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Firefly Luciferase Assay for Adenosine Triphosphate in Cultured Cells*)

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ABSTRACT

A method for determining intracellular content of adenosine triphosphate (ATP) in the cultured cells was investigated with a firefly luciferase reagent, and a micro assay method was established. The method minimized the size of both samples and luciferase reagent employed.

The per cell amount of intracellular ATP of HeLa 229 and McCoy cells was estimated to be 7.20×10^{-11} M and 8.74×10^{-11} M, respectively. The method could be applied to a direct measurement of the ATP content in cultured cells grown in each well of a 96-well micro tissue culture plate.

INTRODUCTION

Since Lundin et al.[5,6] developed a highly purified firefly luciferase reagent, great advances have been made in sensitivity and reliability of assay methods for determination of adenosine triphosphate (ATP) content.

ATP is an essential constituent of all living cells and is contained almost equally in each kind of cell species. As ATP takes part in various metabolic reactions, not only ATP contents but also metabolites as well as enzymic activities involved in ATP-converting reactions may be quantitated by the firefly luciferase assay[2,7].

An application of the firefly luciferase ATP assay method in microbiology seems mostly confined to determining the total number of microorganisms contained in such clinical specimens as urine[10] and blood[8]. To our knowledge, no report has appeared about any variation in the ATP level of mammalian cells infected with microorganisms. There might be a bit of difference in ATP level of the cultured cells following the intracellular growth of microorganisms.

The present paper deals with a perliminary experiment on an ATP assay method for cultured cells using the firefly luciferase reagent.

MATERIALS AND METHODS

1. *Firefly luciferase assay of ATP.*

1) *Analytical equipment*

Light emission from the bioluminescent assay was measured in Lumicounter ATP-237 (Toyo Kagaku Sangyo).

2) *Analytical reagent*

An ATP monitoring kit (ATP bioluminescence CLS) was perchased from Boehringer Mannheim GmbH. All other reagents were of tissue culture grade. The distilled water used was sterilized through a membrane filter (TM-4: Toyo Roshi), followed by autoclaving at 121°C for 15 min.

Firefly luciferase reagent was prepared by dissolving the lyophilisate of ATP bioluminescence CLS kit in 25 ml of the distilled water, and kept at room temperature for about 30 min. Crystallized ATP disodium salt (MW=605.2) was dissolved in the distilled water to make 1 mM stock solutiom, a 0.5 ml-portion of which was distributed in a Cryotube (48×12.1 mm : Nunc) and stored at −80°C until use.

3) *Luciferase assay of standard ATP solution*

Standard ATP solutions were prepared at

*) 清谷克寛，金本康生，松尾吉恭：ルシフェラーゼ試薬を用いる培養細胞のＡＴＰ測定法

Table 1. Various methods for firefly luciferase ATP assay

Ingredient	Assay method		
	Micro	Semimicro	Standard
ATP standard	50 μl	50 μl	500 μl
Distilled water	150	450	
Luciferase reagent	200	500	500

concentrations from 10^{-5} to 10^{-9} M ATP. Determination of ATP was performed with three different assay methods shown in Table 1. Each standard ATP solution was pipetted into a siliconized glass cuvette (55 × 16.5 mm: Pyrex). The cuvette was placed in the Lumicounter and an equal volume of firefly luciferase reagent was added. The value of light intensity was measured and printed every 10 sec.

2. *Determination of intracellular ATP of cutured cells.*

1) *Cell culture*

Monolayers of HeLa 229 and McCoy cell lines were grown in a CO_2-incubator at 37°C in Eagle minimum essential medium (MEM (1): Nissui Seiyaku Kogyo) supplemented with 10% heat-inactivated fetal calf serum (FCS: Flow Laboratories), per ml 0.292 mg of L-glutamin, 1.5 mg of sodium bicarbonate and 10 μg of Gentacin (Schering Corporation).

2) *Extraction of ATP*

Intracellular ATP of cultured cells was extracted by adding ATP releasing reagent (Extra-light type B-2: Niti-on Irika Kikai Seisakusho) to the cell suspension or monolayer at room temperature for an appropriate period before the assay of cellular ATP.

3) *Determination of ATP*

ATP of cultured cells was measured by the micro assay method.

a. The cells grown in a 25-cm^2 culture flask (Nunclon: Nunc) were harvested by trypsinization, washed with and suspended in PBS (−) in the cold. A 50 μl-portion of the cell suspension was pipetted into a siliconized glass cuvette containing 50 μl of distilled water and then a 100 μl-volume of ATP releasing reagent was added. After shaking the mixture thoroughly, the cuvette was placed in the Lumicounter and a 200 μl-volume of luciferase reagent was added.

b. The cells grown in a 96-well micro tissue culture plate (Falcon 3072: Becton Dickinson Labware) were washed three times with PBS (−). A 100 μl-portion of distilled water was added to each well, kept for 15 sec and an equal amount of ATP releasing reagent was added. The contents of each well were mixed thoroughly for 30 sec to extract cellular ATP, and a 100 μl-portion of the mixture was used for ATP assay.

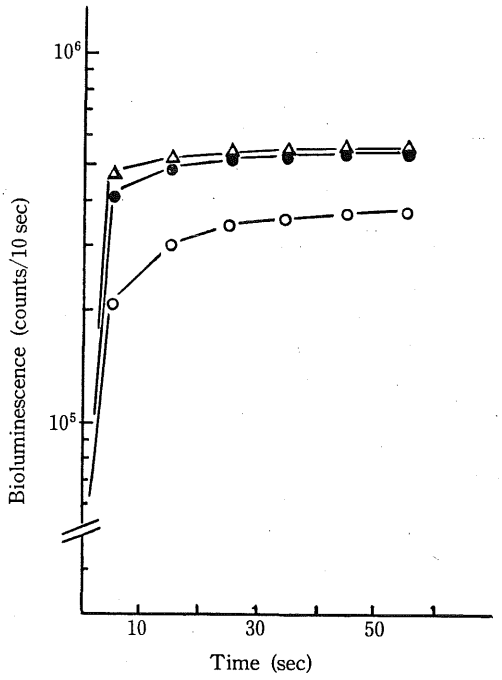

Fig. 1. Time course of bioluminescent responses of ATP-standard solutions by means of various assay methods tested. Open circles represent bioluminescent counts determined by the micro assay method using ATP solution at 10^{-6}M, solid circles those by the semimicro assay method using at 10^{-6} M and open triangles those by the standard assay method using ATP at 10^{-7} M.

RESULTS

1. *Sensitivity and accuracy of various assay methods.*

Time courses of the bioluminescent response determined by the different assay methods are shown in Fig. 1. With the micro assay method, bioluminescent response gradually increased and reached the maximum level approximately 30 sec after the addition of the firefly luciferase reagent, while the responses with the semimicro and standard assay methods bursted instantly and maintained constant level of light signals.

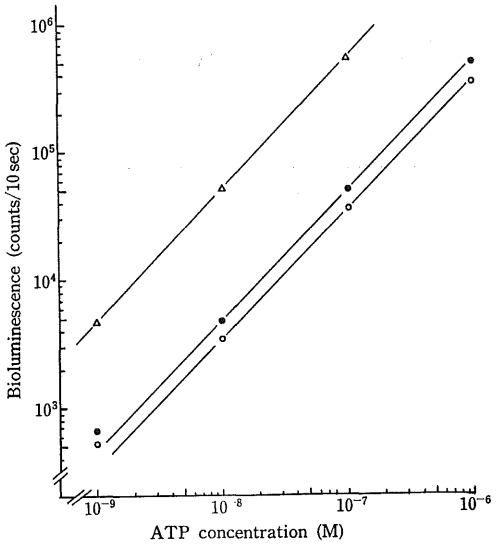

Fig. 2. Standard calibration curve for ATP with various assay methods. Symbols: the micro assay method (—○—), the semimicro assay method (—●—) and the standard assay method (—△—).

From the results, ATP levels were decided to express as the bioluminescent intensity values for a period of 10 sec between 40 and 50 sec after the addition of the reagent. Bioluminescent intensities of various concentrations of ATP ranging from 10^{-5} to 10^{-9} M were measured with each assay method, and the standard calibration curve for ATP was prepared. As shown in Fig. 2, the sensitivity with the semimicro assay method was one-tenth of that with the standard assay method, and that with the micro assay method was 30% less compared with the semimicro assay method. However, a good correlation was obtained between the bioluminescent intensity and concentration of ATP in any method.

2. *Stability of firefly luciferase reagent.*

Stability of the reagent was investigated with the micro assay method. The working luciferase reagent was stored at 4°C in a refrigerator for periods indicated on Table 2.

The reagent was stable at 4°C for at least 7 days, when tested with 10^{-6} M ATP solution (Table 2).

The reagent retained its ability fairly well after 2 cycles of freezing and thawing procedure (Table 3).

3. *Optimal condition for extraction of intracellular ATP from cultured cells.*

The suspension of HeLa 229 cells at a concentration of 1×10^5 cells/ml in PBS (−) was mixed with the ATP-releasing reagent for various periods and the bioluminescent intensity was determinined by the micro assay method. The highest extraction of ATP was achieved 30 sec after the treatment with the ATP-releasing reagent (Table 4). The extraction efficacy with PBS (−) or distilled water was 28% and 47%, respectively.

Almost the same results were obtained with McCoy cells (the data not shown).

4. *Measurement of intracellular ATP of cultured cells.*

Bioluminescent intensity of serially diluted suspensions of HeLa 229 and McCoy cells was determined by the micro assay method. The values were converted to ATP contents according to the calibration curve shown in Fig. 2. Finally, the ammount of intracellular ATP per cell was calculated to be 7.20×10^{-11} M with HeLa 229 and 8.74×10^{-11} M with McCoy cells, respectively (Table 5).

Subsequently, the direct measurement of total ATP content of the monolayer of HeLa 229 and McCoy cells grown in a 96-well micro tissue culture plate was made. Total ATP content of HeLa 229 and McCoy cell mololayer was calculated to be 5.2×10^{-6} M ATP/well and 7.4×10^{-6} M ATP/well, respectively (Table 6). The level of ATP spontaneously released from each cell line in the culture supernatant was negligible.

From the results in Tables 5 and 6, the mean number of cells grown in a well of micro tissue culture plate (0.2826 cm^2) was estimated at 7.22×10^4 cells/well with HeLa 229 and 8.46×10^4 cells/well with McCoy cells, respectively.

Table. 2. Stability of firefly luciferase reagent at 4°C

Storage period (days)	ATP (M)	Bioluminescent intensity		
		counts/10 sec*	% recovery	
0	10^{-6}	416,576	100	
	10^{-7}	38,830	100	
	10^{-8}	3,440		100
3	10^{-6}	401,839	96	
	10^{-7}	39,201	101	
	10^{-8}	2,970		86
5	10^{-6}	415,748	100	
	10^{-7}	37,655	97	
	10^{-8}	3,024		88
7	10^{-6}	439,213	105	
	10^{-7}	34,367	89	
	10^{-8}	2,404		70
10	10^{-6}	350,094	84	
	10^{-7}	32,271	83	
	10^{-8}	2,213		64

* Determined by the micro assay method

Table 3. Stability of firefly luciferase reagent by freezing and thawing

Freezing and thawing	ATP (M)	Bioluminescent intensity		
		counts/10 sec*	% recovery	
None	10^{-6}	362,099	100	
	10^{-7}	37,342	100	
	10^{-8}	3,492		100
1	10^{-6}	471,390	130	
	10^{-7}	43,022	115	
	10^{-8}	3,488		100
2	10^{-6}	456,092	126	
	10^{-7}	43,082	115	
	10^{-8}	1,221		35

* Determined by the micro assay method

Table 4. Extraction of ATP from cultured cells

Treatment*		Bioluminescence (counts/10 sec)	Elution %
ATP-releasing reagent (Extralight type B-2)	5 sec	128,171	94
	10	117,879	87
	15	123,472	91
	30	135,698	100
	45	129,876	96
	60	131,435	97
	120	128,361	95
PBS (−)	30	37,752	28
Distilled water	30	63,455	47

* HeLa 229 cells at 1×10^5/ml in PBS (−) was treated with ATP-releasing reagent for the periods indicated and the resulting bioluminescnce was determined by the micro assay method. The cells suspended in PBS (−) or distilled water served as controls.

Table 5. Intracellular ATP of cultured cell suspensions

Cell line	No. of cells/ml	Bioluminescent intensity[1] (counts/10 sec)	ATP content[2] (M)	
HeLa 229	1×10^5	142,128	3.6×10^{-7}	
	1×10^4	14,202	3.8×10^{-8}	7.20×10^{-11} [3]
	1×10^3	1,212	3.4×10^{-9}	
McCoy	1×10^5	155,411	4.2×10^{-7}	
	1×10^4	15,493	4.4×10^{-8}	8.74×10^{-11}
	1×10^3	1,552	4.5×10^{-9}	

1) Determined by the micro assay method
2) Calculated by the calibration curve for ATP shown in Fig. 2
3) Amount of intracellular ATP per cell

Table 6. Intracellular ATP of cultured cells grown in a 96-well micro tissue culture plate

Cell line	Bioluminescence		ATP content per well (M)
	counts/10 sec*	mean	
HeLa 229	1,158,611	1,015,542	5.2×10^{-6}
	962,651		
	1,022,194		
	923,560		
supernatant		96	
McCoy	1,300,599	1,365,183	7.4×10^{-6}
	1,542,689		
	1,432,018		
	1,245,178		
	1,454,695		
	1,231,105		
supernatant		467	

* Determined by the micro assay method

DISCUSSION

A method for determining intracellular ATP content of cultured cells was investigated in order to obtain information about applicability of the method for evaluating interaction between such obligate intracellular microbes as viruses, rickettsias or chlamydias and their host.

The results obtained with the micro assay method showed slower development and less sensitivity of bioluminescence than those with both the semimicro and standard assay methods. Constant levels of light signal, however, could be obtained (Fig. 1) and a good correlation was demonstrated between the bioluminescent intensity and ATP concentration (Fig. 2). Thus, the micro assay method minimizes the amount of samples to one-tenth and that of the luciferase reagent to two-fifth of those with the standard assay method without any loss of reliability for the measurement of ATP concentration as low as 10^{-9} M. The minor difference in sensitivity between the micro and semimicro assay methods may be attributed to the difference in the height of reaction mixture in the cuvette. The height of the former was 2.6 mm and that of the latter 6.5 mm. Although the Lumicounter ATP-237 is designed to permit the detection of bioluminescence over a relatively wide range of height of reaction mixture, the possibility that decrease in the height of content affects the sensitivity cannot be denied.

The working solution of firefly luciferase reagent was stable for a relatively long period at 4°C and against 2 cycles of freezing and thawing. It would be desirable, however, to keep the reagent at −80°C to avoid microbial contamination as well as degradation in activity of the reagent during prolonged storage.

The activity of the ATP releasing reagent, Extralight type B-2, was sufficient enough to release intracellular ATP from the cultured cells in suspension. Released ATP maintained a constant level and was not affected by cellular ATPase for a relatively long period. For the direct extraction of ATP from the monolayer of the cells in a 96-well micro tissue culture plate, the ATP releasing reagent was used in combination with the equal amount of distilled water. The extraction efficiency was satisfactory. The difference in ATP content per well was within 20% from well to well. The per cell amount of ATP was 7.20×10^{-11} M with HeLa 229 and 8.74×10^{-11} M with McCoy cell lines, respectively. Reversely, the number of cells in each well of the micro plate could be estimated.

Our ultimate is to apply the ATP assay method to cultured cells infected with intracellular microbes, since a simple method for detecting their growth in host cells has not been developed except the immune fluorescent techniques[1,3,4,9]. The method is required to satisfy the followings at least; 1) a number of samples can be handled at a time, 2) only a small quantity of samples is needed, 3) the technique is simple, 4) the method is not costly and time-consuming, and 5) the results obtained are accurate and reproducible. The micro assay method with a 96-well micro tissue culture plate for evaluating the intracellular ATP in cultured cells may become a useful tool along this line.

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