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Detection of Bacteriuria by Luciferase Assay of Adenosine Triphosphate

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A selective method for distinguishing bacterial and nonbacterial adenosine triphosphate (ATP) in clinical bacteriological specimens was studied. The method involved incubation of samples with the detergent Triton X-100 and the ATP-hydrolyzing enzyme apyrase. The incubation selectively destroyed ATP in suspensions of various human cells while not affecting the ATP content in microbial cells. ATP remaining in the sample after incubation was extracted in boiling buffer and assayed by the firefly luciferase assay. Application of the method to 469 clinical urine specimens showed that the ATP level after treatment with Triton/apyrase was correlated to bacterial counts and that the sensitivity of the assay was sufficient for the detection of 10^5 bacteria/ml. The ATP levels per bacterial cell remaining in the urine specimens after treatment with Triton/apyrase were close to values observed in laboratory-grown cultures. The specificity and sensitivity of the luciferase assay for the detection of urinary bacteria and its possible use as a bacteriuria screening method are discussed.

Recently the assay of adenosine triphosphate (ATP) with the firefly luciferin/luciferase system has been discussed in connection with microbiological work. The most frequently proposed application has been the use of ATP assays as a means of quantitation of microbial cells in various samples (4, 10-14, 18; G. L. Picciolo and E. W. Chappelle, personal communication). Numerous studies have been made to determine cellular levels of ATP in both bacteria and other microorganisms (1, 3-5, 7, 9, 10, 12, 15), demonstrating that variations in cellular ATP content rarely exceed one order of magnitude (1, 6, 10, 12). Thus, it would seem that, theoretically, the accuracy of the assay is adequate for many microbiological applications.

The luciferase assay has been shown to have a sensitivity allowing the detection of approximately 10^{-14} M ATP, corresponding to the ATP content of fewer than 10 bacteria/ml (1, 10). This extreme sensitivity, combined with the simple and rapid assay procedure, would make the luciferase assay an attractive alternative to existing methods for quantitation of bacteria.

A major problem in the application of ATP assays to quantitative bacteriology has been the frequent occurrence of large amounts of nonbacterial ATP emanating from cellular material present in many types of bacteriological specimens. If nonbacterial ATP cannot be removed prior to the assay, erroneous results will be obtained (13, 14). The present study was performed to investigate the occurrence of nonbacterial ATP in urine and possible ways of its elimination. It has been suggested that the action of the detergent Triton X-100, selectively lyzing nonbacterial cells, combined with the ATP-hydrolyzing enzyme apyrase, might be used for this purpose (Picciolo and Chappelle, personal communication). The procedure was worked out in a model system including several types of human cells representative of cells likely to occur in urine specimens. The analytical system arrived at through these studies was applied on urine specimens sent for routine culture to a clinical bacteriological laboratory.

MATERIALS AND METHODS

Analytical equipment. The analytical equipment used in the luciferase assay of ATP was a modification of a device originally described by Chappelle et al. (2). It consisted of a dark chamber into which the sample to be analyzed was introduced, so that it faced a photomultiplier, RCA 931 A, operated at 1,000 V. The light signal obtained upon mixing luciferase reagent and sample was recorded on a potentiometric chart recorder (Electronic 194 Lab/Test Recorder, Honeywell), and the peak light emission was used in the calculations. The total time required for assay and registration of the result was in the order of 15 s per sample.

Analytical reagents. Firefly luciferase was purchased from Sigma Chemical Co., St. Louis, Mo. Of the crude firefly lantern extract, 50 mg was made up in 20 ml of 0.1% bovine serum albumin containing 10 mM $MgSO_4$ and 1 mM ethylenediaminetetraacetate (EDTA), and the pH was adjusted to 7.4. The suspension was centrifuged at 27,000 × *g* for 15 min to remove particulate matter. To reduce background light emission, the solution was allowed to age overnight in a refrigerator before use. The luciferase reagent could be kept at room temperature for several hours without appreciable loss of activity and could be used for 2 to 3 days if kept refrigerated.

Apyrase, grade II (crude), was purchased from Sigma Chemical Co. Stock solutions containing 1% apyrase made up in distilled water were kept frozen until use.

Other chemicals were of analytical grade.

Microorganisms and human cells. In model studies, strains of *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Streptococcus* sp., and *Candida albicans* recovered from clinical urine specimens were used. Cultures were grown in sterile urine at 37 C overnight for use in the experiments. Human blood cells (red blood cells, mononuclear white blood cells [16], and polymorphonuclear granulocytes) and tissue culture cells (HeLa [epitheloid carcinoma] cells and human embryonic lung fibroblasts) were also used in model studies. The tissue culture cells were grown in basal medium (Eagle; Flow Laboratories) supplemented with 10% human and 5% calf serum. The cells were detached with trypsin (crystallinic, 0.025%) and washed twice in phosphate-buffered saline before use.

Clinical specimens. Urine specimens were obtained from 469 patients, including clinic as well as hospitalized patients. Of the 469 specimens, 290 yielded $< 10^4$ microorganisms/ml and were designated as *negative*, 56 showing 10^4 to 10^5 microorganisms/ml were designated as *borderline specimens*, and the remaining 123 specimens with counts of $\geq 10^5$ microorganisms/ml were designated as *positive*. The qualitative yield of these specimens is summarized in Table 1.

Quantitative culture. Quantitative culture was performed on all bacteriological specimens by plating on blood agar plates 0.2 ml of 10-fold serial dilutions in sterile saline. In Fig. 4 and 5, results are also included from experiments in which bacterial counts were only approximately estimated by gross examination of the plate from the first dilution.

Pretreatment of urine specimens and bacterial cultures before assay. When nonbacterial ATP was to be removed, samples were incubated with apyrase in the presence of Triton X-100. The procedure finally adopted was as follows: 1 ml of the bacteriological specimen was made up to contain 0.1% Triton X-100, 2 mM $CaSO_4$, and 0.1% apyrase (0.4 units) in a total volume of 2 ml. The mixture was then incubated for 10 min at 37 C, after which a 1-ml sample was pipetted into 4 ml of boiling 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.75, containing 2 mM EDTA to extract ATP. From non-pretreated samples, 0.5-ml portions were taken directly from the sample and extracted in 4.5 ml of boiling Tris buffer.

TABLE 1. *Qualitative yield of microorganisms in 123 clinical urine specimens with* $\geq 10^5$ *microorganisms/ml*

No. of specimens	Type of microorganism
54	*Escherichia coli*
16	*Proteus* sp.
12	*Streptococcus* sp.
9	*Klebsiella/Enterobacter*
4	*Pseudomonas* sp.
3	*Staphylococcus* sp.
2	*Candida albicans*
14	Mixed infections
9	Unidentified bacteria

After heating for 90 s, the extracts were cooled and kept on ice or frozen until the assay was performed. This extraction procedure gave reproducible results with all bacteria tested. The recovery of ATP was 76 to 99% compared with acid extraction procedures such as $KClO_4$ or trichloroacetic acid extractions. Nevertheless, the Tris-EDTA extraction was preferred because of its great technical advantages.

Assay procedure. The assay was performed on 1-ml portions of the boiling buffer extracts which were transferred to 4-ml polystyrene Ellerman tubes and placed in the dark chamber of the luminometer. Luciferase reagent (0.4 ml) was then injected into the extract by means of a 1-ml syringe driven by a linear motor. Standards with known amounts of ATP and reagent blanks were assayed in each series.

Calculation of assay results. The ATP levels of the urine samples were calculated by using assays of standard amounts of ATP as reference and correcting for reagent background values. The ATP concentration arrived at in this way had to be further corrected for effects of interfering components present in the extracts. In the model experiments with pure bacterial cultures and human cells, a correction factor was obtained by the addition of a known amount of ATP to the extract followed by repeated assay.

In the clinical urine specimens, an average correction factor was determined to be 2.7 ± 1.1 (95% confidence level), corresponding to an inhibition of the analytical reaction of about 60%, and all assay results were corrected by multiplication with this factor. Thus, the ATP concentrations determined for the urine specimens include a statistical error of ±40% (95% confidence level).

RESULTS

In the first series of experiments, the Triton/apyrase treatment was developed and applied to model systems of human and bacterial cells. In Fig. 1, the time course of the effects of the treatment on ATP levels in human red blood cells and *E. coli* is shown. The treatment resulted in the complete elimination of ATP from the red blood cells within 2 min. The rate of ATP breakdown was identical to that ob-

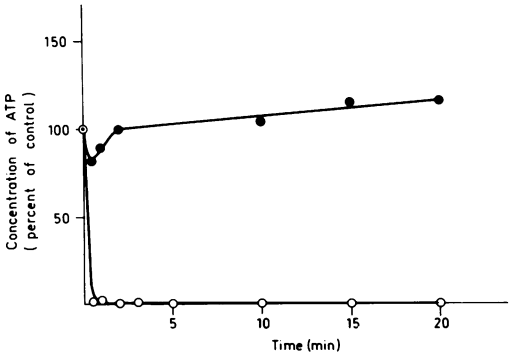

FIG. 1. *Time course of changes of ATP levels during treatment of red blood cells and E. coli with Triton/apyrase. Washed red blood cells* (○), *5 × 10⁶ cells/ml, and E. coli* (●), *10⁹ cells/ml, were incubated in sterile urine (diluted 1:1 in distilled water) containing apyrase (0.2 unit/ml) and Triton X-100 (0.1%) at 37 C. At the times indicated, 1.0-ml portions were extracted and assayed.*

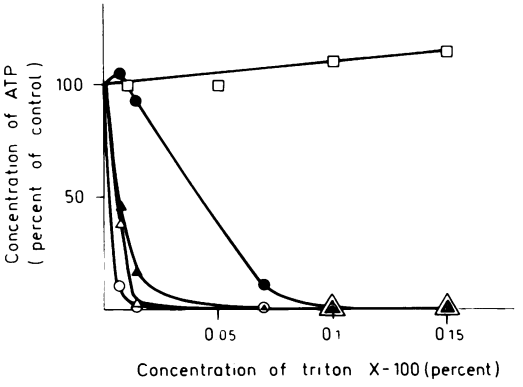

FIG. 2. *Influence on ATP level of concentration of Triton X-100 on treatment of various types of cells with Triton/apyrase. Experimental conditions as in Fig. 1 except that various concentrations of Triton X-100 were used and the incubation was for 10 min. The following numbers of cells/ml were added to the incubation medium: red blood cells, 5 × 10⁶* (○); *mononuclear white blood cells, 10⁵* (●); *HeLa, 10⁵* (△); *human embryonic lung fibroblasts, 3 × 10⁵* (▲); *E. coli, 10⁹* (□).

served with free ATP; i.e., the detergent-induced lysis of red blood cells was not rate limiting. The upper curve in Fig. 1 demonstrates the effect of the same treatment on *E. coli*. With the bacteria, essentially no effect on the ATP level was observed, except for a slight decrease at the start of the incubation, possibly resulting from metabolic disturbance caused by the transfer of the bacteria to the incubation medium. In the following experiments, an incubation time of 10 min was used.

In the experiment shown in Fig. 2, four different types of human cells were treated at various concentrations of Triton X-100 in the presence of a constant amount of apyrase. For comparison, a strain of *E. coli* was included. At 0.1% Triton X-100, the loss of ATP was complete with all types of human cells, whereas *E. coli* was not affected even at higher concentrations. A concentration of 0.1% Triton X-100 was chosen for use in all of the following experiments.

In Table 2, the effect of Triton/apyrase treatment on various cells of human and microbial origin is summarized. The treatment resulted in an almost complete loss of ATP in all human cells, whereas the microbial cells were more or less resistant to the treatment. In the absence of Triton X-100, no effect of the apyrase was observed on either type of cells. Thus, in all of the model experiments, the Triton/apyrase treatment resulted in the desired differential effect. Subsequent experiments were performed to evaluate the effect of Triton/apyrase treatment in a clinical material.

To determine whether nonbacterial ATP in urine was free or intracellular, we treated 26 culture-negative specimens with apyrase in the presence and absence of 0.1% Triton X-100. The results are presented in the histogram of Fig. 3A, depicting percentage ATP remaining after

TABLE 2. *Cellular ATP levels of various microbial and human cells before and after treatment with Triton/apyrase*

Cell	Cellular ATP level[a] (moles × 10^{18}/cell)	
	Before treatment	After treatment
E. coli	1.6	2.1
Proteus mirabilus	0.9	0.9
S. aureus	2.3	1.1
Streptococcus sp.	1.6	0.8
Candida albicans	26	26
Red blood cells	60	0
Mononuclear white blood cells	50	0.5
Polymorphonuclear granulocytes	170	0
HeLa cells	8,800	0
Human embryonic lung fibroblasts	450	0

[a] The experiment was performed as in Fig. 2 with a Triton X-100 concentration of 0.1% and an incubation time of 10 min. The following numbers of cells/ml were added to the incubation medium: bacteria, 5×10^8 to 10×10^8; *C. albicans*, 2×10^6; polymorphonuclear granulocytes, 1.5×10^6; other human cells as in Fig. 2.

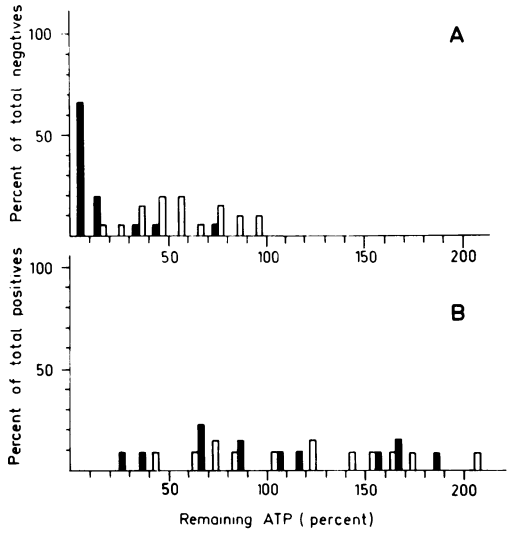

FIG. 3. *ATP assay in 26 culture-negative (A) and 14 culture-positive (B) clinical urine specimens. After a 1:1 dilution in distilled water specimens were subjected to the following treatments: nontreated controls (100% remaining ATP); incubated with apyrase (0.2 unit/ml), 10 min, 37 C (open bars); as above but in the presence of 0.1% Triton X-100 (filled bars).*

the treatments. Both types of treatments resulted in decreased levels of ATP in all specimens. However, in most specimens the treatment was considerably more efficient when Triton X-100 was included. Thus, it appears that in many specimens a major part of the nonbacterial ATP may be cellular since detergent is required for the breakdown of ATP by apyrase.

As shown in Fig. 3B, the effects of the same treatments on culture-positive urine specimens are quite different from the effects on negative specimens. On the average, these specimens increase rather than decrease their ATP content as a result of the treatments, and there is also no obvious difference between treatment with or without Triton X-100.

The histogram of Fig. 4A presents the ATP levels before Triton/apyrase treatment in a larger part of the material studied, 319 specimens in total. It is evident that there are differences in ATP content between negative and positive urine specimens. Median levels of ATP were 2.5×10^{-8} and 1.4×10^{-7} M, respectively. However, considerable overlapping occurs, and the ATP assays under these conditions do not give a reliable indication of the occurrence of bacteria.

In Fig. 4B, the same clinical material was analyzed after treatment with Triton/apyrase. In this experiment, the difference between positive and negative urine specimens is pronounced and most of the negative specimens fall below the detection limit of the assay, which was approximately 10^{-10} M ATP. Positive specimens are not affected or even show slightly increased ATP levels, as also shown in Fig. 3B and several of the model experiments. The median ATP levels in the Triton/apyrase treated urine specimens were $< 10^{-10}$ and 1.5×10^{-7} M in the negative and positive specimens, respectively.

The diagnostic utilization of the difference in ATP level between culture categories necessitates the determination of the ATP concentration limit resulting in the most effective discrimination between culture-positive and -negative specimens. To facilitate the determination of this limit, the data of Fig. 4B were replotted as shown in Fig. 5, which also includes 150 additional urine specimens. The ordinate describes the percentages of each of the three culture categories which would be classified as "luciferase positive" if the ATP concentration limit is arbitrarily varied along the abscissa.

In the present material, the ability of the assay to identify the cultivation-positive specimens is adequate up to an ATP concentration limit of approximately 4×10^{-9} M, as shown in Fig. 5. When this ATP concentration is defined as the lower limit for a sample to be regarded as "luciferase positive," 96% of the culture-positive specimens are classified as "positive" by luciferase assay, whereas only 20% of the culture-negative specimens have ATP levels exceeding the value of 4×10^{-9} M. The percentage of borderline specimens classified as "luciferase positive" is always higher than that of the culture-negative specimens.

In Table 3, average and median ATP levels per cell remaining after treatment with Triton/apyrase are shown for different bacterial species in culture-positive urine specimens. As a comparison, results from laboratory experiments with bacterial strains grown in sterile urine are included. In spite of large variations in cellular ATP content within each group of organisms, there is a striking similarity between the average ATP levels per cell in the clinical specimens and those obtained in the model studies. In the case of *Proteus*, there is a tendency to low values, and the lower ATP content of this organism was also observed in the model experiments.

In Fig. 6, the ATP content of the 86 positive urine specimens shown in Table 3 is plotted against the number of viable bacteria detected

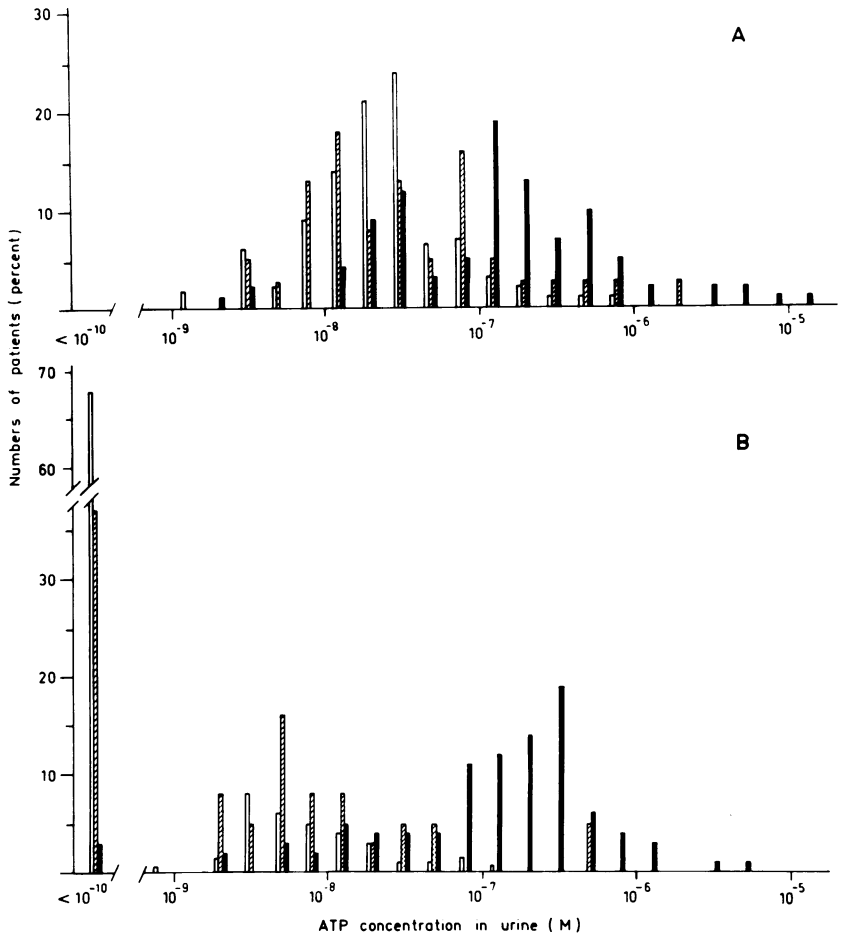

FIG. 4. *Effect of Triton/apyrase treatment on ATP levels in 319 clinical urine specimens. (A) ATP levels measured without Triton/apyrase treatment. (B) Specimens treated with Triton/apyrase as described in Materials and Methods. The specimens were classified according to culture: positive ($\geq 10^5$ cells/ml), filled bars; negative ($< 10^4$ cells/ml), open bars; borderline (10^4 to 10^5 cells/ml), hatched bars. The total numbers of specimens in each of these categories were 94, 187, and 38, respectively. The proportions of specimens in each category containing various levels of ATP are represented in the histogram.*

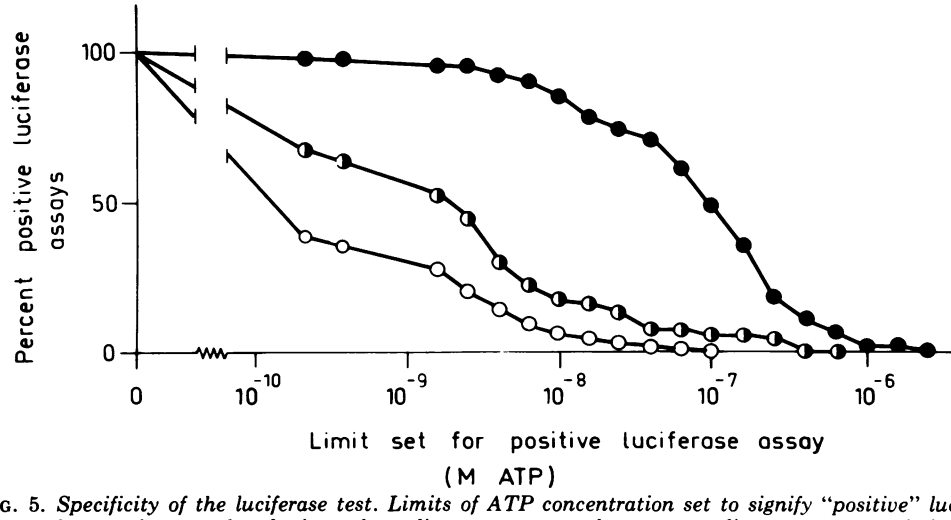

FIG. 5. *Specificity of the luciferase test. Limits of ATP concentration set to signify "positive" luciferase assay results are given on the abscissa; the ordinate represents the corresponding percentages of "luciferase positives" in each of the three cultivation categories: ●, culture positives (123 specimens); ○, culture negatives (290 specimens); ◑, borderline specimens (56 specimens). The material is that presented in Fig. 4B with the addition of 150 other specimens assayed only after treatment with Triton/apyrase.*

TABLE 3. *ATP content in some bacteria identified in urine specimens and grown in the laboratory in sterile urine*

Organism	No. of patients	Cellular ATP level[a] (moles × 10^{18}/cell)			
		Urine specimens		Model expt	
		Avg ± SEM	Median	Expt 1	Expt 2
E. coli	40	3.9 ± 0.9	1.3	2.6	2.1
Proteus sp.	10	1.0 ± 0.8	0.8	1.2	0.9
Streptococcus sp.	8	2.6 ± 0.8	2.6	2.0	0.8
Miscellaneous[b]	28	4.2 ± 1.2	2.0		
Total	86	3.6 ± 0.6	1.6		

[a] The urine specimens and laboratory cultures were treated with Triton/apyrase as in Table 2 and Fig. 3 and 4B. Only specimens are included in which culture counts were accurately determined. One culture-positive specimen (*E. coli*) not containing measurable amounts of ATP was excluded.

[b] Including *Klebsiella* (4), *Staphylococcus* sp. (3), *Pseudomonas* sp. (4), various mixed infections (10), and unidentified bacteria (7).

by quantitative culture. The range of variation in ATP content enclosed by the broken lines is of about one order of magnitude at each level of magnitude. In spite of the large variation, a relationship between bacterial level and ATP concentration may be inferred. However, the relationship does not appear to conform to the direct proportionality described by the theoretical solid line calculated from the average ATP level per cell (3.6×10^{-18} mol; cf. Table 3). It may be noted that in specimens containing high levels of bacteria there is a tendency towards ATP levels lower than the average, in accordance with results on laboratory-grown bacteria (3).

DISCUSSION

A serious obstacle in the application of the luciferase assay to many microbiological problems has been the inability to differentiate between bacterial and nonbacterial ATP. The present study shows that in model systems this may be achieved by a simple chemical technique based on the combined action of a detergent. Triton X-100, and an ATP-hydrolyzing enzyme, apyrase.

When applied to urine specimens, the Triton/apyrase treatment strongly enhances the difference in ATP level between culture-positive and -negative specimens. Furthermore, the amount of ATP remaining in the treated urine specimens is relatively well correlated with bacterial counts, and the ATP content per bacterium is similar to results obtained in model experiments. This is an indication that the differentiating effect of the Triton/apyrase treatment obtained in model systems may be representative also of the effect in the clinical material. Thus, it appears likely that the ATP remaining in the Triton/apyrase treated urine specimens is mainly of microbial origin.

Variations in ATP level per viable unit observed in treated urine specimens may, among other things, be caused by differences in metabolic rate and bacterial cell size, affecting cellular ATP pools (3, 10, 12). Furthermore, cell aggregation, causing erroneous culture results, would be expected to be common in this type of material.

A level of 10^5 bacteria/ml of urine has been shown to be an important criterion for the identification of cases of significant bacteriuria (8). The average ATP content in a single viable bacterial cell was shown to be approximately 3.6×10^{-18} mol/cell. Thus, a level of 10^5 bacterial cells/ml of urine would correspond to an ATP concentration in the urine of 3.6×10^{-10} M, approximately fourfold higher than the lowest ATP concentration detectable in the assay as set up by us.

For practical purposes, however, the criterion for a "luciferase-positive" assay may be shifted towards somewhat higher levels of ATP, resulting in a lower number of "false positives," as shown in Fig. 5. Thus, in any particular clinical material it may be worthwhile to establish empirically the concentration limit of ATP resulting in the highest degree of specificity.

"False-positive" results obtained with the luciferase assay might partly be caused by a failure to detect all bacteria by culture because of unusual nutrient or anaerobic requirements or because of the presence of antibiotics in the urine. In the borderline group, the reason for the relatively high numbers of "false positives' might, in addition, be a lack of precision in the reference method, making the correct classification of these specimens somewhat uncertain.

The present study has shown that luciferase assay of ATP may be a potentially useful method for bacteriuria screening. The sensitivity is sufficient to detect the presence of 10^5 bacteria per ml of urine, and the specificity allows the elimination of a majority of the culture-negative specimens, in a totally unselected clinical material. The assay procedure is rapid, inexpensive and simple. Commericial equipment for the bioluminescence assay is available, and the technique may be further

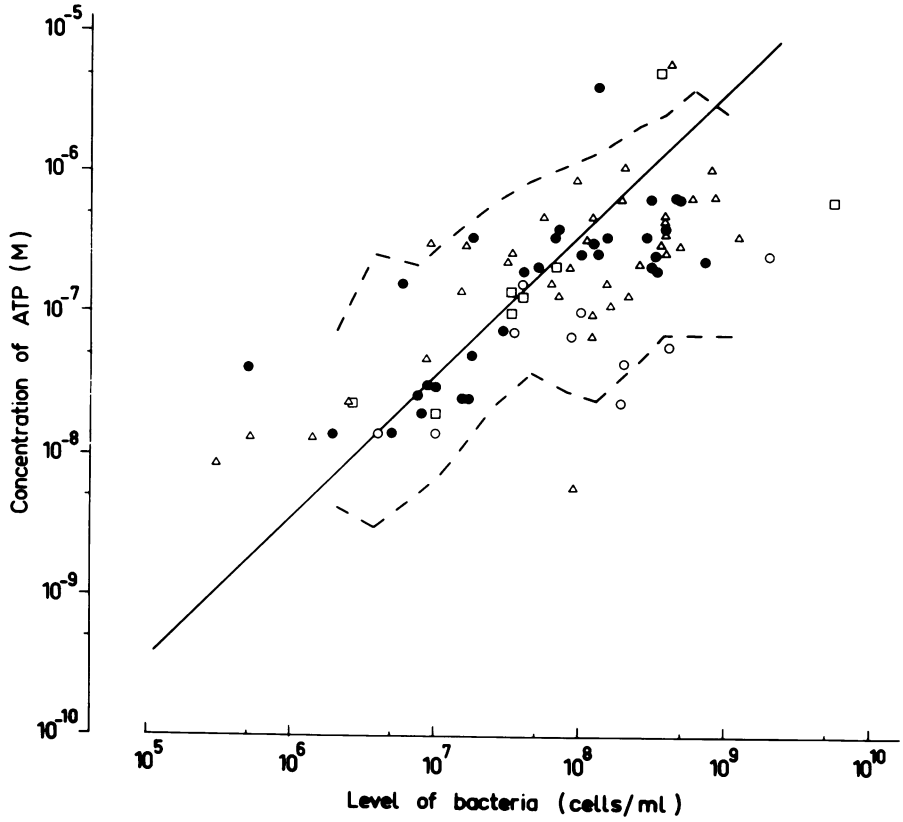

FIG. 6. *Urinary ATP levels as a function of bacterial content. The data in this figure correspond to the data of Table 3 obtained from 86 culture-positive clinical urine specimens treated with Triton/apyrase. The solid line is calculated from the average ATP content per cell in all of the 86 specimens; the broken lines connect 95% confidence intervals for calculated average ATP levels in groups of specimens not further than four times apart with respect to bacterial counts. Several such calculations were made by regrouping the values, and the lines connect the end points of the intervals. Symbols:* ○, *E. coli;* △, *Proteus sp.;* □, *Streptococcus sp.; and* ●, *miscellaneous bacteria as defined in Table 3.*

simplified by automation (17). Further studies on the luciferase assay as a bacteriuria screening method are underway. They include the investigation of a larger clinical material and the development of equipment and assay procedures suited for routine work. An investigation is also being made to determine the cause of remaining discrepancies between ATP assays and culture.

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