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Evalution of an Adenosine 5'-Triphosphate Assay as a Screening Method to Detect Significant Bacteriuria

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The bioluminescent reaction of adenosine 5'-triphosphate (ATP) with luciferin and luciferase has been used in conjunction with a sensitive photometer (Lab-Line's ATP photometer) to detect significant bacteriuria in urine. This rapid method of screening urine specimens for bacteriuria was evaluated by using 348 urine specimens submitted to the clinical microbiology laboratory at the University of Minnesota Hospitals for routine culture using the calibrated loop-streak plate method. There was 89.4% agreement between the culture method and the ATP assay, with 7.0% false positive and 27.0% false negative results from the ATP assay using 10^5 organisms/ml of urine or greater as positive for significant bacteriuria and less than 10^5 organisms/ml as negative for significant bacteriuria.

Quantitative urine cultures are routinely performed on all urine specimens submitted for culture to distinguish between urines with significant bacteriuria (100,000 bacteria/ml or greater) (8, 10, 12) and urines that are sterile or have lower counts. Culture methods require 18 to 24 h for incubation before the number of organisms can be assessed. Immediate approximation of the number of organisms can be obtained by Gram staining freshly voided urine or by using phase-contrast microscopy (3, 19), but the evaluation of negative specimens by these methods is time consuming. Since more than 60% of the urines received for culture at the University of Minnesota Hospitals do not contain significant numbers of organisms, it seemed important to evaluate a rapid and immediate means of determining significant bacteriuria: the enzymatic bioluminescent reaction of bacterial adenosine 5'-triphosophate (ATP) with luciferin-luciferase.

This assay was originally developed by the National Aeronautics and Space Administration for quickly determining the purity of drinking water supplies during long space flights (11), but can be made quantitative for bacterial ATP in the urine by using three basic preparatory steps. (i) Nonbacterial ATP must be released and destroyed. (ii) The chemical that destroys the ATP in step (i) must be removed prior to releasing the bacterial ATP. And (iii) the bacterial ATP must be released into its free soluble state ready to react with luciferin and luciferase to produce light and other products (5). The purpose of this paper is to report the results of our evaluation of the ATP assay method for determining significant bacteriuria.

MATERIALS AND METHODS

Reagents. Octyl phenoxy polyethoxyethanol was obtained from Rohm and Haas Corp. and is a nonionic detergent used for the selective lysing of the erythrocytes, leukocytes, and epithelial cells found in urine. Dimethyl sulfoxide (Me_2SO) of reagent grade quality was obtained from the J. T. Baker Chemical Co. Trizma-7.7, a tris(hydroxymethyl)-aminomethane and hydrochloride buffer of reagent grade, was obtained from the Sigma Chemical Co. (no. T-4378) and diluted to a concentration of 0.05 M, giving a pH of 7.7 at 25 C. Potato apyrase, an ATPase, was also obtained from Sigma Chemical Co. (grade I, A6132).

Firefly lantern extract. Firefly lantern extract (FLE) was purchased from Worthington Biochemical Corp., in Freehold, N.J. Each vial of this lyophilized water extract of 50 mg of firefly lanterns was reconstituted with 5.0 ml of sterile distilled water, resulting in a solution containing luciferin, luciferase, 0.05 M $KAsO_4$, and 0.02 M $MgSO_4$, at pH 7.4. Each vial was mixed thoroughly in a Vortex mixer, and, depending on the number of samples to run, either two or three vials were reconstituted, vortexed, mixed together, and vortexed again, resulting in a homogenous FLE solution (containing both the enzyme and the substrate) for all the samples to be analyzed in that run. The FLE was stored in a dark container at −20 C until it was reconstituted. When reconstituted, it was stored either on ice or in a 4 C refrigerator and kept away from bright light.

ATP standards. ATP was obtained from Sigma Chemical Co. (stock no. FF-ATP) as a disodium salt.

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Each vial contained 1 mg of ATP, disodium, and 40 mg of magnesium sulfate and was stored at −20 C away from the light. It was reconstituted by adding 10 ml of sterile trizma buffer to each vial, mixing, and then in a sterile manner dispensing the 0.1-mg/ml solution in 1-ml aliquots into 10 separate vials, which were stored at −20 C.

Equipment and supplies. The Lab-Line ATP photometer, no. 9140, was obtained from Scientific Products, Inc., on a rental basis. Disposable liquid scintillation vials were from Wheaton Laboratory Products and were the Vitro "180" brand. Biopette, a semiautomatic pipette from the Schwarz/Mann Co., was used for dispensing 1-ml aliquots of urine. The reusable tips were autoclaved in a physiological (0.9 N NaCl) saline solution before use. Membrane filters (Millipore Corp.) of 25 mm in diameter and 0.45-μm in pore size were used. The 100-μl Eppendorf pipette was used, and the tips were sterilized between uses.

Standard curve using ATP standard. Standard ATP calibration curves were prepared twice daily with serial dilutions of ATP prepared immediately before use from a stock 0.1-mg/ml solution. The FLE used in the assay was reconstituted 1 to 2 h prior to the assay time and was dispensed into the scintillation vials 5 to 20 min before assay. It was found that the enzyme solution achieved more stability, evidenced by the smoother, more reproducible standard curves, if reconstituted more than an hour before assay.

The procedure for assay with the ATP photometer was kept uniform for both the test samples and the standard solutions of ATP. Using a 100-μl Eppendorf pipette and sterile tips, 0.1 ml of the test sample or ATP standard was injected into a scintillation vial containing 0.1 ml of FLE and 0.4 ml of trizma buffer. This was swirled and then placed in the photometer, and the light emission value in counts per minute was recorded. This value is the count taken during the minute after an exactly timed 15-s delay from the injection time (the delay being automatically timed by the instrument). Urines assayed by the photometer were interpreted as positive or negative for bacteriuria by comparing the light emission units value with the ATP standard curve. Milligrams of ATP in 100,000 organisms was calculated to be 3×10^{-8} by using the value 3×10^{-10} μg of ATP per cell, which is the mean ATP content of a bacterial cell (5).

Standard curve using bacteria. Several strains of *Escherichia coli* and *Klebsiella* species were inoculated into tryptic soy broth (GIBCO) and incubated for 18 to 24 h. Assuming there were approximately 10^9 organisms/ml by the end of the incubation period, the broth cultures were then diluted with sterile water so that the numbers of organisms per milliliter ranged from 10^9 to 10^3. One milliliter of each of these dilutions was extracted with Me_2SO and assayed with the photometer.

Evaluation of enzyme activity. Changes in the enzyme activity of the FLE through time, and variations from one batch of FLE to another, have been encountered by many researchers (1, 4, 6, 13, 14, 16). To offset the deleterious effects of the decreasing activity of the FLE with time, one standard curve was prepared prior to the processing of a group of test samples, and another standard curve was prepared after completing the assay of the test samples. The test samples, however, were assayed when the enzyme activity was intermediate to these extremes. Therefore, the difference between the "before" and "after" standard curves was divided into several sections, and several "new" standard curves were drawn. The test sample could then be assigned to its appropriate standard curve by knowing the time when the test sample was assayed. Conversion from a light emission value of the test sample to a reading in organisms per milliliter was then accomplished from the standard curve. Since the difference between the standard curves prepared with the same enzyme solution at two times, 3 to 4 h apart, was significant, these measures were necessary. Sectioning the standard curve was done under the assumption that enzyme activity decays approximately linearly with respect to time.

ATP assay of urine specimens. Releasing and destroying the nonbacterial ATP was accomplished by adding 0.3 ml of a 2:1 mixture of 0.5% octyl phenoxy polyethoxyethanol and a 10-μg/ml potato apyrase solution to a test tube (13 by 100 mm), to which a 2.0-ml aliquot of the well-mixed urine specimen was then added (using the Biopette). This mixture was allowed to stand 15 to 30 min, during which time the octyl phenoxy polyethoxyethanol selectively lysed any cell that might be in the urine (leukocytes, erythrocytes, or epithelial cells), but not the bacterial cells (15).

Removing the ATPase before releasing the bacterial ATP was done by filtering the urine through a membrane filter (Millipore Corp.). The filter was washed with 2 ml of buffer (trizma) to remove any remaining ATP, ATPase, and other interfering substances, leaving only the bacteria on the filter.

Extracting bacterial ATP with Me_2SO was chosen as the method for releasing bacterial ATP because of its convenience. A 2-ml amount of Me_2SO was placed in a plastic disposable weighing boat to which the filter membrane from the membrane filter was then added. The membrane was removed aseptically from the filter apparatus by using a forceps flamed three times with ethanol. The filter membrane stood 5 to 15 min in the Me_2SO before pouring the Me_2SO-ATP lysate into a sterile test tube and freezing at −20 C until the time of assay with the photometer.

Clinical specimens and reference method. The calibrated 0.001-ml loop method was used as the reference method in evaluating the ATP assay (2–7). A total of 348 urine specimens received at the University of Minnesota Hospitals were tested in the ATP photometer immediately after the clinical microbiological laboratory had cultured them by the calibrated loop method. Care was taken to process the urines as soon as possible, or to refrigerate them until the time when they could be assayed by the photometer. Samples were collected at all times of the day or night, and no special preparation or selection of the urine specimens was made. The organisms were identified according to the procedures used in this clinical microbiological laboratory (2).

RESULTS

Figure 1 is an example of a standard curve made by plotting arbitrary light emission values against concentrations of ATP from 10^{-5} to 10^{-9} mg/ml. This figure illustrates the loss of sensitivity of the bioluminescent reaction in the critical range (equivalent to 10^5 organisms/ml) using the materials and instrumentation described earlier. An emission curve made by extracting the ATP from various dilutions of an overnight broth culture of *E. coli* is shown in Fig. 2. Similar curves were obtained when other strains of *E. coli* and *Klebsiella* species were assayed.

The results of quantitative urine cultures compared with bacterial ATP assays on the same 348 specimens showed a total agreement of 89.4%. Of the 37 urines which were not in agreement, the ATP assay gave 20 false positives and 17 false negatives, or 7.0 and 27.0%, respectively. A total of 63 urines, or 18.1%, were positive for bacteriuria, as determined by the culture method. The sensitivity of the ATP assay was 73.0%, whereas the specificity was 93.0%.

Table 1 presents a more detailed analysis of both the positive urine specimens that were in agreement and of 37 urine specimens that were not in agreement. In all three classes of results, whether false positive, false negative, or positive by both methods, there seems to be a general agreement with the distribution of organism identities given by the average of 5 years of urine cultures at the University of Minnesota Hospitals.

Table 2 indicates the correlation between the culture method and the ATP assay method at

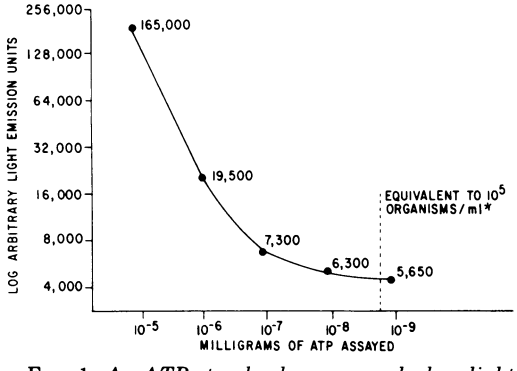

FIG. 1. *An ATP standard curve graphed as light emission units versus concentration of ATP in the standard solution. *The value of 10^5 organisms/ml equals 3×10^{-8} mg of ATP per ml, using 0.1-ml assay sample size.*

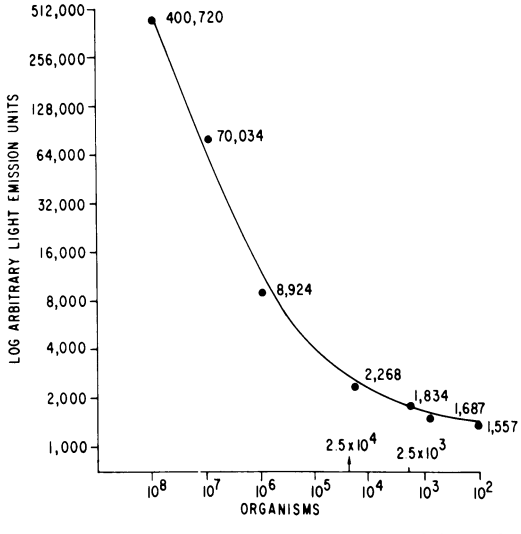

FIG. 2. *A light emission curve obtained from the ATP assay of serially diluted E. coli.*

TABLE 1. *Identification of the organisms in urine samples positive by the culture method*

Predominant species	False negatives		False positives		Positive by both methods	
	No. of samples	%	No. of samples	%	No. of samples	%
Escherichia coli	4	23.5	2	10.0	19	41.3
Klebsiella	3	17.6	1	5.0	5	10.9
Proteus species					1	2.2
Streptococcus	3	17.6	1	5.0	6	13.0
Proteus mirabilis	2	11.8			3	6.5
Pseudomonas aeruginosa					1	2.2
Staphylococcus	2	11.8	3	15.0	4	8.7
Candida albicans			2	10.0	3	6.5
Gram-negative rods	1	5.9	1	5.0	1	2.2
Other gram-negative rods	2	11.8	3	15.0	3	6.5
No growth			7	35.0		

TABLE 2. *Correlation of urine specimens with standard culture methods and ATP assay*

Concn of organisms	No. of samples with growth by culture method	No. of samples positive by ATP assay	No. of samples negative by ATP assay	Agreement (%)
0 to $<10^2$	178	7	171	96.1
10^2 to $<10^3$	4	0	4	100
10^3 to $<10^4$	50	2	48	96
10^4 to $<10^5$	53	11	42	79.2
$\geq 10^5$	63	46	17	73.0

the various levels of organism content. There was 96.1% agreement between the two methods when the number of organisms per milliliter being analyzed was less than 10,000. However, agreement dropped to 73.0% when greater than 10^5 organisms were present, due to the number of false negative results. Over one-half of the false positive results occurred in the 10^4 organisms range.

Figure 3 presents a profile of the 46 urines designated positive by both quantitative culture and by the ATP assay. Over 75% of the urine cultures designated as greater than or equal to 100,000 organisms/ml by the quantitative culture method are quantified as 10^7 organisms/ml or greater by the ATP assay, and only 9% are quantitated as 100,000 organisms/ml by the ATP assay.

Data on the light emission values of the false negatives show that several are borderline (quite near the cut-off point), but most are well within the "negative range." The urine specimens negative by the ATP assay could unfortunately not be quantitated more exactly because of the lack of sensitivity of the standard curve in the concentrations below 10^{-9} mg of ATP per ml.

DISCUSSION

A screening test for significant bacteriuria should have few false positive results (when compared with an accepted culture method) and, ideally, not any false negative results. The cause of the significant number of false negatives given by the ATP assay does not seem to be related to the kind of organisms, since Table 1 shows no striking pattern in terms of the organisms involved.

Patients whose urine demonstrated a false negative result consistently gave results in agreement with the culture method on a later urine sample. Records were also kept of the antibiotics being used by the patients from which the specimens were taken and on the length of time the urine specimens were refrigerated. Neither factor appeared to influence results, with both groups being proportionately represented in the agreeing specimens and in those not in agreement.

In searching for a cause of the false negatives, it can be suggested that all the apyrase solution is not rinsed from the filter membrane by the 2-ml aliquot of buffer. The apyrase might then destroy the ATP released by the bacterial cells, thus giving rise to a false negative value. However, there is evidence that this is not the case. Many urine specimens had bacteria so plentiful that the organisms clogged the filter. On two urines it was possible to filter only a few drops of buffer through; yet these urines gave positive results by the ATP assay. Nevertheless, urines with fewer bacteria might be affected by the remaining apyrase, though this is merely speculation.

Profiles of the false positive results show no striking unifying features either. No one organism group seems responsible for the false positive results. It is significant that 35% of the false positive results are on cultures that are sterile by the quantitative culture method. This indicates that it is unlikely that all the false positives were due simply to quantifying borderline cases (10^4 organisms/ml) as higher, although the latter did contribute at least 50% of the false positives.

Positive results from the ATP assay were converted to the number of organisms per milli-

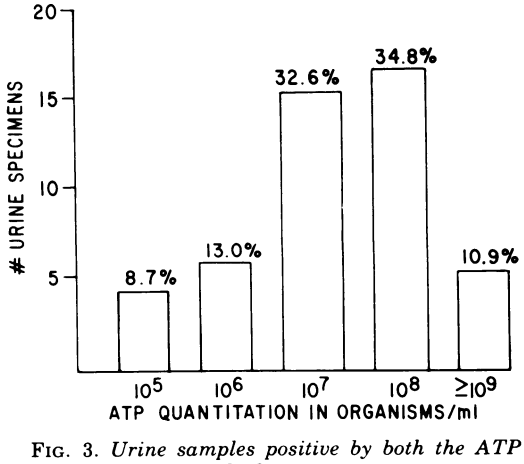

FIG. 3. *Urine samples positive by both the ATP assay and culture method.*

liter by using the ATP standard curve. Results are in agreement with other published data in that generally the ATP assay gave a very high number of organisms per milliliter for the urines with bacteriuria compared with the number given by the culture method. The concentrations given by the ATP assay were primarily in the range of 10^7 to 10^9 organisms/ml. Chappelle and Picciolo obtained 17 positive bacteriuria results from their ATP assay, whereas only eight of these were positive by the pour plate method, and all 17 of the positives by the ATP assay were in the range of 10^7 and 10^9 organisms/ml (5).

Thore et al. (15) have evaluated an ATP bacteriuria assay similar to the one evaluated in this paper. The major difference is that they used boiling TRISS buffer containing ethylenediaminetetraacetate to extract the ATP from bacterial cells, whereas we used Me_2SO. They obtained 4% false negatives and approximately 30% false positives at an ATP concentration limit of 4×10^{-10} M ATP. Our values of 27% false negatives and 7% false positives are not strictly comparable, because we used an ATP concentration limit of 6×10^{-11} M ATP. However, our data agree with that of Thore et al. in that: (i) the number of false positives and false negatives are inversely related; (ii) these proportions are determined by the ATP concentration limit; and (iii) most importantly, one cannot eliminate the false negatives without incurring an unreasonably large number of false positives.

Both limits (4×10^{-10} for Thore et al. [15] and 6×10^{-11} for our studies) correspond to 10^5 organisms/ml. There is a 10-fold difference between their estimation and our estimation as to the ATP content of a single bacterial cell, leading to the differences in the actual ATP limits used in the two studies.

The primary advantage for considering the ATP assay method for screening for bacteriuria is that quantitative results can be obtained immediately. This advantage is offset by two problems with the assay at this state of its development. Firstly, approximately 15 min of a technologist's time is required for processing a specimen, and, secondly, the ATP assay method, even though now commercially available, does not appear to be sufficiently accurate for clinical use since false negative results can be obtained. Even when the sensitivity of the procedure is improved and the possibility of false negative results are eliminated, the procedure will be costly to consider for routine use because of the initial cost of the instrument, the cost of materials per test, and the amount of technologist time required for processing. This latter problem might be resolved if the procedure were automated.

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LITERATURE CITED

1. Aledorf, L. M., R. I. Weed, and S. B. Troup. 1966. Ionic effects on firefly bioluminescence assay of red blood cell ATP. Anal. Biochem. 17:268–277.
2. Blazevic, D. J. 1974. Laboratory procedures in diagnostic microbiology. p. 44–46. Telstar Productions, Inc., St. Paul, Minn.
3. Brody, L., M. C. Webster, and R. M. Kark. 1968. Identification of elements of urinary sediment with phase-contrast microscopy. J. Am. Med. Assoc. 206:177.
4. Chappelle, E. W., and G. V. Levin. 1968. Use of the firefly bioluminescent reaction for rapid detection and counting of bacteria. Biochem. Med. 2:41–52.
5. Chappelle, E. W., and G. W. Picciolo. 1971. Bacterial adenosine triphosphate (ATP) as a measure of urinary tract infection. NASA publ. TSP1-10051. Technology Utilization Office, Goddard Space Flight Center. National Aeronautics and Space Administration, Greenbelt, Md.
6. Johnson, R. A., J. G. Hardman, A. E. Boadus, and E. W. Sutherland. 1970. Analysis of adenosine 3′,5′-monophosphate with luciferase luminescence. Anal. Biochem. 35:91–97.
7. Kass, E. H. 1956. Asymptomatic infections of the urinary tract. Trans. Assoc. Am. Physicians 69:56–63.
8. Kass, E. H. 1957. Bacteriuria and the diagnosis of infections of the urinary tract. Arch. Intern. Med. 100:709–714.
9. Kunin, C. M. 1961. The quantitative significance of bacteria visualized in the unstained urinary sediment. N. Engl. J. Med. 265:589–590.
10. Kunin, C. M. 1972. Detection, prevention, and management of urinary tract infections, p. 1–99. Lea and Febiger, Philadelphia.
11. Levin, G. V., E. Usdin, and A. R. Slonim. 1968. Rapid detection of microorganisms in aerospace water systems. Aerosp. Med. 39:14.
12. Merritt, D. A., and J. P. Sanford. 1958. Sterile-voided urine culture. J. Lab. Clin. Med. 52:473.
13. Rasmussen, H., and R. Nielsen. 1968. An improved analysis of adenosine triphosphate by the luciferase method. Acta Chem. Scand. 22:1745–1756.
14. Stanley, P. E., and S. G. Williams. 1969. Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. Anal. Biochem. 29:381–392.
15. Thore, A., S. Ånséhn, A. Lundin, and S. Bergman. 1975. Detection of bacteruria by luciferase assay of adenosine triphosphate. J. Clin. Microbiol. 1:1–8.
16. VanDyke, K., R. Stitzel, T. McClellan, and C. Szustkiewicz. 1969. An automated procedure for the sensitive and specific determination of ATP. Clin. Chem. 15:3–14.