

THE EFFECT OF GROWTH PHASE AND MEDIUM ON THE USE OF THE FIREFLY ADENOSINE TRIPHOSPHATE (ATP) ASSAY FOR THE QUANTITATION OF BACTERIA

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ABSTRACT

The luciferase assay for adenosine triphosphate (ATP) has been suggested for use as a rapid method to determine the number of bacteria in a urine sample after nonbacterial components of the urine are removed. Accurate cellular ATP values, determined when bacteria are grown in an environment similar to that in which they are found in urine, are necessary for the calculation of bacterial titer in urine. Cellular ATP values vary depending on the method of extraction, the growth phase of the cells, and the growth conditions of the cells. ATP per cell values of stationary phase *E. coli* grown in urine were two times greater than ATP per cell values of cells grown in trypticase soy broth. Glucose and urea were examined as possible components responsible for the cellular ATP variation. Glucose was added to sterile glucose-free pooled urine, and urea was added to trypticase soy broth. The cells in which urea was added to trypticase soy broth had ATP per cell values similar to those found when cells were grown in urine.

The luciferase assay for adenosine triphosphate (ATP) has been extensively applied to areas of bacterial detection. In this work, an *E. coli* population in different physiological states and grown in different media was examined to determine variations in ATP per viable cell values. This work was done to determine ATP per cell values of bacterial cells obtained from clinical urine samples which would reflect the physiological state of the cells at the time when the luciferase ATP assay would be done.

All work was done using a clinical urinary tract isolate of *E. coli*. *E. coli* was selected for use because it is the cause of the majority of urinary tract infections.

A logarithmically growing culture of *E. coli* in trypticase soy broth was added to two growth media: (1) trypticase soy broth, a general purpose medium, and (2) urine. The urine was collected at random from healthy male and female adults. All samples were initially tested with Uristix and were rejected if glucose or protein was detected using this measurement. These samples were usually the second-morning specimens. The urine was filtered through a 0.22-micrometer Millipore filter after prior filtration which removed larger particulate material in the urine. *E. coli* was grown in the two media at 310 K (37°C) without shaking. At periodic intervals, the population was analyzed for intracellular ATP content and the number of viable *E. coli* present.

Procedure 1 shows the procedure used to extract ATP from *E. coli*. The ATP from the bacterial cells was extracted using nitric acid, after a prior treatment with apyrase, an ATPase, to remove any nonbacterial ATP present.

Procedure 1
Procedure to Extract ATP from Bacteria

1. 0.5 ml bacterial cells with their growth medium +0.1 ml 40 mg apyrase/ml 0.03 M CaCl₂ are mixed and allowed to sit for 15 minutes.
2. Add 0.1 ml 1.5 N HNO₃. Allow to sit 5 minutes.
3. Volume brought to 5 ml with deionized H₂O.

Procedure 2 shows the procedure used to treat standard ATP. Instead of using the bacterial cells and their growth medium as the starting sample, the bacteria were filtered out of the growth medium using a 0.22-micrometer Millipore filter. The filtrate was treated with apyrase and nitric acid in the same manner as the bacterial sample. A known amount of ATP was then added to the reaction mixture.

Procedure 2
Procedure to Treat Standard ATP

1. 0.5 ml growth medium (bacterial cells removed) +0.1 ml 40 mg apyrase/ml 0.03 M CaCl₂. Mix. Wait 15 minutes.
2. Add 0.1 ml 1.5 N HNO₃. Mix. Wait 5 minutes.
3. Add 0.1 ml ATP (1 µg/ml).
4. Volume brought to 5 ml with deionized H₂O.

The procedure to treat the blank to extract any ATP is shown in procedure 3. The growth medium from which bacterial cells were filtered was treated with apyrase and nitric acid.

Procedure 3
Procedure to Treat Blank to Extract ATP

1. 0.5 ml growth medium (bacterial cells removed) +0.1 ml 40 mg apyrase/ml 0.03 M CaCl_2 . Mix. Wait 15 minutes.
2. Add 0.1 ml 1.5 N HNO_3 . Mix. Wait 5 minutes.
3. Volume brought to 5 ml with deionized H_2O .

After the ATP was extracted from the bacterial sample, standard ATP, and blank, the assay to measure the amount of ATP was performed as follows. One-tenth milliliter of the treated sample was injected by needle and syringe into 0.1 ml of rehydrated DuPont luciferase (3 ml 0.2 M TRIS, 0.01 M MgSO_4 , pH 8.25 per vial). The luciferase was located in a cuvette in the light-tight chamber of the Chem-Glow (Aminco) instrument, which was attached to an X-Y recorder (Hewlett-Packard) to obtain a permanent record of the amount of ATP present in each sample. Using the blank, standard, and sample, the amount of ATP per milliliter in the bacterial sample was determined.

The number of viable cells was determined by pour plating in which serial dilutions of the population were made and then duplicate 0.1-ml aliquots of each dilution were added to 18 ml of melted trypticase soy (TS) agar. The plates were incubated at 310 K (37°C) for 24 hours, and the total number of colonies was counted. The number of viable cells per milliliter was then calculated.

Figure 1 shows the ATP per viable cell values when *E. coli* was grown in TS and urine for various time lengths. The growth curves of *E. coli* in TS and urine during the logarithmic phase are very similar. The number of cells that each medium will support during the stationary phase is about 1.2×10^9 cells per ml in TS broth and 4×10^8 cells per ml in urine. This pooled urine is a good growth medium for *E. coli*; the factors limiting the growth of *E. coli* in urine as compared to the growth which can be obtained in TS broth were not investigated.

Examination of the ATP per viable cell value of *E. coli* grown in both urine and TS broth shows a reduction in value as the cells grow logarithmically. The lowest ATP per cell values of *E. coli* grown in both urine and TS broth occur during the stationary phases of the populations.

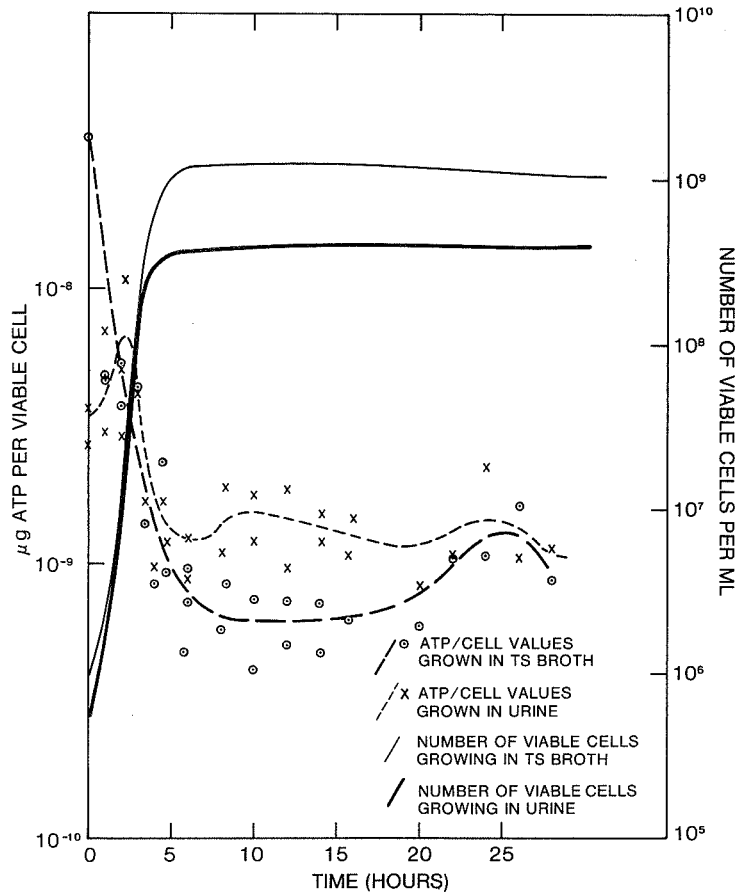


Figure 1. Effect of growth medium and the length of time grown in this medium on ATP per viable *E. coli* values.

In comparing the ATP per cell values of stationary phase populations of *E. coli* in TS and urine, a two-fold difference is found between the cells grown in these two media. The average amount of ATP per viable cell of cells grown in urine is approximately $1.2 \times 10^{-9} \mu\text{g}$ ATP per viable cell and $8 \times 10^{-10} \mu\text{g}$ ATP per viable cell when grown in TS broth. The mean difference between TS and urine ATP per viable cell values when the two populations are in stationary phase is $4.88 \times 10^{-10} \mu\text{g}$ ATP per cell.

Our next investigation was to determine the cause of the large difference in ATP per cell values found in the stationary phase between urine and TS grown *E. coli* populations. Possible explanations for this phenomenon were (1) a growth factor contained in urine or TS that is unique to that medium or (2) a metabolite produced as a consequence of growth that is affecting the ATP per viable cell value. We decided to examine two factors,

glucose and urea, to see their effects on the ATP per viable cell values of stationary phase cultures of *E. coli*. The urine we used had no detectable glucose in it as measured by the Uristix method which can detect 0.1 percent glucose levels. TS broth had a glucose content of 2.5 g per liter. We added various concentrations of glucose to the urine prior to the addition of *E. coli*. The cells were then grown in the same manner as previously described and the same measurements made on stationary phase cells. The effect of glucose addition to urine on ATP per viable cell values of stationary *E. coli* is shown in figure 2. As more glucose was added to urine, there was a decrease in ATP per viable cell values. When the glucose concentration in urine was similar to the glucose concentration in TS broth, there was very little difference in ATP per cell values when cells were grown under these two conditions.

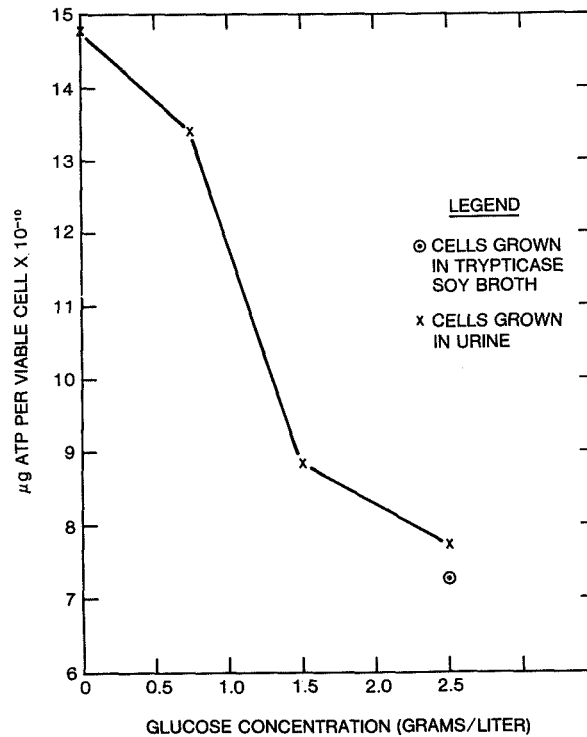


Figure 2. The effect of glucose addition to urine on ATP per viable cell values of stationary *E. coli*.

In investigating the effect of urea on ATP per viable cell values, we added urea to trypticase soy broth so that its concentration would be comparable

to that found in urine. *E. coli* was inoculated into these growth media and grown for 16 to 18 hours at 310 K (37°C). The addition of urea to TS broth caused an increase in ATP per cell value to a level higher than that found in cells grown in urine as shown below.

Effect of Urea on ATP per Viable Cell Values
of a Stationary Phase Population of *E. Coli*

<u>Growth Medium</u>	<u>μg ATP per Viable Cell</u>
Trypticase soy broth	5.29×10^{-10}
Urine	9.14×10^{-10}
Trypticase soy broth +0.025 g/ml urea	1.22×10^{-9}
Urine +0.025 g/ml urea	1.76×10^{-9}

Adding the same concentration of urea to urine resulted in about a two-fold increase in ATP per cell value above the value found in urine alone.

Further investigations have not been made to date to determine the explanation for these observed ATP per cell value differences. It may be that the presence of glucose or urea may be the factor regulating ATP per cell values or there may be a metabolite produced when glucose is metabolized by *E. coli* or a chemical such as ammonia or carbon dioxide produced during the spontaneous breakdown of urea in the medium that is regulating intracellular ATP values (Hempfling, 1970; Pastan and Perlman, 1970).

In summary, if use is to be made of a determined ATP per cell value in calculating the number of bacteria present in a sample such as a clinical urine sample, this determined ATP per cell value must take into account the following factors:

- The bacteria found as urinary tract pathogens should be grown under various conditions to get a range in ATP per cell values.
- Temperature, pH, nutrients in the medium, and oxygen tension are a few factors which must be considered (Forrest, 1965; Cole et al., 1967; Strange et al., 1963; Bailey and Parks, 1972).

As shown in this work and by previous investigators (Hamilton and Holm-Hansen, 1967), the age of the population makes a difference in the amount of ATP found in each cell. It is also important that the same procedure that will be used to treat the sample (such as urine) is followed to arrive at ATP per cell values.

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