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New Procedure for Assay of Lactobacilli using the Luciferin-Luciferase System[1]

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An enzymatic method was developed for the simple and rapid assay of lactobacilli. A glucose-hexokinase system was used to remove extracellular ATP, and intracellular ATP was assayed with the luciferin-luciferase system using luciferase commercially prepared from fireflies. Freeze-dried powders of *Streptococcus faecalis* (SF), *Lactobacillus bifidus* (LB) and erythromycin-resistant *Lactobacillus acidophilus* (LA-E) were used as the samples. Prior to assay the freeze-dried powder of lactobacilli was incubated at 37° in a glucose-peptone-yeast extract medium to increase the amount of intracellular ATP.

Using the proposed system, the intracellular ATP levels of SF, LA-E and LB were about 100, 80 and 800 times those obtained with the conventional system, respectively. The detection limit was 1.0×10^5 cells/0.1 ml. Reproducibility was 6.0—9.9% (coefficient of variation). This method was found to be extremely sensitive compared with the conventional luciferin-luciferase system and is convenient because it does not require sterilization and can be completed within a day. This method seems suitable for routine application in survival tests of lactobacilli.

Keywords——freeze-dried powder of lactobacilli; *Streptococcus faecalis*; *Lactobacillus acidophilus*; *Lactobacillus bifidus*; luciferin-luciferase method; extracellular ATP; intracellular ATP; survival tests; bioluminescence; plate-counts

Adenosine triphosphate (ATP) is thought to be present in all living organisms, and it has been reported by many workers that ATP can be measured by the luciferin-luciferase method. Hansen found that cell ATP of marine bacteria, algae and zooplankton, which is related to the total cell carbon in all these diverse organisms, could be determined easily and rapidly by the luciferin-luciferase method.[3] Reports have appeared on ionic effects in the measurement of red blood cell ATP with the luciferin-luciferase system,[4] the interference by ATPase in the assay of cell ATP of *Bacillus brevis*[5] and the quantitative extraction of ATP from microbes.[6] In addition, ATP assay using the luciferin-luciferase method has been employed for investigating the growth of bacteria or ATP metabolism.[7,8]

Test samples for the assay of microorganisms by the luciferin-luciferase method have usually been used in aqueous suspension. However, it is difficult to employ this approach for microorganisms in a freeze-dried state, such as freeze-dried lactobacilli, which are often administrated in cases of superinfection. The authors, therefore, developed a new scheme for the assay of ATP in the biomass of viable lactobacilli in a freeze-dried state, based on the

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3) O.H. Hansen, "Determination of Total Microbial Biomass by Measurement of Adenosine Triphosphate," *Estuarine Microbial Ecology*, 1973, pp. 73—89.
4) P. Santillo and J. Szerczes, *Anal. Biochem.*, **17**, 268 (1966).
5) J.A. Davison and G.H. Fynn, *Anal. Biochem.*, **58**, 623 (1974).
6) A.M. Dhople and J.H. Hanks, *Appl. Microbiol.*, **26**, 399 (1973).
7) H.A. Cole, J.W.T. Wimpeny, and D.H. Hughes, *Biochim. Biophis. Acta*, **143**, 445 (1967).
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luciferin-luciferase system, for application to the quality control of pharmaceutical preparations of freeze-dried lactobacilli.

Experimental

Reagents——Hexokinase and luciferase were products of Boehringer Mannheim Gmbh. Other chemicals were of reagent grade.

Lactobacilli——The following freeze-dried powders of lactobacilli, *i.e.*, freeze-dried products of the centrifuged precipitate of a culture medium containing lactobacilli after passage through a 50 mesh sieve, were used after storage at 5°: *Streptococcus faecalis* (SF, 1.22×10^{12} cells/1 g); *Lactobacillus bifidus* (LB, 1.10×10^{11} cells/1 g); erythromycin-resistant *Lactobacillus acidophilus* (LA-E, 5.33×10^{11} cells/1 g). Lactobacilli were also used in an aqueous culture medium (SF, 1.51×10^{9} cells/1 ml; LB, 8.00×10^{8} cells/1 ml; LA-E, 1.16×10^{9} cells/1 ml) within two days after cultivation (stored at 5°). All the lactobacilli were supplied by Wakamoto Pharmaceutical Co., Ltd.

Assay of ATP in Cells of Lactobacilli——a) Media used for the Preparation of Samples: The following four kinds of media were used: (i) GPY-A medium (glucose 4%, peptone 2%, yeast extract powder 1.5% and calcium carbonate 0.3%, pH 6.8—7.0); (ii) adenine–inosine–glucose–phosphate medium (adenine 0.027%, inosine 0.268%, glucose 0.09% and 1/10 M phosphate buffer pH 7.4 10%); (iii) glucose 4%; (iv) tomato juice-A medium (lactose 1%, peptone 1%, yeast extract powder 1%, sodium acetate 1.5%, L-cysteine hydrochloride 0.1%, L-ascorbic acid 0.1% and tomato juice (Kagome Co., Ltd.) 20%).

b) Preparation of Samples: 4.5 ml of medium, 20 μl of hexokinase solution (14 units/1 ml) in 20 mM Tris-HCl buffer) and 0.5 ml of a solution (or suspension) of lactobacilli (in 20 mM Tris-HCl buffer) were incubated at 37° for 10 min for SF and for 270 min for LA-E and LB, and then heated at 100° for 2 min in boiling water. One-tenth milliliter of the solution (or suspension) thus prepared, 0.8 ml of 20 mM Tris-HCl buffer containing 4 mM $MgSO_4$ (pH 7.8) and 0.1 ml of luciferin-luciferase solution (0.1% Luciferase in 20 mM Tris-HCl buffer) were mixed and used as the test sample.

c) Determination of the Intensity of Bioluminescence: The ATP-dependent luminescence was detected with a Aloka liquid scintillation counter, model 501 (^3H-setting, single analyzer in the manual mode; channel 1: 1; count time 30 sec). The time elapsed between injection and initiation was 15 sec.

Assay of Viable Cells by the Plate-Count Method——The culture medium was GPY medium (glucose 1%, peptone 1%, yeast extract 1%, calcium carbonate 0.1% and agar-agar 1.5%, pH 6.5—6.8) for SF and LA-E, and tomato juice medium containing agar-agar 1.5% for LB. After inoculating the lactobacilli into each medium, SF, LB and LA-E were cultured for 24 hr, 72 hr and 72 hr, respectively, at 37°, then the number of colonies was counted. SF and LA-E were cultured aerobically and LB was cultured anaerobically.

Results and Discussion

Medium for Assay of ATP in Cells of Lactobacilli

The determination procedure for lactobacilli in this study required the exclusion of the extracellular ATP by using a glucose-hexokinase system and measurement of the intracellular ATP with the luciferin-luciferase system, using luciferase commercially prepared from fireflies. Initially, in order to exclude the extracellular ATP of microbes, ATP was converted into ADP using the glucose-hexokinase system. This was very convenient compared with the existing method, in which membrane filters are used to remove extracellular ATP. The intracellular ATP of lactobacilli was diffused out of the cells by treatment with boiling water after incubation with hexokinase and used to produce bioluminescence with the luciferin-luciferase solution. The light intensity of the luminescence was proportional to the content of intracellular ATP.

Following this approach, the authors employed a new GPY medium, GPY-A, as described in the experimental section. Using the conventional luciferin-luciferase system (that is, after removing extracellular ATP by membrane filtration, washing the lactobacilli on the filter with Tris-HCl buffer, heating the solution or suspension at 100° for 2 min and measurement as described in experimental method c), it was impossible to determine the freeze-dried lactobacilli accurately because the amount of ATP was so small, compared with that in the cultivated microbes, though they were identical organisms (Table I). This may be because of the different metabolic states of the organisms. Thus, the glucose-hexokinase system was

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Table I. Intracellular ATP of SF, LA-E and LB in Liquid Culture Medium and Freeze-driedPowder as Determined by the Luciferin-Luciferase Method

Occurrence	ATP $\times 10^8$ ng/cell		
	SF	LA-E	LB
A*	2.73± 1.34	6.10±2.83	1.67±0.85
B*	41.8±10.4	37.5 ±7.5	9.1 ±2.9
B/A	15.3	6.2	5.5

A* in freeze-dried powder, B* in liquid culture medium.
Value are given ± S.D. × 1.96 ($p<0.05, n=4$). SF 5.22×10^6, LA-E 5.82×10^6 and LB 2.40×10^7 cells/1 ml were filtered through membrane filters (Toyo Roshi Co. Ltd., 0.45 μ, 25 mm diameter), and lactobacilli on the filter was washed and made up to 10 ml with 20 mM Tris-HCl buffer containing 4 mM $MgSO_4$ (pH 7.8). After heating the sample solution at 100° for 2 min, one-tenth milliliter of this solution (or suspension) was subjected to assay as described in the experimental section.

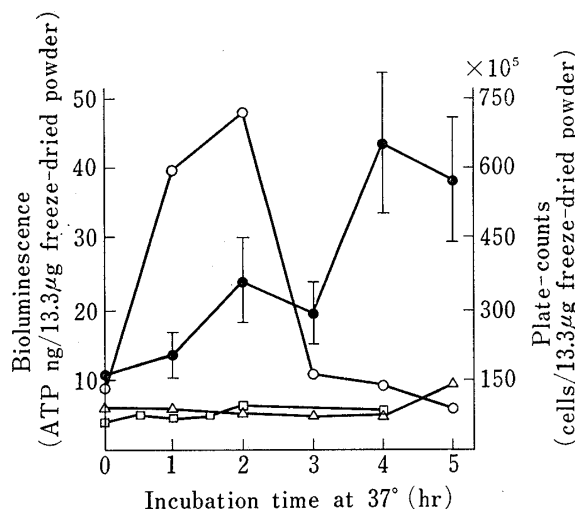

Fig. 1. ATP in Freeze-dried Powder of SF during Growth on Three Kinds of Media, and Growth of SF on GPY-A Medium

—○—, ATP on GPY-A medium, —△—, ATP on adenine-inosine-glucose-phosphate medium, —□—, ATP on glucose medium, —●—, growth on GPY-A medium. I : range for $p<0.05$. SF (15.1 μg/1 ml) was added in a vial to the assay system.

utilized in order to exclude the extracellular ATP, resulting in an increase in the amount of intracellular ATP to give a calibration curve in the range up to about 20 μg/0.1 ml concentration of lactobacilli. It was found that the increase in the amount of ATP using the glucose-hexokinase system was due to the glucose added in this procedure. Therefore, in order to find the best medium for obtaining a large amount of intracellular ATP in the freeze-dried powder, and in the expection of finding a good correlation between the cell ATP and the viable cell count, four kinds of media were examined, as described in the experimental section. GPY-

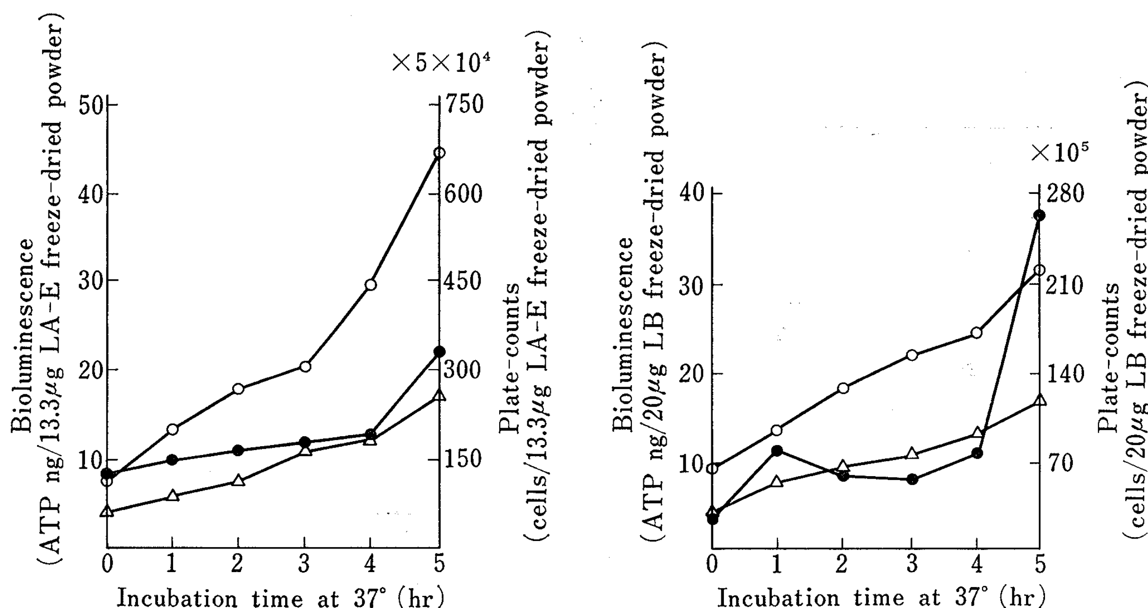

Fig. 2. ATP in LA-E, LB Freeze-dried Powders during Growth on Two Media and Growth of LA-E and LB on GPY-A Medium

—○—, ATP on GPY-A medium, —△—, ATP on tomato Juice-A medium, —●—, growth on GPY-A medium. 13.5 μg/1 ml LA-E or 18.8 μg/1 ml LB was added in a vial to the assay system.

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A medium has been employed as a nutrient medium for microbes. Adenine–inosine–glucose–phosphate medium has been used for enhancing the ATP level in erythrocytes.[9] The microbes were incubated in each medium at 37°, then intracellular ATP was diffused out the microbes by treatment with boiling water, and determined using the luciferin-luciferase system. The results are shown in Fig. 1. A rapid increase of ATP of SF was observed during incubation for 2 hr in the new GPY medium. At that time, the growth of the microbes was smaller than the increase in ATP, though the growth increased gradually. The dip in growth at 3 hr was not considered significant.

Using GPY-A medium and tomato juice-A medium for LA-E and LB, the intracellular ATP increased sharply in GPY-A medium for at least 5 hr, as shown in Fig. 2. It appeared that the ATP was utilized for growth after incubation for 2 hr in the case of SF (Fig. 1), while in the case of LA-E and LB, where the ATP levels continued to increase, sufficient energy for the growth appeared to be supplied by the GPY-A medium.

It was apparent that the cell ATP of these three microbes was increased in GPY-A medium, in accord with the findings reviewed by Decker *et al.*[10]

Incubation Time for the Diffusion of Intracellular ATP from Cells

An incubation time of 100 min was selected for SF because the intracellular ATP decreased rapidly upon incubation for more than 120 min. On the other hand, a 270 min incubation time was selected for both LA-E and LB for experimental convenince, since the cell ATP levels of LA-E and LB after this time were of the same order as that of SF after 100 min. The diffusion patterns of ATP from the cells are shown in Fig. 3; an incubation time of 2 min was

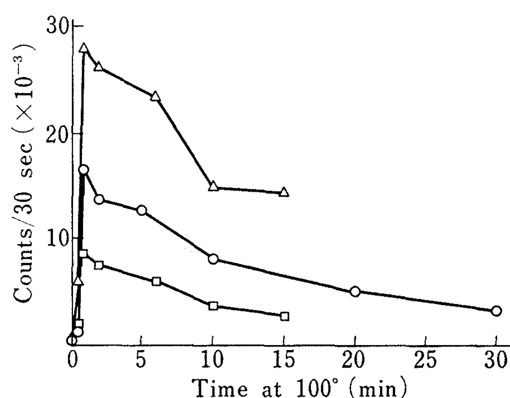

Fig. 3. Diffusion of ATP from SF (○), LA-E (△) and LB with the GPY Medium-Hexokinase System in Boiling Water

These patterns were obtained after incubating freeze-dried powders of lactobacilli at 37°: 100 min for SF, 270 min for LA-E and LB. 13.7 μg/1 ml SF, 15.9 μg/1 ml LA-E or 11.5 μg/1 ml LB was added in a vial to the assay system.

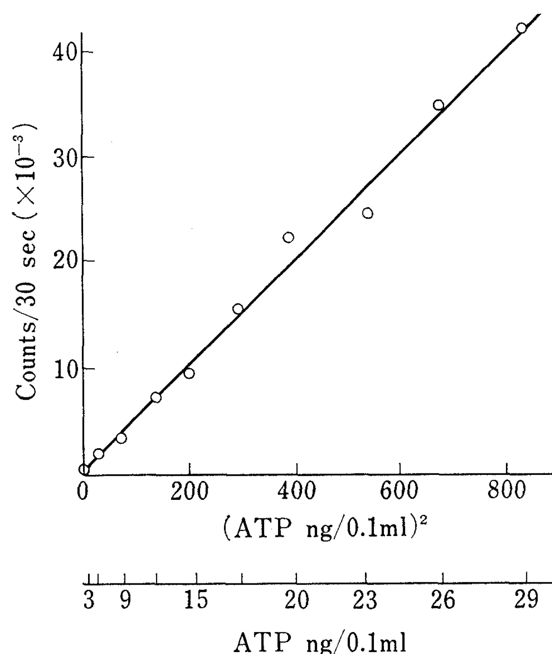

Fig. 4. Light Emission as a Function of the Square of ATP Concentration

The actual amount of ATP used for each point is shown on the lower abscissa scale. Each point is the mean of three determinations. Various amounts of ATP (ATP2Na. $3H_2O$, Boehringer Mannheim GmbH) in 20 mM Tris-HCl buffer (pH 7.8) were used in the assay system described in experimental method c. ATP (ng/0.1 ml) as indicated on the lower abscissa scale was added in a vial.

9) M. Nakao, T. Nakao, S. Yamazoe, and H. Yoshikawa, *J. Biochem.*, **49**, 487 (1961).
10) K. Decker, K. Jungermann, and R.K. Thauer, *Angew. Chem. Internat. Edit.*, **9**, 138 (1970).

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chosen because of the rapid increase during the first minute. Thus, the procedure described in the experimental section was selected for the assay ATP in cells of lactobacilli.

Calibration Curve and Reproducibility in the Assay of ATP in Cells of Lactobacilli

Using the above procedure, the calibration curve of standard ATP is shown in Fig. 4; the light emission was linearly dependent on the square of ATP concentration. This relationship has already been reported by Kimmich *et al.*[11] As much as 50 ng/0.1 ml ATP could

Table II. Reproducibility in the Determination of ATP by the Luciferin-Luciferase Method and of Cells by the Plate-Count Method from Freeze-dried Powders of SF, LA-E and LB

No.	SF		LA-E		LB	
	ATP ng per 13 μg	Cells[a] per 13 μg	ATP ng per 13 μg	Cells[b] per 13 μg	ATP ng per 20 μg	Cells[c] per 20 μg
1	38.2	170	37.4	155	27.3	193
2	36.7	144	35.7	145	25.8	257
3	45.1	120	32.2	142	25.7	306
4	46.5	156	38.4	129	25.4	293
5	45.5	136	33.3	153	23.8	297
6	45.8	152	37.3	145	25.6	227
7	46.0	173	43.2	134	21.8	292
8	41.7	183	38.5	163	24.7	260
9	36.6	167	38.8	159	27.8	231
10	36.7	176	35.0	158	25.6	246
$\bar{X}$	41.8	157	37.0	149	25.4	260
CV(%)	9.9	12.0	8.0	6.6	6.0	13.4

a) $\times 10^5$, *b*) $\times 5 \times 10^4$, *c*) $\times 10^4$.

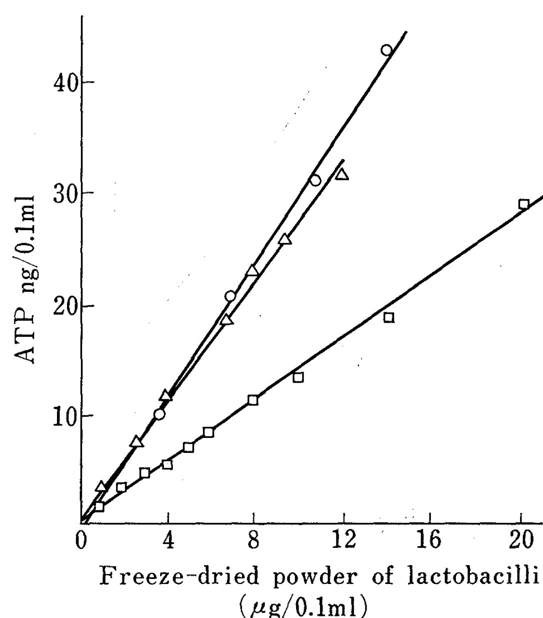

Fig. 5. Calibration Curves for SF (○), LA-E (△) and LB (□)

Various amounts of each lactobacilli were subjected to assay.

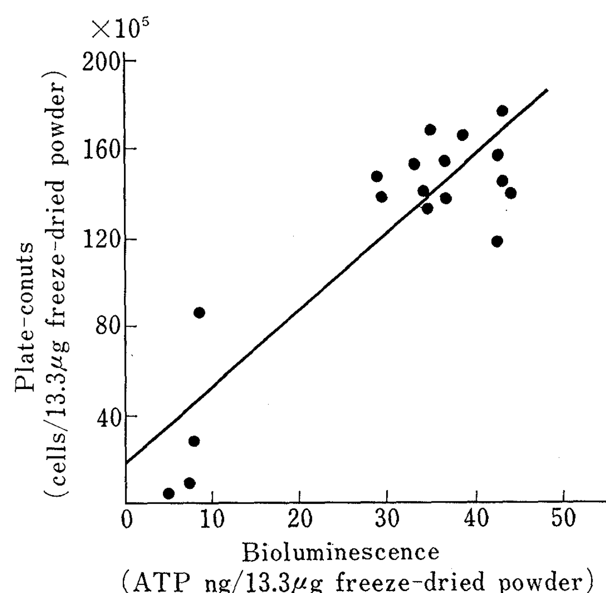

Fig. 6. Correlation of Survival Fractions of SF Freeze-dried Powder as Determined by the Bioluminescence and Plate-Count Methods

$Y=3.50X+17.1$, $r=0.858$, $n=18$.

11) G.A. Kimmich, J. Randles, and J.S. Brand, *Anal. Biochem.*, **69**, 187 (1975).

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be assayed within the scope of the linear relationship. Next, calibration curves to obtain the amount of ATP in freeze-dried powder of lactobacilli were prepared. There was a linear relationship between the concentration of freeze-dried powder of lactobacilli and the amount of ATP determined, as shown in Fig. 5.

The reproducibility of determination of ATP by the luciferin-luciferase method was, as shown in Table II, roughly equal to that of the plate-count method. The bacteria typically had a generation time of thirty minutes, which may account for the rather large values of coefficient of variation (%).

Survival Tests of Freeze-dried Powder of Lactobacilli in Relation to the ATP Assay Method Proposed in This Study

The survival of the freeze-dried powder of lactobacilli on heating was examined in order to investigate the scope of the proposed method. Samples of about 10 g were heated in a

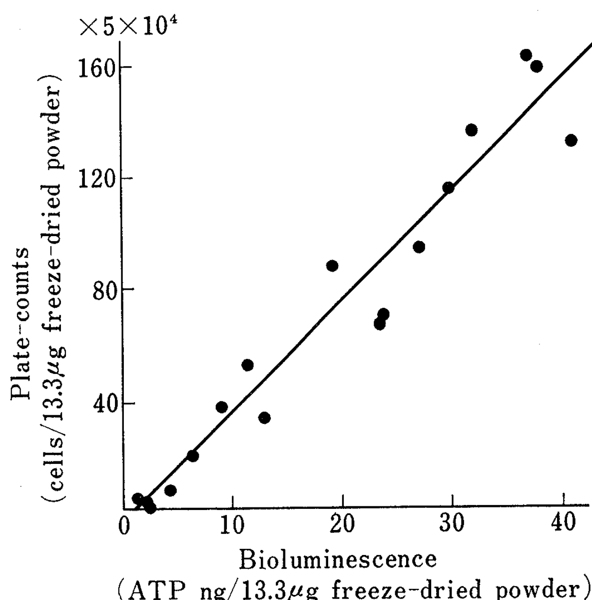

Fig. 7. Correlation of Survival Fractions of LA-E Freeze-dried Powder as Determined by the Bioluminescence and Plate-Count Methods

$Y=3.96X-6.49$, $r=0.973$, $n=19$.

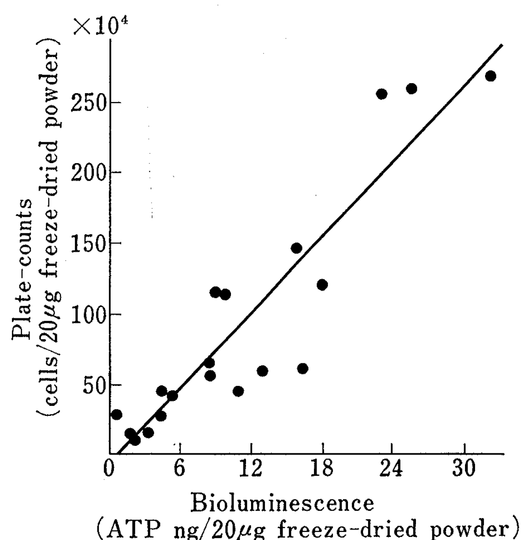

Fig. 8. Correlation of Survival Fractions of LB Freeze-dried Powder as Determined by the Bioluminescence and Plate-Count Methods

$Y=8.86X-7.03$, $r=0.912$, $n=19$.

Table III. Intracellular ATP Levels of Freeze-dried SF, LA-E and LB determined by the Proposed Luciferin-Luciferase System in Comparison with Those determined by the Usual Method

Sample	ATP × 10^8 ng/cell		
	A*	B*	B/A
SF	2.73±1.34	307±60	112
LA-E	6.10±2.83	481±62	79
LB	1.67±0.85	1290±152	772

A*; usual system, B*; proposed system.
Values are given±S.D.×1.96 (p>0.05, n=4).
Numbers of viable cells of SF, LA-E and LB were 5.22×10^6, 5.82×10^6 and 2.40×10^7 cells/1 ml, respectively. Intracellular ATP was determined by the usual system according to the notes under Table I.

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25 ml polystyrene vessel at 32°, 40° and 50°. The cell ATP was determined by the proposed method and the viable cells by the plate-count method, sampling every day. The relationships between the cell ATP and the viable cells obtained from the results of these survival tests are illustrated in Figs. 6—8. There were good linear relationships between cell ATP and viable cells; the coefficients of correlation were as follows: SF 0.858; LA-E 0.973; LB 0.912.

As shown in Table III, the amounts of intracellular ATP per cell of SF, LA-E and LB determined by the proposed system were about 100, 80 and 800 times those determined with the usual luciferin-luciferase system, respectively.

The plate-count method, which has usually been employed for the assay of lactobacilli, requires sterilization of the apparatus, medium and solutions, and requires 20—70 hr for colony formation and counting. On the other hand, the proposed method is simple and rapid, and can be completed within a day. It was confirmed that there was a close relationship between the cell ATP determined by the luciferin-luciferase method and the viable cell count determined by the plate-count method.

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