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

Valerie N. Bush
Department of Biology
Delaware State College
Dover, Delaware

January 1973

This paper summarizes approximately three-fourths of the work done for Nasa Research Grant #08-002-003 held for the period January 15, 1972 to November 15, 1972.

INTRODUCTION

Urinary tract infections are among the most frequent bacterial infections of man in the United States (Hoeprich, 1960). At the present time, the calibrated loop method is used in most laboratories for detecting bacteria in urine. According to Hoeprich (1960) fewer than 10,000 viable bacteria per ml indicates that urinary tract infection is unlikely. Ten thousand to 100,000 viable bacteria per ml indicates urinary tract infection.

Picciolo et al. (1971) estimated bacterial numbers by measuring the adenosine triphosphate (ATP) concentration in a urine sample after removing nonbacterial ATP. Measurement of ATP concentration is based on the bioluminescent reaction of luciferase when mixed with ATP. The mechanisms involved have been discussed by McElroy et al. (1969).

The reactions depend on the concentrations of reduced luciferin, luciferase and ATP. If luciferin and luciferase are present in excess, the reaction is directly dependent on the amount of ATP. The intensity of the initial burst of light is directly propotional to the concentration of ATP when luciferin is in excess, provided other conditions for the reaction are optimal (Chappelle and Levin, 1968).

Picciolo et al. (1971) obtained higher bacterial titers by the ATP assay than by the pour plate or culture colony methods. This discrepancy might be explained by one or a combination of the following possibilities: (1) the amount of ATP per bacterial cell increases after growth or suspension in urine for long periods of time, (2) the ATP assay detects dead, contaminating, or pathogenic bacteria which are not detected by routine laboratory methods, and (3) one or more types of mammalian cells remain after they are

supposedly ruptured and their ATP removed. These cells are ruptured with the bacterial cells and contribute ATP not of bacterial origin.

ATP from epithelial cells, platelets, leukocytes, erythrocytes, sperm cells as well as contaminating fungal and Streptomyces species that may be present in urine might influence results of bacterial assays by the ATP method. This is a report of a study of the effectiveness of the rupturing agents on monkey kidney cells, Candida albicans, a Rhodotorula species and a Streptomyces species in determining whether these cells could contribute ATP to the bacterial ATP value of a urine sample.

MATERIALS AND METHODS

Organisms Used. Candida albicans isolated from a urine sample at the Kent General Hospital, Dover, Delaware, was grown in Malt Extract Broth (Difco) for 24 hours at 37 C. After incubation, the cells were centrifuged (Size 1 Type C International Centrifuge with 240 Head, International Equipment Co., Boston, Mass.) for 15 minutes. The sedimented cells were resuspended in Malt Extract Broth. Viable cells were enumerated by the pour plate method using Malt Extract Agar plates and by direct counts in a Spencer AO Bright Line Improved Neubauer Hemocytometer.

The Rhodotorula and Streptomyces species were provided by Dr. Grace Picciolo of the Goddard Space Flight Center, Greenbelt, Md. Rhodotorula was grown in Sabouraud Dextrose Broth (Difco) at 37 C for 48 hours and then centrifuged for 15 minutes. The sedimented cells were resuspended in saline. Cells were enumerated by direct counts in a hemocytometer. Streptomyces was grown in Trypticase Soy Broth (Baltimore Biological Laboratories) at 37 C for 24 hours, then centrifuged for 15 minutes. The sedimented cells were re suspended in saline.

Kidney cells from a Rhesus monkey were obtained from Flow Laboratories, Rockville, Maryland the day after they were suspended in growth medium containing 5% calf serum penicillin and streptomycin. The cells were held at 5 C for another 24 hours before they were used. Viable cells numbered approximately 10^6 per ml (67 - 83% of the total cell number) as determined by trypan blue staining. The stock cell suspension was diluted in Hanks' BSS containing phenol red (Flow Laboratories). Sterility tests of cell suspensions were negative.

Reagents Used in Luciferase Assay for ATP. Partially purified potato apyrase - Grade I (Sigma Chemical Co., St. Louis, Missouri) at a concentration of 73 mg per ml of 0.55M $CaCl_2$ was prepared fresh each day. One tenth ml apyrase hydrolyzed one ml ATP (0.1 mg/ml) in 15 minutes by the firefly luciferase reaction.

Triton X-100 (Sigma) the registered trademark of the Rohn and Hass corporation's brand of octyl phenoxy polyethoxyethanol was used. Perchloric acid, 70% Reagent Grade (Allied Chemical Co., Morristown, N.J.) was used to make the various perchloric acid concentrations. The concentrations of perchloric acid and Triton X 100 were stored at -18 C until ready for use.

A stock solution of disodium salt of ATP (Sigma) was prepared in deionized distilled water to 1 mg/ml and stored in 1 ml aliquots at -18 C. Immediately before use, ATP was thawed and diluted to the desired concentration. ATP was discarded after sitting at room temperature for two hours.

Luciferin - luciferase (E.I. Dupont Nemours and Co., Wilmington, Del.) packaged in vials containing approximately 220 mg. was used. Three ml of diluent (0.1M Tris, 0.01M $MgSO_4$) was added to each vial.

Firefly Luciferase Assay for ATP. The light measuring instrumentation used in these experiments consisted of a Chem Glow Photometer (Aminco, Silver Spring, Md.) attached to an X Y Recorder (Hewlett Packard Model #7035B). One tenth ml of the treated sample to be assayed for ATP content was injected by needle and syringe into 0.1 ml of the luciferin-luciferase mixture. The difference in light intensity of the test sample and the control sample was compared to the light intensity of known ATP standard diluted in the fluid used for the control in determining the amount of ATP in each sample. There was a direct relationship between ATP concentration and maximum light intensity in the range of ATP content in the samples used.

Procedure for Treatment of Urine Samples. One milliliter of sample, 0.1 ml Triton X-100, and 0.1 ml apyrase were mixed and allowed to stand for 15 min. Then 0.2 ml of 0.5N perchloric acid was added, mixed and allowed to stand for 5 minutes. Finally, 8.6 ml deionized, distilled water was added and mixed. The amount of ATP per sample was determined as described above.

Procedure for Treatment with Triton X-100 to Rupture the Cells amd Release Their ATP. One milliliter of sample and 0.1 ml of 2% Triton X 100 were mixed and allowed to stand for varying lengths of time. One tenth milliliter of this sample was then injected into the luciferin-luciferase as described above. The amount of soluble ATP as determined by injection of an untreated sample into the luciferin-luciferase mixture was subtracted from the amount of ATP after Triton X 100 treatment.

Procedure for Treatment with Perchloric Acid to Rupture the Cells and Release Their ATP. One milliliter of sample and 0.1 ml of apyrase were mixed and allowed to stand 15 minutes. Two tenths

milliliter of varying perchloric acid concentrations was then added and allowed to stand for varying times. This mixture was diluted 1:10 in deionized, distilled water. One tenth milliliter of this diluted treated sample was injected into 0.1 ml luciferin-luciferase in the manner described above.

RESULTS

Monkey Kidney Cell Suspensions. ATP remained after all non-bacterial ATP should have been removed when different numbers of monkey kidney cells in suspension received the same treatment as the urine sample (Fig. 1).

A comparison of ATP released after (1) normal treatment of urine samples and (2) perchloric acid treatment (0.5N) for 5 minutes is shown in Table I. The percentage of ATP not released by Triton X-100 and subsequently destroyed by apyrase remained constant in the concentrations of monkey cell suspensions tested.

The amount of ATP per monkey kidney cell released with varying perchloric acid concentrations and different periods of treatment is shown in Table II. The 0.5N concentration of perchloric acid treatment did not release the maximal amount of ATP from monkey kidney cells.

Lysis of cells and a rise in ATP was observed within 1-3 minutes when 2% Triton X-100 was added to the kidney cell suspension. Immediately following this, the ATP concentration dropped due to the release from the cells of intracellular ATPases. At the end of the 15 minute treatment with Triton X-100, 0.00035 µg ATP per ml could be detected. This was in contrast to 0.8694 µg ATP per ml released by Triton X-100 (extrapolated from Table I) and subsequently hydrolyzed by the added apyrase and intracellular ATPases.

FIGURE 1. Amount of ATP in monkey kidney cell suspensions released after the normal procedure used to treat urine samples.

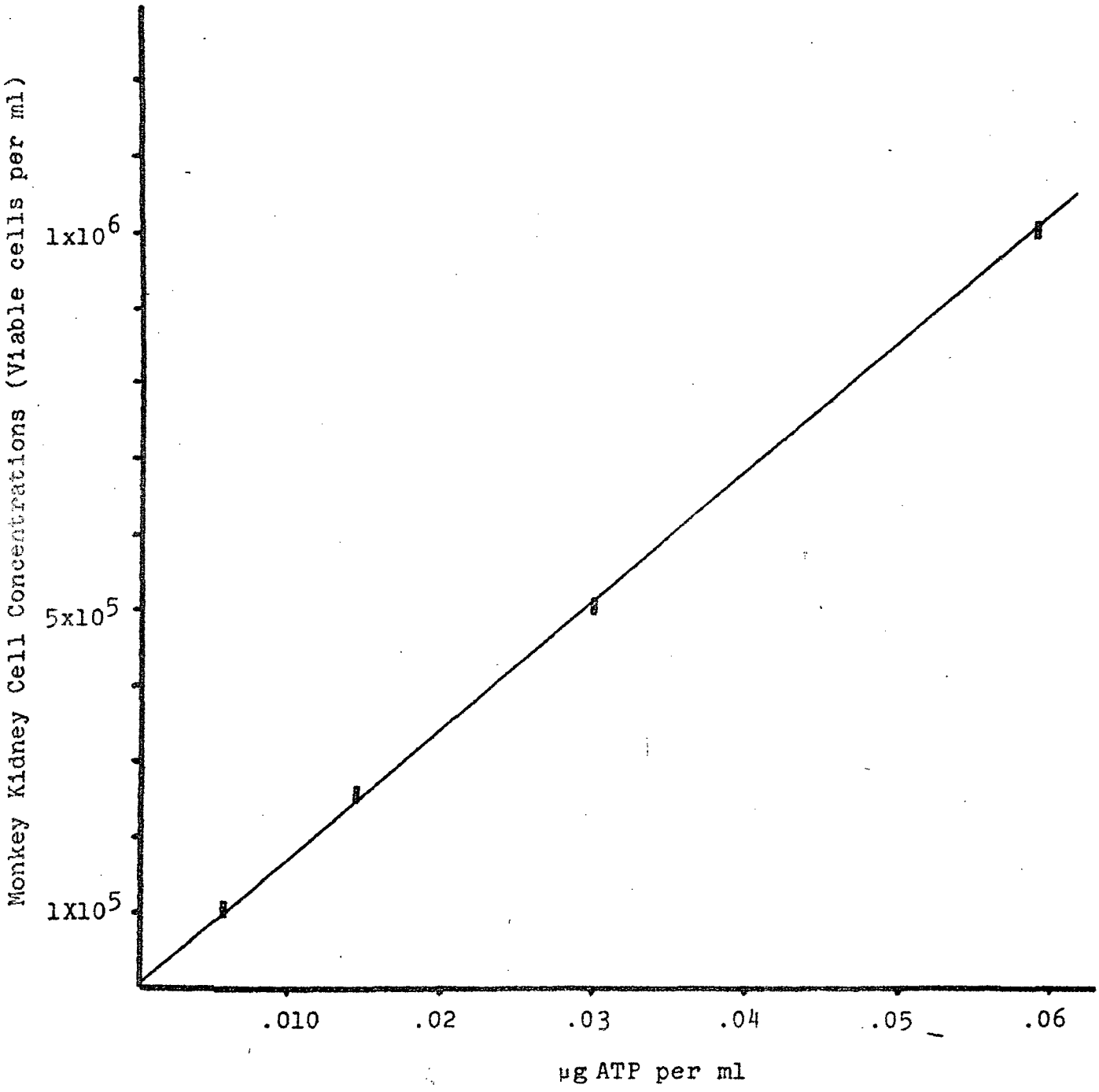

TABLE I

TABLE I. Comparison of ATP released from Monkey Kidney Cell Suspensions after various treatments

Concentration of Viable Monkey Kidney Cells (cells / ml)	Amount of ATP released after 0.5M perchloric acid treatment for 5 min. (µg ATP per ml)	Amount of ATP released after normal treatment used for urine samples (µg ATP per ml)	% not released by 2% Triton X-100 and subsequently destroyed by apyrase before the perchloric acid treatment
1×10^6	.9418	.0716	7.6
5×10^5	.3645	.0331	9.1
2.5×10^5	.2059	.0137	6.7
1.0×10^5	.0341	.0063	8.1

TABLE II: Amount of ATP per cell released from monkey kidney cells following different perchloric acid treatments

Concentration of Perchloric acid	Time of treatment with perchloric acid (min)	µg ATP per cell x 10^{-7}
0.5 N	5	8.34
0.5 N	10	10.8
0.5 N	20	10.2
0.5 N	30	12.5
2.0 N	5	13.7
2.0 N	30	22.7

Streptomyces. These fungal-like bacterial cells are very difficult to mix and quantitate due to their filamentous nature. The amounts of ATP released from various concentrations of Streptomyces after various treatments are presented (Table III). The type of treatment releasing the maximum ATP varied with the cell concentration.

In other studies with Streptomyces 1.3% of the total amount of ATP released by 0.5N perchloric acid treatment for 20 minutes was released by the 2% Triton X-100 treatment for 8 minutes.

Fungal Cells. Amounts of ATP per cell released after various treatments of C. albicans and Rhodotorula species are presented in Table IV. Cell titers used in calculations for this table for C. albicans were determined by the pour plate method and ranged from 6×10^4 to 2×10^7 colonies per milliliter. Very good correlation was obtained between the titers as determined by the direct microscopic counts and pour plate counts; however, only the titers obtained by pour plating are shown. C. albicans was actively budding with an average of one bud for every two cells. Rhodotorula species was also budding with an average of one bud for every 2.8 cells.

DISCUSSION

In the automated treatment of urine samples proposed by Picciolo et al. (1971) chemical treatment to (1) rupture mammalian cells and hydrolyze their ATP and (2) rupture bacterial cells so their ATP content can be measured is preferred over physical treatment. Perchloric acid was a very effective lysing agent for all bacterial cells tested. A critical step in this treatment for urine is the method used to rupture mammalian cells. The agent used must effectively lyse the mammalian cells without affecting the bacterial cells to any appreciable extent.

TABLE III: The amount of ATP (μg ATP per ml) released by different perchloric acid treatments on varying concentrations of Streptomyces suspensions

Perchloric acid concentrations and time of treatment	100%	50%	10%	5%
0.5 N PCA 5 min	1.36	.884	.191	.0219
0.5 N PCA 10 min.	1.89	1.07	.185	.0892
0.5 N PCA 20 min	1.91	1.13	.205	.222
2 N PCA 5 min	1.45	1.03	.277	.112
2 N PCA 10 min	.594	.554	.151	.107
2 N PCA 20 min	1.81	1.71	.356	.226
5 N PCA 5 min	1.58	1.32	.179	.080
5 N PCA 10 min	1.47	.919	.246	.0829
5 N PCA 20 min	1.44	.739	.215	.106

TABLE IV: Amount of ATP (μg per cell) obtained after various treatments of fungal cells

Fungal Cell Type	5N Perchloric Acid treatment for 5 min	0.5N Perchloric Acid treatment for 5 minutes	2% Triton X-100 Treatment for 8 minutes
C. albicans	8.45×10^{-8}	8.70×10^{-9}	1.37×10^{-9}
Rhodotorula sp.	8.93×10^{-8}	2.14×10^{-9}	6.41×10^{-10}

Triton X-100 failed to completely lyse the monkey kidney cells in the suspensions used; the remaining 6.7 - 9.1% of the ATP in the unlysed cells was released by further treatment with perchloric acid. The failure to completely lyse these cells was not due to a saturation effect since the concentration of cells varied while the apyrase perchloric acid, and Triton X-100 concentrations remained constant. No analysis of the contribution of a specific component of the kidney cell suspension to the remaining ATP was made.

The amount of ATP in the Kidney cell suspension which remained after the procedure used for urine samples will only affect the number of bacteria per milliliter of urine sample if (1) large numbers (10^3 cells/ml) of cells similar to those found in the monkey kidney cell suspension are present in the urine sample or (2) if small numbers of these cells as well as contaminating bacteria are present in the urine sample. If large numbers of kidney cells are present in urine, there probably is extensive destruction of the kidney which may be caused by bacterial action. The added ATP from unlysed kidney cells will be found in addition to the ATP from bacteria in the urine. If there is extensive kidney destruction not associated with any bacterial infection, the high ATP content determined for this urine specimen would be false positive.

Trypan blue staining was the only check made of the physiological state of the monkey kidney cells. The role of the nonviable cells in the suspensions was not investigated. It would be of interest to compare results of cells suspended in urine and allowed to remain for periods of time up to 48 hours with the results obtained in this study.

Few conclusions can be made from the studies with Streptomyces due to the filamentous nature of these cells. Triton X-100 was not

very effective in rupturing these cells; 0.5N perchloric acid for 5 minutes was reasonably effective. This urinary contaminant commonly found in the soil would be present in quantities of less than 10,000 colonies per ml. The exact contribution of this contaminant to the present treatment of urine can not be determined adequately.

C. albicans lives and multiplies on the skin and may persist there for some time. It may be found as a urinary contaminant with less than 10,000 colonies per ml. Candida infections are usually secondary infections found in patients under therapy with broad spectrum antibiotics because, without competition, C. albicans multiplies rapidly. If C. albicans is pathogenic, more than 10,000 colonies per milliliter is found.

The 5N perchloric acid treatment is approximately ten times more effective in rupturing C. albicans and releasing the ATP from these cells than the 0.5N perchloric acid treatment. The values from the treatments with Triton X-100 and 0.5N perchloric acid could be used to determine the numbers of bacteria in urine that would be reported if C. albicans were present in varying concentrations.

If one assumes that there are 8,000 colonies per ml of C. albicans in urine (contaminant) the use of 2% Triton X-100 for 8 minutes and 0.5N perchloric acid for 5 minutes would release approximately 5.8×10^5 µg ATP per ml. If one converted this into bacteria per ml, the ATP in these contaminating C. albicans would be about 1.2×10^5 which would indicate a urinary infection. If C. albicans is present as a pathogen and 5×10^5 colonies per ml are present, this would be reported as 7.3×10^6 bacteria per ml in the urine sample which would be considered a urinary infection.

Cryptococcus and Torulopsis, other urinary pathogens, might resemble C. albicans in the amount of ATP per cell and susceptibility to the various rupturing agents since they are all in the same subfamily. Rhodotorula is a rare urinary contaminant. Since Rhodotorula and Candida are in the same family (Cryptococcaceae) the similarity of results using the rupturing agents is not surprising. If Rhodotorula were found at a concentration of 8,000 colonies per ml and the 2% Triton X-100 and 0.5N perchloric acid were used to treat the urine sample, 2.4×10^4 bacteria per ml would be reported. This value would indicate a probable urinary tract infection. This organism with a slightly lower sensitivity to 0.5N perchloric acid would probably not cause a false positive unless both bacteria and Rhodotorula were present.

Levin et al. (1968) reported an average of 1.3×10^{-7} µg ATP per Saccharomyces cerevisiae cell when the cell number was determined by plate counts and 2.8×10^{-8} g ATP per cell when cell number was determined by chamber count. These values are somewhat higher than the values obtained for C. albicans and Rhodotorula reported here. Levin et al. did not give the method used to rupture their yeast cells.

The results of this study show that both fungal cells and kidney cells can contribute ATP to the bacterial ATP value of a urine sample. Examination of clinical specimens in which discrepancies in cell titer between the ATP assay and pour plate or culture colony methods occur will be necessary before the percentage of discrepancies due to fungal and kidney cells can be made.

LITERATURE CITED

Chappelle, E. W. and G. V. Levin. 1968. The Use of the Firefly Bioluminescent Assay for the Rapid Detection and Counting of Bacteria. Biochem. Med. 2: 41-52.

Hoeprich, P.D. 1960. Culture of the Urine. J. Lab. Clin. Med. 56: 899-907.

Levin, G. V., E. Usdin, A. R. Slonim. 1968. Rapid Detection of Microorganisms in Aerospace Water Systems. Aerospace Med. 39: 14-16.

McElroy, W. D., H. H. Seliger and E. H. White. 1969. Mechanism of Bioluminescence, Chemiluminescence and Enzyme Function in the Oxidation of Firefly Luciferin. Photochem. Photobiol. 10: 153-170.

Picciolo, G. L., B. N. Kelbaugh, E. W. Chappelle and A. J. Fleig. 1971. An Automated Luciferase Assay of Bacteria in Urine. GSFC X641-71-163.