A Rapid Method for Determining ATP by the Firefly Luciferin-Luciferase System

Ever since the classic work of McElroy and his colleagues on the mechanism of action of firefly luciferase, there have appeared many papers on the use of this system to determine small amounts of ATP[1–10]. The various investigators have reported on diverse methods to measure the light produced, instrumentation, and preparation of the luciferase.

We report a method that can reliably detect 1 picomole of ATP utilizing pure luciferin and a partially purified preparation of firefly luciferase. The method is rapid, the instrumentation easily obtained, and the data are reproducible. The method has been applied to the determination of ATP in a variety of biological material including bacteria, plant material, and animal tissue.

Materials and methods. Luciferase: Partially purified luciferase was prepared from a stock of dessicated and frozen firefly tails by the following procedure: 10 g of firefly tails were ground in a mortar and pestle to a fine powder. This powder was then placed in a beaker containing 50 ml of acetone that had previously been chilled to −20 °C or below. The powder was well stirred in the acetone and then the mixture was filtered through a Buchner funnel. The powder was dried by driving air through it until there was no odor of acetone. This powder can then be used immediately in the succeeding steps or it can be stored dry in the deep freeze until required.

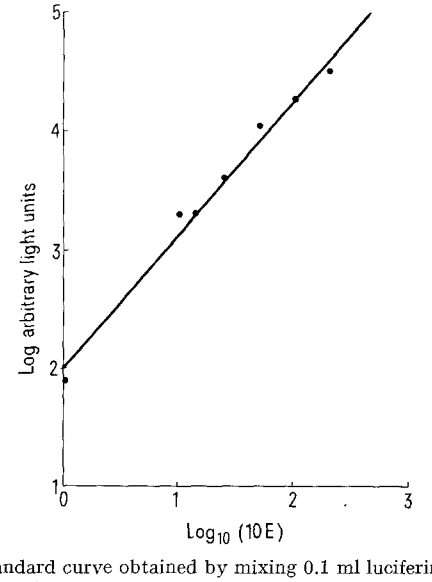

Fig. 1. Standard curve obtained by mixing 0.1 ml luciferin (0.1 mg/ml), 0.1 ml $MgSO_4$ (0.1 *M*) and 0.1 ml of enzyme in appropriate dilutions and adding 0.1 ml ATP (10^{-8} g).

The acetone powder was extracted with approximately 25 to 35 ml of 0.1 *M* glycylglycine buffer, pH 7.8, that contained 1×10^{-3} *M* versene by homogenization in the cold with a Potter-Elvehjem homogenizer. The mixture was then centrifuged in a Sorvall SS-1 centrifuge at 5,000 rpm for 20 min and the supernatant fluid was saved. The precipitate was reextracted with 20 ml of 0.017 *M* NaOH containing 1×10^{-3} *M* versene and re-centrifuged. The supernatant fluid was combined with the first supernatant fluid. The NaOH extraction step was repeated one time with the supernatant fluid combined with the others to give a volume between 50 and 75 ml. The precipitate was discarded.

The combined supernatant fluid was then subjected to stepwise ammonium sulfate fractionation in the following manner: solid ammonium sulfate was added to bring its concentration to 20% of saturation. The resulting precipitate, after recovery by centrifugation, was dissolved in 10 ml of 0.10 *M* glycylglycine buffer, pH 7.8, and labelled 0–20. The ammonium sulfate concentration was now raised to 30% and the resulting precipitate was recovered. This fraction was labelled 20–30. In a similar fashion, fractions 30–40, 40–50, 50–60, and 60–80 were all prepared.

The various fractions were assayed; the results of a typical preparation are shown in the Table. These data show that the fraction precipitating between 40 and 50% saturation contained 65% of the original activity. The fractions were assayed by adding 0.1 ml fraction, 0.1 ml luciferin (0.1 mg/ml), and 0.1 ml 0.1 *M* $MgSO_4$ to a test tube with 0.1 ml of ATP containing 1×10^{-8} g added to initiate the reaction.

Luciferin: The luciferin used in these experiments was synthesized by Mr. George Svarnas of the Biological Sciences Laboratories, Fort Detrick, and by the McCollum-Pratt Institute of The Johns Hopkin University. The pure luciferin is stored until needed in sealed ampules containing 5 mg under nitrogen in the deep freeze.

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Recovery and purity of luciferase fractionation

% as precipitate	Volume (ml)	Activity/ml (mV)	Total units	Recovery (%)	mg protein/ml	Units/mg protein	Relative purity
1[a]	50	1,450,000	72,500,000	100	26	55,800	1
0–20	10	265,000	2,650,000	3.7	27	9,810	<1
20–30	10	225,000	2,250,000	3.1	19	11,800	<1
30–40	10	624,000	6,240,000	8.6	10	62,400	1.1
40–50	10	4,740,000	47,400,000	65.4	25	190,000	3.4
50–60	10	170,000	1,700,000	2.3	13	13,100	<1

[a] Initial extract.

ATP: ATP disodium $5H_2O$ crystalline, Sigma grade 99 to 100%. 5 mg of ATP were diluted to 5 ml with pH 7.8 0.01 *M* glycylglycine buffer; further dilutions were carried out as needed.

Instrumentation: The light produced by the addition of ATP to the reaction mixture was measured in an Aminco Chem-Glow photometer using a Heathkit-Servo recorder. The reaction is initiated by the addition of 0.1 ml of ATP into a test tube containing 20 µl of a mixture of luciferin, luciferase, and magnesium. Following the addition of the ATP, the contents are vigorously mixed on a Vortex mixer for 5 sec and then placed into the chamber. The flash height is read 7 sec after the addition of ATP. It has been ascertained that this method is much more reproducible than the injection procedure used by other investigators.

Optimum conditions for assay: It was first necessary to show that in the presence of all components (ATP, luciferin, Mg^{++}, and O_2) the rate of the reaction was directly proportional to the luciferase concentration. These data, in which the concentration of luciferin, ATP, MG^{++}, and O_2 were kept constant but the amount of luciferase was varied, are shown in Figure 1. These data are plotted as log of arbitrary light units versus log of 10 times the enzyme concentration expressed in ml. It can be seen that the response is directly proportional to the enzyme concentration. Activity is defined as the maximum flash height in mV (or any arbitrary unit) achieved when ATP is added to the mixture.

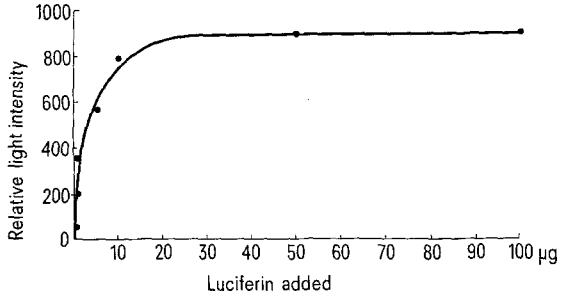

Fig. 2. Effect of luciferin concentration on light emission.

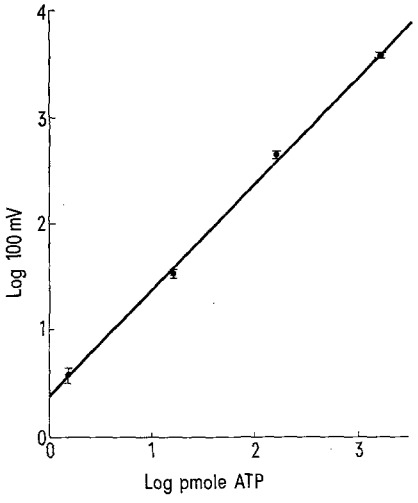

Fig. 3. The effect of ATP concentration on light production.

Effect of luciferin concentration: Figure 2 shows the effect on the light intensity of varying luciferin concentrations in the presence of a fixed amount of enzyme and ATP.

Problem of inherent light: Many methods involving firefly luciferase suffer from the problem of inherent light. We have found that by preparing a premix of pure luciferin and partially purified luciferase and permitting the mixture to age for 24 to 36 h in the cold, that the inherent light decreases to a constant low level with no loss of sensitivity with respect to ATP.

Standardization of the method: The data discussed above have led to the establishment of a standard method for the assay of ultramicro amounts of ATP. These data indicate that the preparation of a premix leads to conditions offering the maximum sensitivity with respect to ATP at the lowest inherent light level. The procedure has been standardized in the following manner: 1 mg of pure luciferin that has been maintained under nitrogen in the deep freeze is dissolved in 10 ml of glycylglycine buffer, 0.01 *M*, pH 7.8, containing 1×10^{-3} *M* EDTA (this can be used immediately or gassed with nitrogen and frozen); the 40–50 fraction of luciferase, as shown in the Table, is prepared and used immediately or frozen; and 0.01 *M* $MgSO_4$ is prepared in demineralized water. The premix is then prepared by adding equal volumes of luciferin, luciferase, and $MgSO_4$. This mixture is then allowed to stand in the cold for a minimum of 24 h before using; it is never frozen. This premix can be used up to a week.

Figure 3 shows the sensitivity of the assay covering a range of 1.56 pmole to 1560 pmoles of ATP.

Zusammenfassung. Eine schnelle Methode zur Bestimmung von ATP in picogramm-Mengen wird beschrieben. Die Grundlage für die Methode ist eine Reaktion zwischen ATP und einem Inkubationsansatz, der Luciferin, Mg^{2+} und partiell gereinigte Luciferase enthält, und ist zur Bestimmung von ATP in biologischem Material von unterschiedlicher Beschaffenheit (Bakterien, Pflanzen- und Tiergewebe) geeignet.

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