. Subtraction of the resulting figures provides an internal standard.

Firefly extract is prepared by grinding the tails of 10 to 14 fireflies (Sigma FFT) into a paste with arsenate buffer which is then made up to 5 ml with more arsenate buffer. This is spun at 5000 G for 10 min. The straw-coloured supernatant is decanted into a plastic container and left at 8°C for 2 h.

The machine, a Packard Tricarb model 3320 liquid scintillation spectrometer, is given the following settings: preset count 900000 or time 20 s; pulse height discriminator window 60 to 65 (see Fig. 1); gain 100%. The coincidence switch is turned off. The counting chamber is at 8°C. The counting window indicates time.

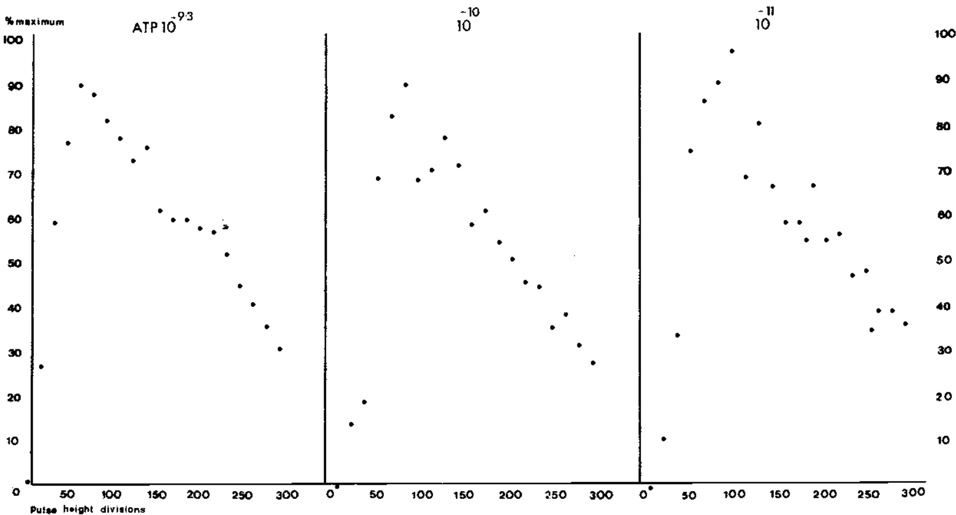



*Fig. 1.* Pulse height spectra for out of coincidence counting of the luciferin/luciferase system with varying amounts of ATP.

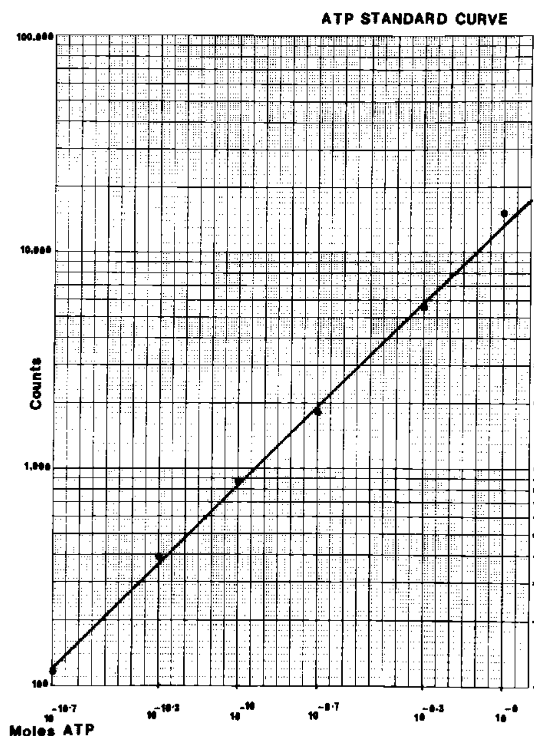



*Fig. 2.* ATP standard curve.

Each vial contains 1 ml of arsenate buffer, 1 ml of phosphate buffer and 1 ml of saline containing 90 mmole/l of sodium chloride. For ATP estimations 'plasma solution' replaces the saline and the volume of phosphate buffer is adjusted to accommodate the addition of standard solutions of ATP.

The vials to be counted include a calibration curve with a range from $10^{-11}$ to $5 \times 10^{-9}$ mole (in triplicate using dilutions of ATP in phosphate buffer, see Fig. 2), the unknown plasmas, an internal standard and four blanks with no plasma nor ATP added. These are left to equilibrate at room temperature for 15 min.

At the commencement of counting an empty vial is placed in the counting chamber. As 10 s is indicated, 0.05 ml of firefly extract is added to the next vial to be counted. The cap is screwed on and the vial is shaken three times and placed in position.

The calibration curve is plotted on semilog paper. The mean value of the counts obtained from the plasma samples are multiplied by an inhibition factor. This is obtained by comparing the counts of the internal standard with the counts of an equivalent amount of ATP in the calibration curve. The corrected figure gives an estimate of the amount of ATP in 1 ml of the plasma solution. The amount in moles can be read from the calibration curve. By applying the formula:

$$X \times 10^{-n} \times V \times \frac{Z+1}{Z} \times 1/3 \times 10^{6}$$

where $X$ = antilog of ATP/ml of plasma solution
$V$ = total volume of plasma solution (usually 11.5 ml)
$n = 10^{-9}, 10^{-10}, 10^{-11}$

$$Z = \frac{100 - \text{packed cell volume}}{100} \times 7$$

a result in $\mu$m/ml of true plasma is obtained, and the result may be expressed in $\mu$g by multiplying by 551.2.

## RESULTS

Using this method the range of normal values for ATP in human plasma is 0.2 to 1.2 $\mu$g/ml. Normal values for ADP in human plasma are 0.05 to 0.7 $\mu$g/ml and there is negligible AMP in resting human plasma.

The method has a standard error of ±13% at 0.2 $\mu$g (one S.D.) and ±7% at 2.5 $\mu$g (one S.D.). As the values for ADP and AMP are obtained by subtraction of repeated estimations of ATP the sensitivity of the method for ADP is a function of the sum of ATP and ADP and the sensitivity of the method for the estimation of AMP is a function of the sum of ATP, ADP and AMP.

When nucleotide is added to whole blood as it is being drawn into the sampling syringe the method gives a 60% recovery of ATP, 80% recovery of ADP and a 60 to 80% recovery of AMP.

## REFERENCE

1 P. E. Stanley and S. G. Williams, *Anal. Biochem.* **29**, 381 (1969).

## DISCUSSION

**P. Stanley**: Have you found that dust and/or micro-organisms in samples and the solutions used for bioluminescence assays present a problem?
I believe that a number of problems have yet to be solved before the estimation of AMP and ADP can be reliably made on samples from a range of systems. Presently internal standards offer the best way out of the problem.
Pulse pile-up is a problem that many people are not aware of in bioluminescence assays. Since large numbers of photons are produced the electronic system in the spectrometer becomes saturated and the pulse height spectrum becomes distended and distorted.

**P. I. Parkinson**: In reply to your first point, we have noticed no such problems. We find that there is minimal degradation of ATP even at low concentrations at room temperature, and what there is, is virtually undetectable for the time of the assay. We were aware of this problem, and although we do not formally sterilize our glassware, it is dried at 200°C and stored in a clean and dust-free cupboard.
Concerning your second point, we have found that the estimation of ADP is quite reliable, but the myokinase reaction for the conversion of AMP tends to vary from assay to assay.