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DETERMINATION OF ATP IN BIOLOGICAL MATERIAL BY A BIOLUMINESCENCE METHOD USING A SCINTILLATION COUNTER

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Abstract

A simple, rapid and inexpensive method for the ATP determination in biological material has been described. The method is based on the measurement of flashes emitted by the firefly luciferin-luciferase system in a liquid scintillation counter. The method permits the routine analyses of fresh as well frozen samples. High sensitivity (10^{-12} M ATP) makes suitable for the ATP analyses in small samples.

1. INTRODUCTION

A number of methods has been described for the determination of ATP (adenosine 5′-triphosphate) in biological material. Most widely used are the methods based on the phosphorylation of sugars using kinase-dehydrogenase system, where either firmly bound phosphate or the absorbance change due to the reduction of NAD or NADP are measured [1, 3, 10, 11]. Sometimes ATP determination is based on the ion-exchange chromatography [4]. Recently, the change of the fluorescence intensity of compounds like 8-aniline-1-naphtalene sulphonate (ANS) as the function of ATP concentration has been introduced [2].

The firefly luminiscence method seems to be the simplest, fastest and most sensitive of the ATP determination methods [6, 10, 9]. It is based on the measurement of flashes emitted by firefly luciferin activated in the presence of the specific luciferase. It requires, however, special equipment such as Farrand spectrofluorimeter or Dupont Luminiscence Biometer, which are not widely distributed in standard biochemical laboratories.

The method described here makes it possible to use a liquid scintillation counter instead of the available equipment mentioned which is not always a standard item in the laboratory.

ATP determination may be done in plant material (marine algae), even of freezed samples, which allows for the collection and freezing of the samples outside the analytical laboratory.

2. MATERIALS AND METHODS

2.1. REAGENTS

Luciferin-luciferase system, the firefly (*Photinus pyralis*) lantern extract, lyophilized, was obtained from SERVA (code Nr 28075). The vial contents (equivalent to 50 mg of dried firefly lantern extract) was dissolved in 5.0 ml of water. ATP and dithiothreitol were from SIGMA. Pure, bovine serum albumin (BSA), was from International Enzymes (Windsor). All other chemicals were of analytical grade from POCH (Gliwice).

Luciferin-luciferase buffer: 50 mM glycine, 10 mM sodium arsenate, 2 mM dithiothreitol, 5 mM $MgSO_4$, 5 mg/ml BSA; pH 7.4.

Standard assay mixture: 100 ml of luciferin-luciferase buffer was supplemented with 1 ml of firefly lantern extract. This assay mixture remained stable at 5°C for several days.

2.2. STANDARD METHOD OF ATP DETERMINATION

The ATP containing sample (0.1 - 0.5 ml) was added to the standard assay mixture (5 ml 20°C) in the scintillation counter vial, quickly mixed and the vial was immediately loaded into the liquid scintillation counter (Beckman LS-3100). This preparatory work should not take longer than 30 s. Counting (1 min) was performed in a tritium channel.

2.3. PREPARATION OF BACTERIAL MATERIAL

Escherichia coli B/r cells were cultivated in 200 ml of rich growth medium [5], by conventional method, to $A=0.4$, chilled in ice and centrifuged off. The sediment was suspended in 4 ml of fresh growth medium and transferred to Eppendorf tubes in 0.2 ml portions (equivalent to 4×10^9 bacteria). The tightly-stoppered tubes were immediately immersed in a water bath at 100°C. The samples were successively removed, after extraction times of from 1 to 20 min, quickly chilled in an ice bath and centrifuged in the cold. The supernatant (0.1 ml) was taken for analysis.

3. RESULTS AND DISCUSSION

3.1. THE CHOICE OF ASSAY MIXTURE COMPOSITION AND THE SENSITIVITY OF THE METHOD

The very high sensitivity of ATP determination with purified luciferin and luciferase (10^{15}M of ATP) induced me to use a buffer of the composition as described Johnson *et al.* [6]. The purified luciferin and luciferase used by Johnson were replaced

by the much cheaper firefly lantern extract. In order to choose the optimal concentration of the enzymatic system, different quantities of firefly lantern extract were added to the buffer and c.p.m. (counts per min) values were read at different ATP concentrations. The concentration of analytically pure ATP in water was tested spectrofotometrically [8].

The results (Fig. 1) indicate that the sensitivity increases with the increase of the enzymatic extract concentration. This may be easily observed by comparing the results for 0.5 and 1.0 ml of the extract per 100 ml of buffer. Further increase of the extract concentration leads to a relatively lower increase of the c.p.m. value for the given amount of ATP. The linear relation between ATP (in the range of 0.1 to 1.0 µg) and the c.p.m. values is obtained for 1 ml, and 2 ml of the enzymatic extract in 100 ml of buffer.

Though the commercial lyophilized enzymatic extract is relatively stable, its solutions are very sensitive to a rise in temperature (*vide* the effect of temperature) and the way in which the buffer is prepared [6]. In some cases, the assay mixture stored for a few days at 5°C exhibited increased activity (results not shown).

The factors mentioned above affect the enzymatic activity of the assay mixture

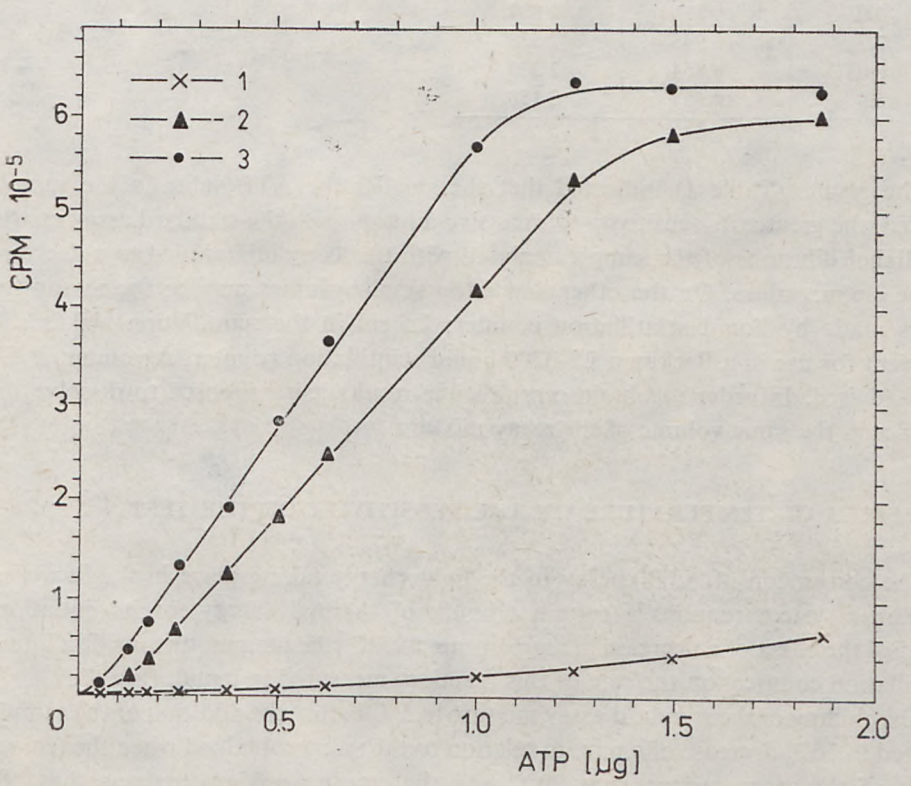

Fig. 1. The effect of lantern extract concentration in the assay mixture on c.p.m. values at different ATP concentrations: *1* – 0.5 ml of extract per 100 ml of luciferin-luciferase buffer, *2* – 1.0 ml of extract per 100 ml of luciferin-luciferase buffer, *3* – 2.0 ml of extract per 100 ml of luciferin-luciferase buffer.

nevertheless 10^{-3} μg of ATP can easily be detected in the standard assay mixture: 5×10^{-2} μg ATP corresponds to about 3×10^{3} c.p.m. The sensitivity may be increased, if needed, by increasing the concentration of the enzymatic extract.

On the basis of my results (Fig. 1), a concentration equal to 1 ml of the enzymatic extract per 100 ml of the buffer was taken as the optimal value. This enabled the preparation of 500 ml of the standard assay mixture from one vial, equivalent to the extract from 50 mg of dried firefly lanterns.

The effect of dilution of the analyzed sample with the standard assay mixture on the c.p.m. values was also studied. Increasing volumes of the standard assay mixture were mixed with a constant volume (0.1 ml) of the ATP solution.

Table 1. The effect of the dilution of the sample analysed with the standard assay mixture on c.p.m. values

Buffer volume [ml]	c.p.m. values (mean value from three readings)	
	0.125 μg ATP	0.05 μg ATP
2.5	16 920	2 898
5.0	16 886	2 839
7.5	16 945	2 538
10.0	16 854	2 380
15.0	14 654	2 158

The results (Table 1) indicated that the smaller the ATP value in the sample studied, the greater its sensitivity to excessive dilution with the standard assay mixture. Higher dilutions of the sample analysed with the assay mixture led to a decrease of the c.p.m. values. On the other hand, too small volumes may be the reason for errors made by liquid scintillation counter; 2.5 ml in the scintillation vial is still sufficient for use in a Beckman LS-3200 liquid scintillation counter. A volume of 5.0 ml is advised. In order to obtain reproducible results it is proposed to dissolve the samples in the same volume of the assay mixture.

3.2. EFFECT OF TEMPERATURE ON THE SENSITIVITY OF THE TEST

The conversion of ATP energy to the light energy taking place in the luciferin-luciferase system requires a certain amount of thermal energy for its initiation. I studied the effect of a decrease in temperature to 5°C (the temperature inside a liquid scintillation counter) on the rate of this reaction, measured in c.p.m. (Fig. 2).

The chilling of the standard assay mixture to 5°C before the addition of the sample resulted in 70% decrease of c.p.m. in relation to the values obtained when the temperature of the assay mixture was 20°C and the counter refrigerator was shut off. When the temperature of the assay mixture was 20°C and the determinations were performed in a refrigerated scintillation counter, the values were only 5% lower (data not shown). When higher enzymatic extract concentrations were used (2 ml

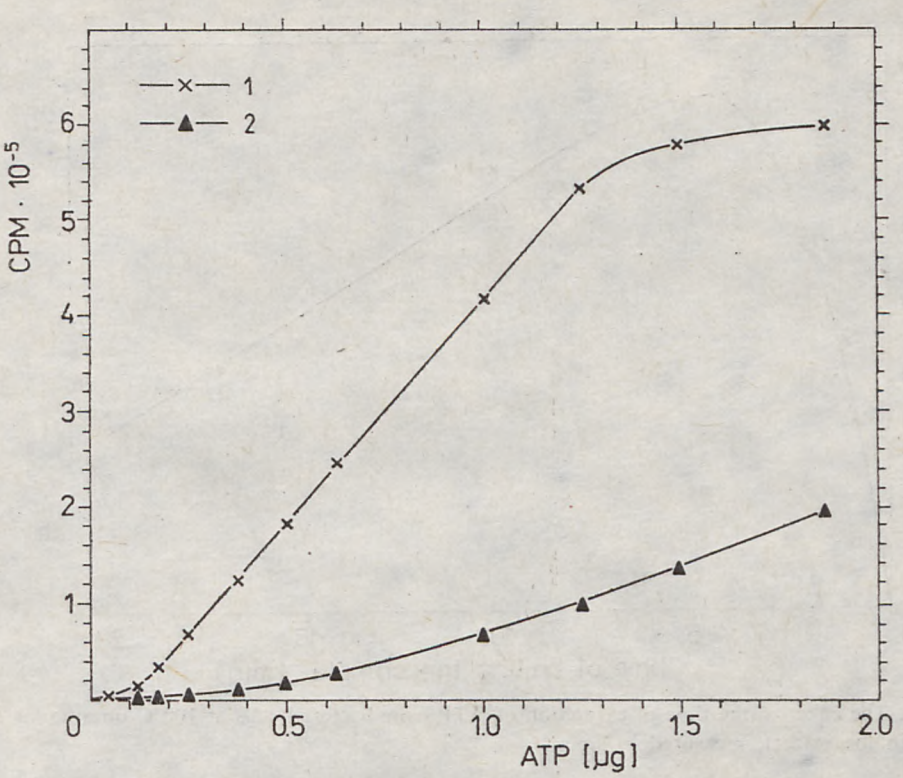

Fig. 2. The effect of temperature on c.p.m. values at different ATP concentrations: *1* — temperature of standard assay mixture 20°C, refrigeration of the counter switched off, *2* — temperature of the standard assay mixture 5°C, refrigeration of the counter switched on.

of extract per 100 ml of buffer) the c.p.m. values obtained at 5°C were only about 10% lower than those obtained at 20°C (data not shown).

The enzymatic extract is unstable at higher temperatures. Heating of the standard assay mixture for 10 min at 30°C leads to a 50% decrease of the enzymatic activity, while heating at 40°C for the same time destroys activity totally (data not shown). The standard assay mixture is, however, stable for a few days when tightly sealed and stored at 5°C.

3.3. ATP EXTRACTION FROM BACTERIAL CELLS

During ATP extraction from biological material, the possibility of rapid inactivation of enzymes has to be taken into account. Good results were obtained for the extraction with trichloroacetic [10], perchloric [2] or formic acid [7]. These acidic extracts cannot, however, be used for ATP determination by methods based on the luciferin-luciferase system, as the acids mentioned inactivate the enzymatic system even at low concentration. The removal of acids from the samples is fairly troublesome.

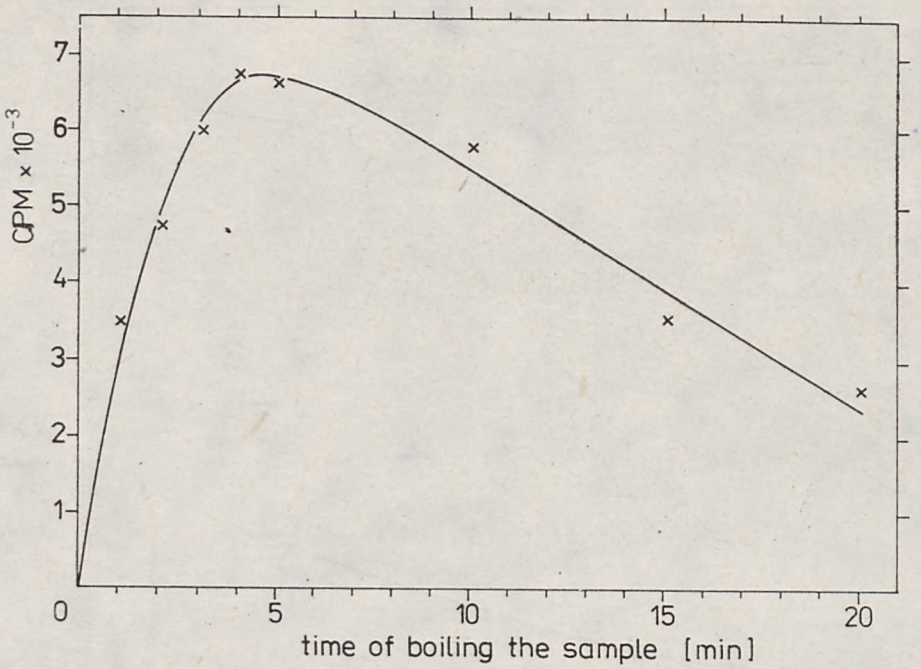

Fig. 3. The effect of the time of extraction of ATP from bacterial cells at 100°C on amount of ATP in the extract, measured in c.p.m.

For this reason, heating of the analyzed material at 100°C is the most convenient method of ATP extraction [10]. Quick denaturation of proteins is achieved by injection of the sample into 2 - 3 volumes of water at 100°C. Extraction time depends on the type of material analysed. The dilution of the sample with water represents a drawback in this procedure, hence I analysed the conditions of ATP extraction from bacterial suspensions without dilution. The bacterial cells in growth medium were sedimented and suspended in a smaller volume of the same medium. It is worth mentioning, that the ATP contents in bacteria sedimented from the growth medium and suspended in buffer were considerably lower, although all manipulations were carried at low temperatures as quickly as possible. A small sample (0.2 ml) placed in a water bath at 100°C quickly reaches this temperature, which results in the inactivation of cell enzymes and the rapid extraction of ATP. In order to prevent the evaporation of water from the samples, this process was carried out in tightly-stoppered Eppendorf tubes.

As shown in Fig. 3, the optimal time of ATP extraction amounted to 4 min. Longer heating resulted in lower ATP values, probably due to the thermal decomposition of this compound.

When the ATP contents are determined in a relatively small quantity of bacterial material (e.g. 5×10^8 bacteria in 0.2 ml of growth medium), the centrifugation step after extraction may be omitted. Low turbidity of the assay mixture does not significantly affect the c.p.m. values (result not shown), which agrees with previous observations [10]. The results obtained by the presented method of ATP determination

in the bacterial material were reproducible and in agreement with the literature [6].

The use of luciferin-luciferase system for ATP determination in plant material has been described earlier [10]. High sensitivity of ATP determination (10^{-12} M) presented here allows for the processing of small samples and hence the negative effect of coloured substances in plant material is practically avoided. The method may be used for freezed material provided that samples are thawed in water bath at 100°C (the fast inactivation of ATP-ases).

The simplicity of the described method allows for the serial analyses, economical buffer composition lowers the analysis cost while the adaptability for the processing of freezed material greatly simplifies the field work.

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