PROBLEM AREAS IN THE USE OF THE FIREFLY LUCIFERASE ASSAY FOR BACTERIAL DETECTION

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INTRODUCTION

Every child has enjoyed the spectacle of flashing yellow-green lights from fireflies on warm summer nights; now the understanding of the chemical nature of this light production, known as bioluminescence, has enabled scientists to apply this phenomenon of cold light to many fields and this includes determination of the presence and quantity of bacteria. Building upon E. Newton Harvey's (1965) extensive and enthusiastic investigations of numerous types of luminescences displayed by organisms in nearly every phylum in the living world, McElroy and his associates (1969) and subsequent workers extracted the light producing substances from fireflies and described the reaction mechanism and kinetics as follows (Plant et al., 1968):

$$E + LH_2 + ATP \xrightarrow{Mg^{++}} E \cdot LH_2 \cdot AMP + PP$$

$$E \cdot LH_2 \cdot AMP + O_2 \longrightarrow E + AMP + CO_2 + h\nu + T$$

where:

E = firefly luciferase enzyme

LH_2 = reduced luciferin

ATP = adenosine triphosphate

AMP = adenosine monophosphate

PP = pyrophosphate

T = thiazolinone

$h\nu$ = light (550 nm)

The amount of light produced is proportional to the reactants when each is limiting. A light measurement capability could be used as an assay method for any of the reactants. Since ATP is a metabolite significant in all energy exchanges within living cells, its assay has implications for many parameters that describe the biota. ATP is known to be present in all forms of life, so its measure could establish the presence of living things. By purifying the firefly luciferase extract and adding all necessary chemicals but ATP in excess, an assay for ATP can be performed by measuring the amount of light produced when a sample containing soluble ATP is added to the luciferase reaction mixture. Thus the amount of light is proportional to the amount of ATP added (Strehler, 1965). When an unknown sample containing living organisms is processed to remove all exogeneous ATP, and the ATP within the organisms is released, a measure of the resultant light indicates the amount of organism ATP. If it is further demonstrated that within groups of organisms, their ATP content is relatively constant, then luciferase-ATP-initiated-light could be used to estimate the numbers of these organisms. This has been shown for bacteria, and procedures have been established to use the luciferase method to quantitate bacteria (Chappelle and Levin, 1968; D'Eustachio and Levin, 1967).

NASA/Goddard Space Flight Center personnel developed procedures for the determination of extraterrestrial life on other planets several years ago and subsequently evolved a program for applying these technological developments to public sector needs, particularly in the area of Health Care Delivery. One area, that of infection detection in urine specimens, is described in this paper.

The paper by Dr. Vellend describes an extension of this work, which was suggested by Dr. David Rutstein of the Harvard Medical School, for the determination of antimicrobial susceptibilities.

INSTRUMENTATION

Many types of photometric instruments exist for quantitating light. Those suitable for discriminating changes in low levels of light as is emitted by the small amounts of ATP present in bacteria, picomoles (10^{-9} moles) of ATP, employ a photomultiplier (PM) tube as a detector and a d.c. amplifier or equivalent. Several companies manufacture light measuring instruments including Schoeffel, Inc., Gamma Scientific, Inc., and Photovolt, Inc.; however, only a few have provisions for measuring light from solutions, such as Scintillation Counters, Packard, Inc., and Technicon, Inc., while those of American Instrument Co. (Aminco), DuPont de Nemours, and JRB, Inc. (SAI, Inc.), provide a means to inject the sample into the luciferase while both are in a light-tight configuration in place before the photocathode

surface. Some investigators have assembled their own instruments from off-the-shelf amplifiers and photomultipliers with the addition of a reaction chamber with capability for injection of liquid (Chappelle and Levin, 1968).* A sensitive instrument has been produced that uses pulse counting. In this instrument, "Diogenes," the PM tube can be cooled to improve the signal-to-noise ratio, an automatic injection system is provided, and there is selection of analog or digital readout (Chappelle and Levin, 1966). With this instrument, 10^{-9} μg of ATP produces a signal above the noise but it is not linear. Because the light from unknown samples may differ by several decades, an automatic electronic amplification switching capability or digital readout in several digits is very useful and eliminates repeating the assay and thereby using more of the luciferase reaction mixture. Instruments that provide this capability are the DuPont Biometer, scintillation counters, the JRB ATP Photometer, and the Aminco Chem-Glow with integrator; while Hewlett Packard makes a picoammeter with automatic ranging that can be coupled with a photomultiplier tube with its power supply.

The light production upon injection of a sample containing ATP into the luciferase reaction mixture rises to a maximum intensity and then decays exponentially. Both the maximum intensity (determined by measuring peak height) and the total light output are proportional to the amount of ATP added. Therefore, several types of data display are suitable. When used in conjunction with a capacitance circuit, the peak height can be sampled and a digital output produced and recorded. A rate function can be produced on a strip chart recorder and a peak height measured. Analog circuitry can be introduced, digitized, and printed to show the total light output. Both the scintillation counter and one version of the JRB instrument use a measurement made during the decay period, since there is no provision for injecting sample into luciferase while positioned in front of a PM tube. However, the JRB provides an attachment for making this modification and also for reading the peak light. As an outgrowth of space research, originally intended for the detection of life on Mars, a prototype instrument was developed to perform chemical processing of a sample with subsequent injection of the luciferase automatically (Picciolo, 1971).

APPLICATIONS

Numerous areas of application are appropriate for using the luciferase assay for ATP of itself as well as to measure organism levels. Since it is specific for ATP and can be performed in the presence of other purine compounds, it does not require isolation of the ATP, so it could be measured in cell extracts

* See Wampler, J.E. paper in this document.

and body fluids. The ATP level is an indicator of metabolic changes in living organisms and therefore can be a monitor of cell integrity, genetic variants, substrate utilization (Klofat et al., 1969), attack by a virus (Chappelle and Levin, 1968; Levin et al., 1964), and cell growth. With further research, these types of measurements could have applications in cancer research (Vlodavsky et al., 1973; Levin et al., 1964) and immunology as well as organ and tissue viability and transplant rejection.

When used as a measure of bacterial levels, the ATP assay can be used for pollution monitoring in air and water supplies, such as drinking water, sewage treatment effluent, river and stream pollution levels, and industrial water supplies and effluents. Closed environment monitoring could be performed for spacecraft, space stations, clean rooms, operating rooms, and so forth. Microbial levels could be measured in dried foods, cereals, spices, milk, beer, wine, liquors, canned and bottled foods, pharmaceuticals, cosmetics, ointments, creams, paints, gasolines, and oils. Agricultural uses include determination of fertility levels, spore viability, and sterility of soils, plants, and animals. Oceanographic monitoring can be performed to determine biomass and effect of pollutants. Medical applications include infection levels in blood, urine, cerebrospinal fluid, wound excretion, joint fluid, and lung and pleural fluid. Evaluation of antibiotic effects on bacteria including synergism as well as antibiotic levels in body fluids could be done using the luciferase assay.

Each of these applications represents areas where research is needed into the problems involved in making the luciferase assay applicable. These include sampling, sensitivity, and background. The advantages in most cases would be speed and specificity.

BASIC CHARACTERISTICS OF THE LUCIFERASE ASSAY

Since the efficiency of light production in the firefly has been shown to be near unity (McElroy and Glass, 1961), it is expected that the in vitro reaction could be made fairly efficient also, thus providing sufficient light signal when measuring small amounts of the other reactants. When all of the chemicals necessary to the reaction are present in excess and when the limiting chemical has been removed from the luciferase reaction mixture, the addition of that chemical stimulates the production of light that is quantitatively related to the concentration of that chemical. When that chemical is ATP, it has been demonstrated that both the peak light output, which occurs within one-half second after proper mixing, and the total light output are proportional to the amount of added ATP (Chappelle and Levin, 1968; St. John, 1970). Within practical limits of reagent concentration and instrument amplification, ranges of ATP can be measured from 5×10^{-2} to 5×10^{-7} μM/ml. With careful manipulation, linearity and reproducibility will range between 5 and 25 percent coefficient of variation, where measuring standard ATP in the absence of inhibitors can be done very accurately, while measurements of bacteria from unknown specimens produce the most fluctuation.

Under ordinary growth conditions, changes in ATP/bacterium have been shown to vary by about a factor of 10 (figure 1). However, for each type of specimen, this variation should be checked if it is considered that the environmental conditions could affect the ATP/bacterium.* Various conditions have been shown to lower the ATP/bacterium; removing 0_2 from the medium gives a rapid, dramatic decrease in ATP levels (Klofat et al., 1969).

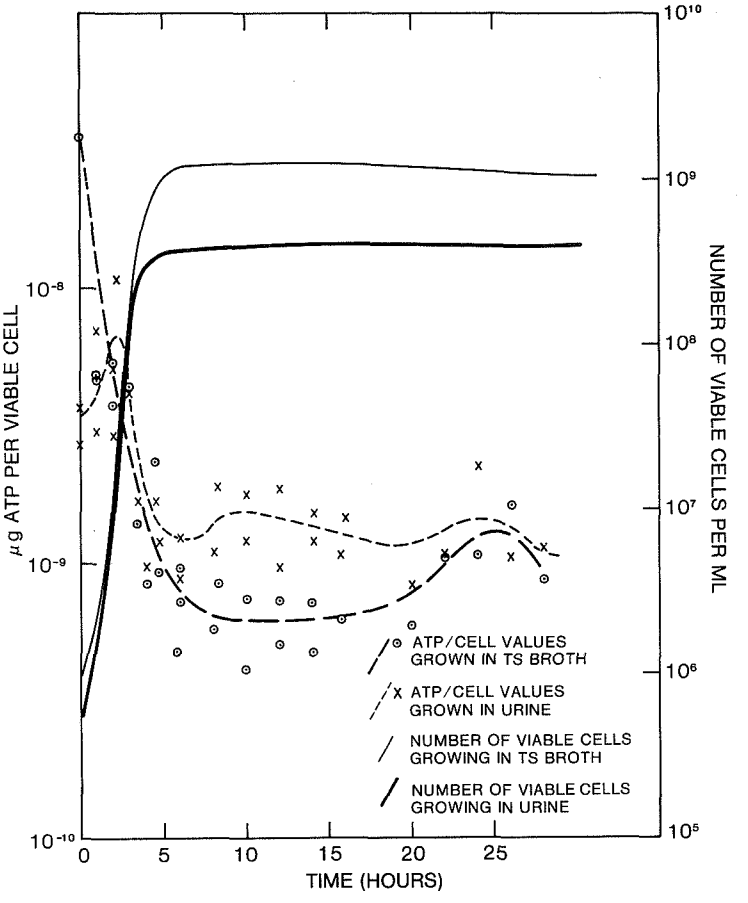

Figure 1. Effect of the growth medium and the length of time grown in this medium on ATP per viable *E. coli* values.

With proper handling, the luciferase reaction mixture is reliable and reproducible. When purification of the luciferase is carried out consistently, the activity can be standardized from batch to batch. The activity decays

* See Holm-Hansen et al. paper in this document.

only slightly at room temperature for up to two hours. Care must be exercised that the sample and luciferase are at the same temperature at measurement.

The luciferase reaction mixture emits light without the addition of any ATP. This is called inherent light. Preincubation will reduce the level of inherent light as well as action of an ATPase, but since the activity also decreases, a compromise time must be selected to achieve an optimum level. It is suggested that the inherent light is due to small amounts of ATP in the enzyme mixture that are not removed during the purification.

When we inject a processed sample containing no ATP into the luciferase, there is a light production which limits the sensitivity of the assay. This "blank" reaction is elusive and, unless proper attention is paid to technique, can be variable. The best of deionized, sterile water must be used for all reagents to keep the blank low and consistent. The blank problem is more severe when using an acid extractant. It can be minimized when the final pH of the luciferase reaction is 7.75 and when there is minimal dilution of the luciferase by the injected sample. This is accomplished by dilution of the extracted samples to contain 0.005 milliequivalents of nitric acid. It is diluted with the appropriate concentration of Na_2SO_4 to achieve an ionic strength equal to that of the luciferase buffer. The starting luciferase buffer pH and molarity is selected to ensure a final pH of 7.75 after use of the acid extractant.*

Chemically purified ATP is used to standardize the reaction for a given light level. The ATP is added at the time in the processing of the sample that it would be released from the bacteria, representing an extraction standard.

Since the chemical environment affects the light production, control of ionic strength and pH must be exercised. This effect can be measured by performing a recovery-type experiment, that is, adding higher amounts of standard ATP after assaying the sample and repeating the assay. Mathematical adjustment can then be made for uncontrollable variations in the chemical environment. This is the same method that is called an internal addition standard (St. John, 1970).

The sensitivity of the luciferase assay is determined by the activity of the luciferase under the specific chemical assay conditions and is limited by the blank response. Increase in ionic concentration decreases the activity and increases the blank response. Specific ions stimulate or inhibit activity and blank response correspondingly.

The response of commercially prepared ATP of various concentrations minus the blank response is plotted as a function of ATP concentration in figure 2. Reproducibility, sensitivity, and linearity of the luciferase assay is given in table 1. The disodium salt of ATP is diluted in distilled water, and the purified luciferase is supplied by DuPont, Inc., and is reconstituted in 0.01 M

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* Chappelle, E. W. and G. L. Picciolo, C. A. Curtis, E. A. Knust, D. A. Nibley, R. B. Vance, "Laboratory Procedures Manual for the Firefly Luciferase Assay for Adenosine Triphosphate (ATP)," NASA-GSFC TM X-70926, 1975.

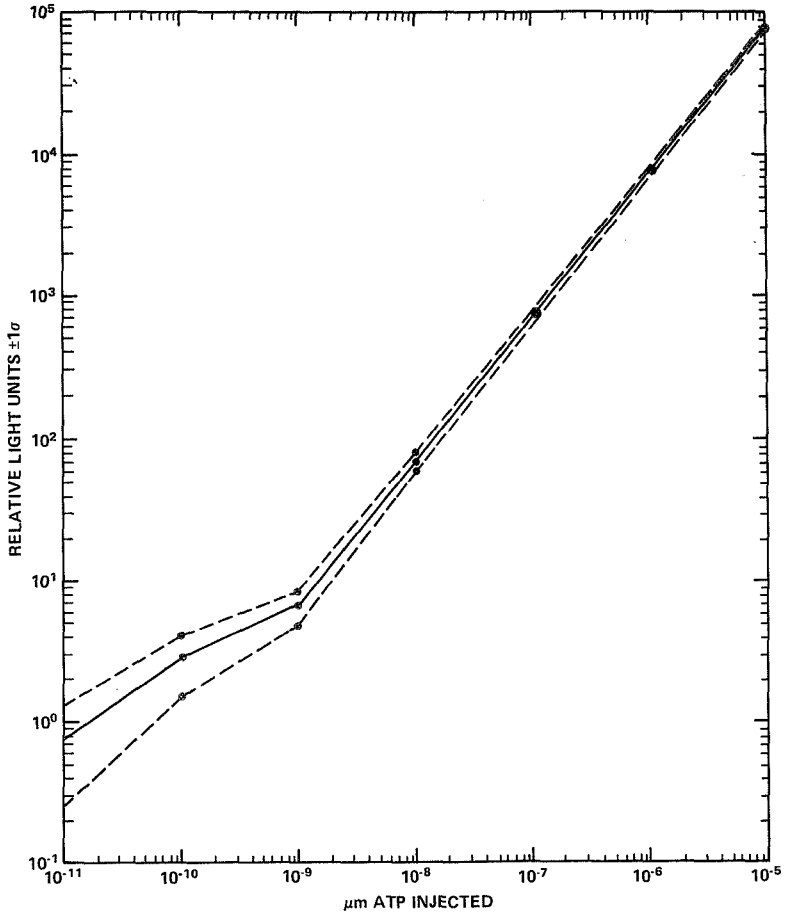

Figure 2. ATP concentration curve showing relative light units $\pm 1\sigma$ versus μM ATP injected (0.1 ml) using the Aminco Chem-Glow Photometer with recorder. The ATP is diluted in water and the DuPont luciferase is reconstituted in 0.01 M TRIS, 0.01 M $MgSO_4$, pH 8.0, using 0.1 ml/cuvette.

TRIS buffer and 0.01 M $MgSO_4$ at pH = 8.0. The conditions represent minimal inhibition and therefore maximum activity.

However, when chemical processing must be performed on the sample, reagents are added to the sample which result in inhibition and therefore decrease in acitivity, which may result in loss of sensitivity. Table 2 shows the linearity and sensitivity of the response to standard ATP when diluted in saline, broth, and urine. This may be compared with the uninhibited condition (table 1). The conditions used here would be used when measuring bacterial ATP in the presence of mammalian cells and other nonbacterial

Table 1

Data for Figure 2*

μM ATP Injected (0.1 ml)	Relative Light Units Less Blank From Chem-Glow with Recorder	MEAN $\bar{X}$	Standard Deviation σ	Coefficient of Variation
10^{-5}	76,000 82,000 78,000 74,000	77,500	3,415	4%
10^{-6}	7,200 7,000 7,400 7,500	7,275	222	3%
10^{-7}	780 740 740 760	755	19	2.5%
10^{-8}	78 78 68 68	73	5.8	7.9%
10^{-9}	5 9 6 7	6.7	1.7	25%
10^{-10}	3 3 1 4	2.7	1.2	46%
10^{-11}	1 0 1 1	0.7	0.5	66%

* The dotted line dividing the data indicates the cutoff point below which the results were not linear and were not used in linear regression analysis.

Linear regression analysis showed: using logarithms of the numbers when n = 20, the slope interval is $0.97 < 0.98 < 0.99$; the intercept is close to 0; the F ratio = 1.7×10^4 when table $F_{0.95} = 19$; and correlation coefficient (r) is 0.999.

Table 2
Linearity and Sensitivity Response to Standard ATP

A. Results from the Centrifuged Procedure using Commercial ATP Diluted in Saline, Trypticase Soy Broth, or Urine				
μM ATP/ml Original Sample	μM ATP Injected	Light Units Less Blank		
		Saline	Broth	Urine
4.4×10^{-4}	1.1×10^{-3}	Saturated	Saturated	Saturated
4.4×10^{-5}	1.1×10^{-4}	62,000	58,000	54,000
4.4×10^{-6}	1.1×10^{-5}	6,000	6,000	6,500
4.4×10^{-7}	1.1×10^{-6}	600	550	600
4.4×10^{-8}	1.1×10^{-7}	58	70	54
4.4×10^{-9}	1.1×10^{-8}	28	15	7
4.4×10^{-10}	1.1×10^{-9}	2	4	0
B. Results from the Nonconcentrated Procedure using Commercial ATP Diluted in Saline, Trypticase Soy Broth, or Urine				
1×10^{-2}	1×10^{-4}	45,000	55,000	52,000
1×10^{-3}	1×10^{-5}	6,500	6,500	5,400
1×10^{-4}	1×10^{-6}	700	600	530
1×10^{-5}	1×10^{-7}	61	45	44
1×10^{-6}	1×10^{-8}	22	5	9
1×10^{-7}	1×10^{-9}	2	0	4
1×10^{-8}	1×10^{-10}	4	0	9
1×10^{-9}	1×10^{-11}	0	0	3

sources of ATP. Results are given when reagents are allowed to remain in the sample (nonconcentrated procedure), and when they are mostly removed by the centrifugation procedure. On the basis of actual ATP injected, the light response for the two methods is approximately the same. It thus follows that in the centrifugation procedure, where one injects the equivalent of 2.5 ml of original sample instead of 0.01 ml as in the nonconcentrated procedure, the overall sensitivity should increase by about 250 times. This is shown in the first column of table 2, micromoles of ATP per milliliter of

the original sample. The upper level of ATP which can be measured is limited by the saturation of the photocathode of the photomultiplier or by the concentration of other reagents, such as luciferase and luciferin. The lower level is limited by the blank level which must be subtracted from each reading. One area of work is to develop a means of measuring a blank for each unknown sample or eliminating the blank. Since the nature of the sample can produce variation in blank levels, this is a very critical and difficult problem.

The presence of each of the assay reagents introduces inhibition of the luciferase activity in varying amounts. For a constant amount of ATP, the percent of inhibition for each of the complete procedures when compared with a procedure with no inhibiting reagents included for ATP in H_2O, luciferase in 0.01 M TRIS, and 0.01 M $MgSO_4$ is:

Nonconcentrated with malate buffer	
ATP in saline	42%
ATP in urine	33%
Centrifuged with malate buffer	
ATP in saline	20%
ATP in urine	30%

When it is expected that fluctuations will be introduced by the unknown samples, each sample must be evaluated for its inhibition. This is done by assaying the unknown and then adding a negligible volume of standard ATP at a concentration far in excess of the sample ATP and repeating the assay. By this recovery method, an exact correction can be made for all inhibitors known and unknown in each sample. Coefficient of variation on the inhibition of individual urine specimens is about 32 percent. The sample reading is subtracted from the recovery reading and then the ratio of sample reading to net recovery reading is equivalent to the ratio between the amount of ATP in the sample and the amount of ATP added to the recovery.

In order to quantitate bacteria in a sample by measuring the ATP in that sample, the ATP from nonbacterial sources must not interfere. The effective ratio of bacterial ATP to nonbacterial ATP is small and is a limit on the resolution of the analysis. Biological fluids may contain soluble ATP and also ATP contained within nonbacterial cells such as mammalian cells in a urine specimen. A description follows of the procedure we have developed to measure bacterial ATP in biological fluids such as urine specimens.

FUNCTION OF REAGENTS USED IN THE PROCEDURE

Samples containing tissue cells can be processed by the addition of a nonionic detergent such as Triton X-100, which releases tissue cell ATP without affecting bacterial ATP at the concentration used. This was verified using 0.17 percent Triton X-100 on the group of urinary bacteria listed below. This was done with a higher Triton X-100 concentration than is used in the procedures. The only organism that gave a significant loss of ATP was β

Streptococcus. A concentration of 0.1 percent Triton X-100 will release the ATP from the number of blood cells contained in 10 percent blood. Other types of tissue cells could be present in a urine sample such as epithelial and kidney cells. We subjected 1×10^6 monkey kidney cells per ml to the Triton X treatment and removed the released ATP by apyrase. Subsequent treatment with perchloric acid recovered up to 9 percent of the kidney cell ATP.* This was done without the pH drop that we found necessary to remove bound ATP from blood cells; therefore, we will repeat this experiment incorporating the malic acid procedure which is the pH drop.

Effect of 0.17 percent Triton X-100 on Bacterial ATP from 10 Species of Bacteria

Organism	Loss of ATP
Proteus mirabilis	0 %
Proteus morganii	0 %
Serratia marcescens	0 %
Streptococcus faecalis	0 %
Staphylococcus aureus	0 %
Herellea species	8.8%
Pseudomonas aeruginosa	0 %
Klebsiella pneumoniae	12 %
Escherichia coli	0 %
β Streptococcus	90 %

Any ATP that is present in solution can be removed by the addition of an ATPase, such as potato apyrase, which hydrolyzes the ATP to a form that is inactive in the luciferase reaction. Potato apyrase requires Ca^{++} as a cofactor at a reacting concentration of 5×10^{-3} M when using up to 6.7-mg purified apyrase/ml of reacting solution. The activity of the Sigma purified apyrase used is given as 2.5 units per mg, where a unit is that amount that will liberate one micromole of inorganic phosphorus per minute at pH 6.5 at 303 K (30°C) from ATP. The optimal pH is 6.5, but there is some activity as low as pH 3.0.

Residual traces of ATP remain in the sample at this point; these are bound to large molecules (such as proteins) and particulates. This ATP can be removed by lowering the pH to dissociate ATP from its binding sites, allowing the apyrase to work at this time. Since the pK_4 of ATP = 4.0, we drop the pH to the lowest value possible to still retain marginal apyrase activity, pH = 3.75.† Malate buffer is used to buffer the sample down to this pH.

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* Bush, V. N., "The Examination of Urine: Samples for Pathogenic Microbes by the Luciferase Assay for ATP. I. The Effect of the Presence of Fungi, Fungal-like Bacteria and Kidney Cells in Urine Samples," NASA Grant Report No. N73 18096, 1973.

† Since the work reported in this paper, we have changed the malate buffer to a pH = 4.25 whereby there is no rupture of urinary pathogenic bacteria.

The sample now contains only ATP contained within the bacterial cell. This is released by an acid extractant.

Organic solvents and inorganic acids were compared for their efficiency on some of the urinary bacteria. Conditions were established for acetone and nitric acid that gave the highest recovery.* For ten of the urinary pathogens, the effect of increasing concentrations of HNO_3 is shown in table 3 as the relative amount of ATP extracted, and is shown as a function of the milli-equivalents HNO_3 (on the pellet of stationary phase organisms). We then chose 0.1 N HNO_3 as the extracting concentration of the acid for urinary pathogens as a compromise between extraction efficiency and inhibition.

Table 3

Relative Amount of ATP Extracted from Various Bacteria using Various Concentrations of Nitric Acid on the Pellet

Organism	Normality of Nitric Acid			
	0.0625	0.100	0.150	0.200
Escherichia coli	1.6	1.7	2.0	2.0
Klebsiella pneumoniae	1.9	2.1	2.0	2.0
Staphylococcus aureus	1.4	1.8	1.9	1.8
Pseudomonas aeruginosa	0.98	1.9	2.7	1.9
Proteus mirabilis	1.8	2.0	2.0	2.1
Enterobacter cloacae	1.2	2.1	3.7	3.3
Streptococcus faecalis	0.38	0.88	1.1	1.0
Serratia marcescens	88.1	91.5	60.3	66.4
Proteus vulgaris	77.6	99.3	79.6	78.4
Staphylococcus epidermidis	41.6	41.0	45.0	38.6

The use of the acid extractant also holds the pH below 2.0 which inactivates the apyrase. The sample is diluted so that the amount of acid injected into luciferase is 0.005 meq in a final volume of sample luciferase mixture of

* See Knust, Elizabeth A., et al., paper in this document.

0.2 ml with a final TRIS buffer concentration of about 0.13 M. The final pH of the sample luciferase mixture must be at 7.75. In order for this to hold, the starting pH of 0.25 M TRIS must be 8.2. The optimal range of pH after each reagent addition is given in the following list. The nonconcentrated procedure is given in procedure 1.

Range of pH Tolerance

After Addition to Sample of:	Acceptable pH Range
Apyrase-Ca ± Triton X-100	5.0 to 7.8
Malate buffer	4.00 ± 0.25
Nitric acid	1.15 to 1.20
H_2O or Na_2SO_4	1.8 to 2.1
Luciferase-luciferin	7.75 ± 0.1

Procedure 1

Nonconcentrated Method for Luciferase Assay of Bacteria:
Malate-Nitric Acid Procedure

0.5 ml sample: Urine, bacterial culture, or other.

Add 0.1 ml apy-TX-Ca: 10 mg apyrase/ml 0.03 M $CaCl_2$
(0.6% TX-100 if contaminating mammalian cells are present).
Wait 15 minutes, vortexing frequently.

Add 0.1 ml malate buffer: 0.5 M malic acid 0.005 M Sodium Arsenate, pH 3.75.
Wait 15 minutes, vortexing frequently.

Add 0.1 ml 1.5 N HNO_3
Vortex well, wait 5 minutes.

Add 4.2 ml 0.15 M Na_2SO_4

Assay: Inject 0.1 ml of above into 0.1 ml of luciferase (DuPont), reconstituted with 1.5 ml 0.25 M TRIS, 0.01 M $MgSO_4$ pH 8.2 per vial.

Recovery: 0.05 ml of ATP (10 μg/ml) or 0.5 ml of ATP (1.0 μg/ml), depending on desired accuracy of delivery, is added to a measured amount (0.3 ml) of the remainder of the treated sample.

CONCENTRATION METHODS

By concentrating the sample one not only increases sensitivity, but removes endogenous inhibitors as well.

Standard ATP under these conditions would be assayed by the following procedures.

The reproducibility, sensitivity, and linearity for standard ATP concentrations, when assayed after the addition of 0.1 N HNO_3 and diluted 1:1 with H_2O and 0.1 ml of this mixture is injected into 0.1 ml of luciferase-luciferin mixture in 0.25 M TRIS, 0.01 M $MgSO_4$, pH = 8.25, are shown in table 4.

Two methods have been optimized using centrifugation: The pure species short centrifugation procedure (procedure 2) is designed to be used in bacterial studies where there are not large amounts of nonbacterial cells. It uses only one centrifugation. It is intended to take approximately 1 hour, thereby allowing greater ease in the execution of timed experiments as well as maintaining the accuracy of the longer centrifugation procedure.

Procedure 2

Pure Species Short Centrifugation Procedure

5.0 ml sample

Add 1.0 ml Tx-Apy-Ca: 10 mg/ml purified apyrase in 0.6% Tx-100, 0.03 M $CaCl_2$.
Vortex well.

Centrifuge 15 minutes at 8000 rpm (10400 RCF X G) and 20°C.
Decant inverted on filter paper 5 minutes.

Add 0.2 ml 0.1 N HNO_3.
Vortex, wait 5 minutes.

Add 0.2 ml H_2O, sterile, deionized.
Vortex.

Assay: Inject 0.1 ml into 0.1 DuPont luciferase reconstituted with 1.5 ml 0.2 M TRIS, 0.01 $MgSO_4$, pH = 8.4.

The second, longer centrifugation procedure (procedure 3) is used when the sample contains other than bacterial cells which interfere with ATP measurement. Any sample of biological fluid can be used that does not contain more than 10^6 leucocytes or more buffering capacity than 10 percent blood or 100 percent urine or 100 percent trypicase soy broth. Any volume up to the capacity of the centrifuge tube used can be accommodated, with proper adjustments for concentration.

Table 4

Reproducibility, Sensitivity, and Linearity for Standard ATP Concentrations When Assayed*

Instrument: Biometer Procedure: Analogous to the centrifuge procedure omitting all reagents and steps except nitric acid and the final water†		
μg ATP Injected	CV% (Blank not subtracted)	Light Units less blank †
3.5×10^{-1}	10%	8.9×10^{9}
3.5×10^{-2}	17%	9.12×10^{8}
3.5×10^{-3}	7%	1.09×10^{8}
3.5×10^{-4}	7%	9.29×10^{6}
3.5×10^{-5}	13%	1.29×10^{6}
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3.5×10^{-6}	------	1.01×10^{6}
3.5×10^{-7}	------	6.70×10^{5}
Blank	0.15%	4.21×10^{6}
Linear Regression Analysis: Slope interval, $\beta = 1.06 > 1.02 > 0.98$ and intercept = -1.06×10^{1} when P = 0.05 and N = 15; coefficient of correlation (r) = 9.98×10^{-1} F ratio = 2.78×10^{3} when table $F_{0.95} = 2.69$		

* See text.

† Water was used as a diluent here, giving a higher blank than is expected when the diluent is Na_2SO_4.

‡ The dotted lines through the data are the cutoff points below which the results are not linear and were not used in linear regression analysis.

Procedure 3 involves the use of 17- X 100-mm disposable plastic test tubes of 12-ml capacity. Centrifuge well adaptors were fabricated to fit a 250-ml-volume well drilled to hold five plastic tubes per well. This allows a total of 30 tubes per centrifugation.

The light measured is calibrated by adding known amounts of chemically pure ATP to a duplicate sample (internal addition standard). After subtracting the sample light value from the internal addition standard light value,

Procedure 3

Centrifugation Method for Luciferase Assay of Bacteria:
Malate-nitric Acid Procedure

10 ml sample: urine, bacterial culture, or other.

0.2 ml 6% Triton X-100
Vortex well.
Centrifuge at 10,400 RCF X G, 293 K (20°C)

Add 1.0 ml Apy-Ca: 10 mg apyrase/ml 0.03 M $CaCl_2$.

Vortex well.

Add 5.0 ml normal saline (0.9%).

Mix well.
Wait 15 minutes.

Add 1.0 ml malate buffer: 0.25 M malic acid 0.005 M Na.

Mix well.
Centrifuge at 10,400 RCF X G, 293 K (20°C).
Discard supernatant and invert tube to drain on paper toweling for 5 minutes.

Add 0.2 ml 0.1 N HNO_3.

Vortex well.
Wait 5 minutes.

Add 0.2 ml 0.15 M Na_2SO_4.

Assay: Inject 0.1 ml of above into 0.1 ml of luciferase (DuPont) reconstituted with 1.5 ml 0.25 M TRIS with 0.01 M $MgSO_4$ pH 8.2 per vial.

Recovery: 0.05 ml of ATP 1.0 μg/ml is added to the remainder of the treated sample.

a direct proportion is made between the unknown sample and the known quantity of ATP added. The ATP value then can be converted to number of bacteria per milliliter by assuming an average ATP per bacterial cell or used directly as a measure of bacterial ATP levels.

Table 2 shows the results obtained when urine, saline, or broth are taken through the centrifugation procedure and dilutions of chemically synthesized ATP added and assayed. In order to summarize the reproducibility and sensitivity of the assay for detecting bacteria, we ran the following experiment where we determined *Escherichia coli* cell number by the luciferase centrifugation assay and the nonconcentrated assay, microscopic counting, and agar plate colony counts. *E. coli* cultures were grown to log phase in trypticase soy broth, centrifuged, and equal aliquots resuspended in normal saline, filtered, pooled urine and fresh trypticase soy broth. Microscopic and spread plate counts were run on all three aliquots. Serial dilutions were made on the three suspensions and the nonconcentrated and the long centrifugation procedures were run on the dilutions. The microscopic counts agreed closely with the plate counts indicating that all the bacteria were dividing and therefore were viable. Data in table 5 give the bacteria per milliliter of stock solution obtained by averaging the microscopic and plate counts, the coefficient of variation for each set of measurements, the ATP per milliliter stock solution, the number of bacteria per milliliter that gives a response of one unit above the blank value, and the average ATP per bacterium calculated from this experiment. The graphs in figure 3 show the relative light units by the luciferase assay versus bacteria per milliliter by microscopic and plate counts.

Table 5

Results from Long Centrifugation Procedure with *E. coli* in Saline, Urine, and Broth (see text)

	Saline	Urine	Broth
Average of microscopic and colony counts-bacteria/ml	1.6×10^{8}	1.6×10^{8}	1.9×10^{8}
Coefficient of variation	18%	14%	19%
μM ATP/ml	3.0×10^{-5}	2.7×10^{-4}	5.4×10^{-4}
Coefficient of variation	25%	25%	19%
Sensitivity-bacteria/ml	3.6×10^{3}	4.1×10^{2}	2.3×10^{2}
μM ATP/bacterium	1.8×10^{-13}	1.6×10^{-12}	2.8×10^{-12}

The lowest number of cells which can be detected by the nonconcentrated procedure has been found to be between 5×10^{5} and 1×10^{6}, depending on the species and media, as compared with 3×10^{3} to 2×10^{2} for the centrifugation procedure.

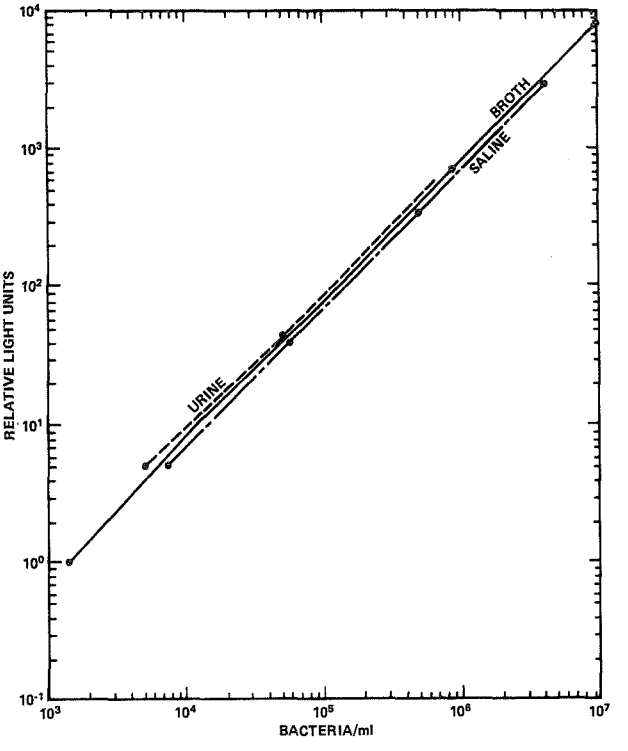

Figure 3. *E. coli* concentration curve showing relative light units versus bacteria per milliliter by microscopic and plate counts. The long centrifugation procedure was run on the bacteria diluted in urine (- - -), saline (— - —), and broth (—).

The noncentrifuged procedure would be more applicable because of its simplicity in situations where the cell number is high, that is, greater than 10^5, and where the media does not contain luciferase inhibitors, for example, pure cultures in defined growth media.

SENSITIVITY AND REPRODUCIBILITY OF THE ASSAY: SHORT CENTRIFUGATION

Using trypticase soy broth as samples, the short centrifugation procedure (procedure 2) was run and standard ATP added after use of nitric acid. The DuPont luciferase was reconstituted either with 0.15 M TRIS, 0.01 M $MgSO_4$ pH 8.5 or 0.2 M TRIS, 0.01 M $MgSO_4$, pH 8.4. Figure 4 shows the results when a 4-1/2-hour culture of *E. coli* is diluted in urine, saline, or broth and a short centrifugation procedure run, where large numbers of nonbacterial cells are not expected. Numbers of bacteria per milliliter, determined by Coulter Count of original sample, are plotted versus μg ATP per milliliter measured.

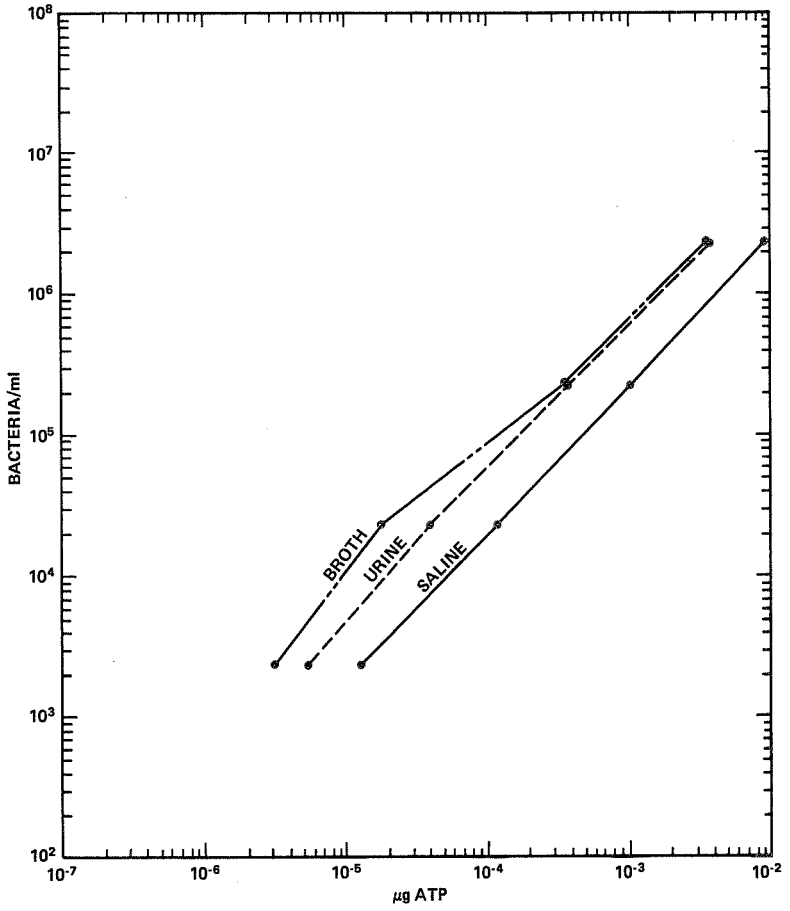

Figure 4. A 4-1/2-hour culture of *E. coli* was diluted in urine (- - -), saline (—), and broth (— - - —). The micrograms of ATP, measured using the short centrifugation procedure, are plotted versus the number of bacteria per milliliter as determined by Coulter Counter.

Table 6 shows the ATP per bacterial cell for some of the urinary pathogens. The bacteria were in stationary phase of growth and the centrifugation procedure was used to determine ATP levels. A microscopic or Coulter Count was done to determine total cell count, and drop plate colony counts were done using trypticase soy agar for viable counts. The percentage of dead cells was calculated as the difference.

Table 6

Urinary Pathogens in Stationary Phase of Growth

Microorganism	Micrograms ATP per Bacterial Cell*	Percent Dead Cells
Escherichia coli	4.44×10^{-10}	39%
Staphylococcus aureus	5.16×10^{-10}	63%
Staphylococcus epidermidis	2.26×10^{-10}	11%
Proteus mirabilis	1.27×10^{-10}	35%
Pseudomonas aeruginosa	3.04×10^{-10}	30%
Streptococcus faecalis	5.47×10^{-10}	34%

* Based on Total Cell Count

ASSAYS ON URINE SPECIMENS

The amount of ATP per cell will affect the sensitivity of the assay as well as the accuracy for unknown samples. Environmental and metabolic factors are known to affect the ATP levels of bacteria. To evaluate this for urinary bacteria, the μg ATP per viable cell was measured as a function of growth phase in both trypticase soy broth and urine for *E. coli*.* When grown in urine, the ATP per cell was on the average two times higher than when grown in trypticase soy broth (figure 1), however, if glucose was added to urine or urea added to trypticase soy broth, the effect was reversed.

The centrifugation procedure was used to evaluate the correlation between colony counts and the ATP assay on infected urine specimens. On 183 urines routinely submitted to a bacteriology hospital laboratory, 0.1 ml of saline serial dilutions were plated on blood agar for colony counts. A scatter diagram of these points is given in figure 5, showing the femptograms of ATP per milliliter versus the colony forming units per milliliter.

The grid lines can be moved to indicate decision levels for calling a urine positive for infection or negative and reading its corresponding ATP level. The clustering at the extremes indicate either saturation or minimum sensitivity

* See Bush, V. N. et al., paper in this document.

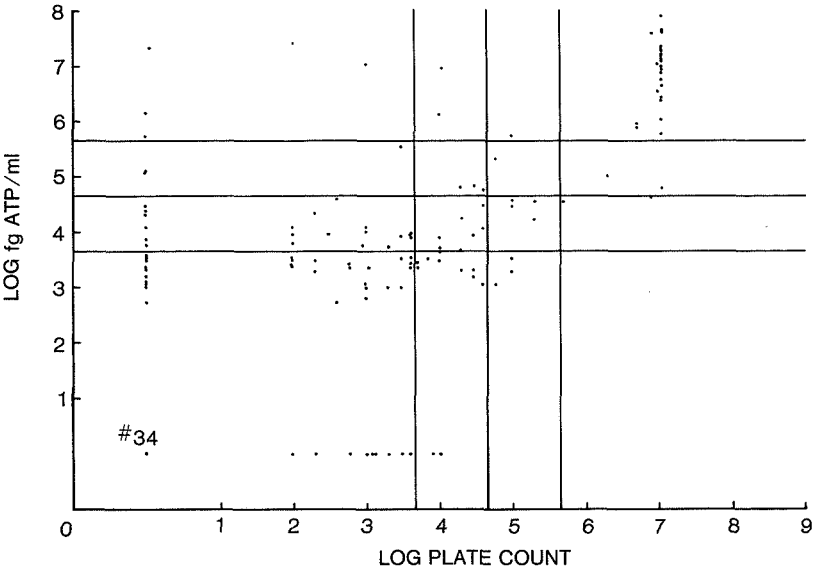

Figure 5. Correlation of ATP and colony counts in urine specimens.

levels for each of the measurement techniques. The #34 at a zero plate count and a zero ATP indicates that there were 34 urines that showed no colonies and blank level ATP measurement.

In order to determine whether a specimen is infected, a blank value is subtracted from each sample. A blank is obtained by using filtered, pooled urine and adding all the reagents used in the sample processing. We know, however, that the sample itself may affect the blank value and vary from sample to sample and therefore represents an uncertainty. In treating the data, therefore, subtract this blank value from each measurement, and then correlate these resulting values with bacteriologic counts. We can establish a number of light units which represents a lower limit around our blank value that gives the desired correlation, that is, choosing the trade-offs of false positives in deference to false negatives.

According to Kass (1957), 10^5 bacteria/ml indicates a urinary infection. When those specimens with a plate count below this level are considered to be negative, figure 6 shows the interpretation errors for a given ATP cutoff point when used to distinguish between ATP positive and ATP negative samples. The other two lines show this for a plate count infection level of 10^4/ml.

By grouping these data by categories representing a range of colony forming units per milliliter versus a range of femptograms of ATP per milliliter, table 7 shows the number of specimens that fell in each category and the percent of the total number of specimens.

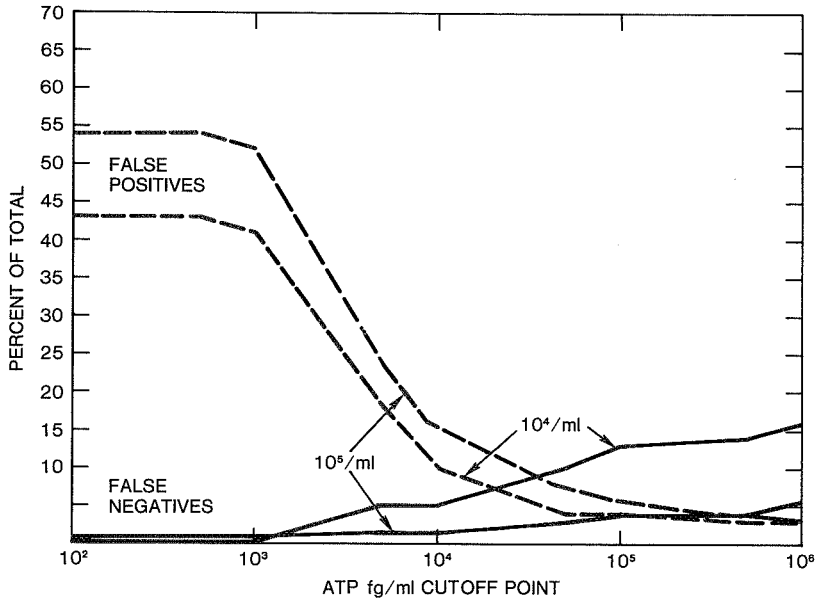

Figure 6. Malate buffer-nitric acid centrifugation procedure on urine specimens with a plate count greater than 10^4/ml and 10^5/ml.

Table 7

Number of Specimens and Percent of Total Number Tested from the Malate Buffer-nitric Acid Centrifugation Procedure on Urine Specimens

Plate Count Colony Forming Units/ml	ATP (fg/ml)				
	0 to 1.0×10^3	1.1×10^3 to 1.0×10^4	1.1×10^4 to 1.0×10^5	1.1×10^5 to 10^{10}	Total
0 - 1.0×10^3	(45)24.6%	(37)20.2%	(8)4.4%	(7) 3.8%	(97)53 %
1.1×10^3 - 1.0×10^4	(12) 6.6%	(19)10.4%	(0)0 %	(2) 1.1%	(33)18 %
1.1×10^4 - 1.0×10^5	(0) 0 %	(9) 4.9%	(9)4.9%	(3) 1.6%	(21)11.5%
1.1×10^5 - 10^{10}	(0) 0 %	(0) 0 %	(6)3.3%	(26)14.2%	(32)17.5%

ANAEROBIC COMPARISON

During the early trials of the ATP assay procedure it became apparent that roughly 10 percent of the assayed urines were negative by streak plate culture but positive by the ATP assay. However, in most of these cases microscopic counts showed better agreement with the ATP assay results. This can

be observed in the results shown in table 8. Thirty-three urine specimens were assayed for bacteria using the nonconcentrated ATP assay, 19 were strongly positive, and 14 were negative. The 19 positive specimens and 2 of the ATP negatives were cultured aerobically and anaerobically and counted microscopically. Five of the specimens were also tested for metabolic activity using BacTec (1972). The specimens were obtained directly from the patient clinics and anaerobic cultures immediately begun by Drs. Moore and Holdeman from the Virginia Polytechnical Institute's Anaerobe Laboratory. In many cases the anaerobic count was appreciably higher than the aerobic count and two cases which appeared negative via aerobic culture had at least 100,000 bacteria/ml when cultured anaerobically. Thus these results show that there are substantial numbers of organisms in urine that culture anaerobically and that in some cases these organisms do not culture aerobically, and the ATP assay does detect them.

Once it is realized that there can be substantial quantities of anaerobic bacteria in urine, the next problem is to determine the clinical significance of anaerobic bacteria in urine. Urine specimens were collected from 40 persons with no indication of urinary infection (midstream collection from 20 males and catheter collection from 20 females). Bacterial cell number estimates were made by aerobic and anaerobic cultures, microscopic counting, and an ATP assay. In every case but one, all the methods indicated that less than 1000 bacteria/ml were present. The one positive specimen was from a female and measured 1.8×10^8 cells/ml by anaerobic culture, 9.3×10^7 cells/ml by aerobic culture, and 4.8×10^8 cells/ml by ATP assay.

Thus, these two tests have indicated that there can be substantial amounts of anaerobic bacteria in urine with or without there being high numbers of aerobically culturable bacteria, and that anaerobic bacteria are not normally present in urine specimens from asymptomatic people. These are very preliminary results from a small number of samples and as such they are far from conclusive. However, the potential implication of the existence of fastidious anaerobic bacteria in some cases of urinary tract infection is substantial. It would be interesting to also determine anaerobic culture reports in a study such as that of Angell, Relman, and Robbins (1968).

This also indicates good agreement when used as a screening test on the general population, which is not expected to show many cases of positive urine culture.

SUMMARY

Efforts to develop a fast automatable system to detect the presence of bacteria in biological fluids, especially urine, have resulted in the optimization of procedures for use with different types of samples. These procedures have been validated by appropriate challenge systems in the laboratory and by the use of clinical specimens.

Table 8

Bacterial Cell Number Estimates by Various Methods

Number	ATP	Microscopic	Anaerobic	Aerobic	BacTec
1	2×10^6	2.4×10^4	2×10^4	1×10^5	ND*
2	4×10^5	1×10^5	4×10^3	2.3×10^5	ND*
3	1.7×10^6	1×10^5	1×10^2	1×10^2	Low+
4	5.7×10^8	2×10^7	6×10^7	Neg	Neg
5	5.6×10^5	2×10^4	1×10^2	Neg	ND*
6	5.7×10^7	6×10^7	1×10^8	4.4×10^7	High+
7	Neg	4×10^4	6×10^2	Neg	ND*
8	1.6×10^5	2×10^4	1×10^2	1×10^2	ND*
9	2.6×10^6	2×10^4	1×10^2	1×10^3	Neg
10	6×10^5	8×10^5	2×10^6	7×10^3	ND*
11	2×10^5	4×10^4	1×10^2	Neg	ND*
12	Neg	4×10^4	2×10^5	3×10^3	ND*
13	3.4×10^6	1×10^7	2×10^6	6.5×10^3	V.Low+
14	1.3×10^5	1×10^5	1×10^4	5×10^3	ND*
15	1×10^5	4×10^3	1×10^5	Neg	ND*
16	4×10^7	2×10^8	4×10^8	10^8	ND*
17	6×10^6	3×10^6	4×10^5	1.3×10^4	ND*
18	3×10^7	2×10^7	5×10^6	3.4×10^6	ND*
19	1.8×10^6	2×10^4	1×10^2	Neg	ND*
20	1.3×10^6	2×10^6	1×10^6	8×10^6	ND*
21	9×10^5	4×10^4	4×10^2	Neg	ND*
22†					

* ND = Not Done

† No. 22 through No. 33 were negative by all methods.

Improvements in reproducibility and accuracy by reducing interfering substances have resulted, as well as increase in sensitivity by decreasing the background and concentrating the sample.

The procedure for removing up to 10^6 leucocytes/ml of urine and measuring the bacterial ATP from as few as 1000 urinary pathogens/ml of urine when starting with a 10-ml sample and concentrating by centrifugation has been developed and used on a small number of clinical specimens. Correlation with culture procedures shows that the luciferase assay can be used to quantitate bacteria from urine cultures with low percentages of false positives and false negatives.

Further efforts will include evaluation with several thousand clinical specimens and correlation with additional types of measurements, such as miniculture methods, metabolic measurements, and anaerobic culture.

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