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ABSTRACT

The many advances in scientific and engineering knowledge produced by NASA space research often apply to major problems of modern society. To assist in the application of new technology and thus reduce the time interval from space development to direct benefit to society, NASA had established a technology-application effort at the Goddard Space Flight Center. As part of this effort we have been using, in collaboration with GSFC and also independently, technology for the detection of extraterrestrial life to develop a simple, fast and accurate procedure for detecting and counting bacteria in clinical specimens since management of infectious disease continues to be dependent upon primary isolation and culturing of organisms infecting biological fluids.

The medical applications of luminescent systems are a direct outgrowth of technology-application effort. The firefly luciferase assay for adenosine triphosphate (ATP) was a candidate for remote detection of microbes on other planets, such as Mars, as ATP is found in all living organisms on earth. An indication of the presence of ATP on another planet would suggest the existance of extraterrestrial life forms similar to those we know. Thus, ubiquitous distribution of ATP make precise, quantitative measurement of this compound a particularly promising chemical method for rapid detecting and quantitating microorganisms and for the study of cellular integrity and changes associated with various pathological processes, monitoring of inflammatory diseases, and sensitive determination of homograft viability. Recently this method is being adopted for rapid determination of metabolic integrity and viability of leprosy bacillus, since this organism has not yet been grown outside of its natural host and there is no suitable animal model currently available that can be used in endemic areas of the world. Thus, investigations undertaken in the 1960's by the GSFC has given a hope of major breakthrough to control leprosy which has so far inflicted 15 million of the world population.

INTRODUCTION

Among the vast array of questions to which man is seeking answers in his investigations of the upper atmosphere and outer space, there is none more challenging and provocative than that pertaining to the existence of life outside terrestrial boundries. Basic to these studies is the establishment of the parameters which define life. A definition which is amenable to operational manipulation is one which characterizes life as the capicity of a molecular complex to perform certain functional activities including metabolism, growth and reproduction. The detection of life in many instances is not a difficult matter, especially when there are visible manifestations such as characteristic form, movement and reproduction. This, however, becomes increasingly difficult as the size of the organism becomes microscopic, and even more so as the number of organisms being sampled decreases. It is at this point that the classical life detection techniques begin to be dependent upon one of the manifestations mentioned above; namely, reproduction.

All terrestrial life insofar as is presently known, is intimately associated with, and dependent upon, the nucleotide phosphate, adenosinetriphosphate (ATP). The ubiquity of this compound in living organisms renders it an excellent indicator of the presence or absence of life. Furthermore, changes in the metabolic integrity of a living organism are frequently accompanied by variations in the steady state concentration of ATP in the organism. Thus, not only may ATP measurements provide the basis for the detection of life, but may also be exploited for the detection of metabolic changes, both normal and

pathological. In order to realize the full potential of ATP as a monitor for the presence of life in space, it was necessary to select and develop a method with which it could be assayed with the highest degree of sensitivity, accuracy, and rapidity.

METHODOLOGY

The many advances in scientific and engineering knowledge produced by NASA space research often apply to major problems of modern society. To assist in the application of new technology and thus reduce the time interval from space development to direct benefit to society, NASA had established a technology-application effort at Goddard Space Flight Center, Maryland. As part of this effort, we have been using, in collaboration with scientists at GSFC and also independently, technology for the detection of extraterrestrial life to develop a simple, fast and accurate procedure for detecting and counting bacteria in clinical specimens, since management of infectious diseases continues to be dependent upon primary isolation and culturing of organisms infecting biological fluids.

The most sensitive method available for measuring ATP is based on the bioluminescent reaction of luciferase when mixed with ATP, the reaction which also creates the glow in the tail of a firefly. The reaction is described in figure 1. The maximum intensity of emitted light in this reaction has a direct linear relationship to the concentration of ATP added. This relationship exists over a range determined by a ratio of the concentration of luciferin-luciferase to that of ATP and by the sensitivity of the light measuring instruments.

Figure 2 is a schematic drawing of the light-measuring instrumentation used in the ATP assay. This was designed and fabricated by the scientists at the GSFC, Maryland, in the late 1960's, even though today several models are available commercially. It consists of a reaction chamber, photomultiplier assembly, amplifier, power supply, and recorder. The reaction chamber which is coupled to a photomultiplier, is a rotary drum designed so that access to and removal of the reaction cuvette is possible without exposing the photomultiplier to ambient light. The chamber can accomodate a 6 X 50 mm cuvette. Immediately above the cuvette holder, when rotated to the injection position, is a 0.040 inch injection port sealed by a replaceable, light-tight, rubber diaphragm to eliminate light leaks during injection. All surfaces of the reaction chamber assembly, exclusive of the mirror, are painted optic black.

Microbiology has remained a traditional science. One reason is that its tools and techniques are, fundamentally, those developed years ago by Koch, Pasteur, and Gram. Today's common methods for estimating total numbers of microorganisms include direct microscopic counting, colony counting in cultures, measurement of culture turbidity, weighing of cells and electronic particle counting. Each of these methods has inherent sources of error that have been tolerated and often ignored by those who use the methods. In an effort to overcome the limitations of physical cell size and culture techniques on the basis of bacteria counting, investigators have explored, rather successfully, the feasibility of relating cell population to a single biochemical parameter of the individual cell, and that parameter is ATP.

RESULTS

In the United States, where the incidence of urinary-tract infection is second only to that of respiratory-system infection, the examination of urine for bacteria is one of the most frequent and important clinical assays. Normally therapy is instituted only 48 - 72 hours after a specimen is collected and processed in the laboratory. Obtaining quick and reliable answers to such questions as, "Is an organism present?", "How many are present?" and "What is the antibiotic of choice for treatment?" would lead to a more rational approach to therapy. Using ATP measurements, the early detection of a significant bacteriuria coupled with a direct antimicrobial susceptibility determination offers the potential for initiation of therapy within 4 - 6 hours after a clinical specimen is received in the microbiology laboratory.

With this in mind, a comparison of the ATP assay to other commonly used methods of urine examination was made. Data in figure 3 show that when a colony count showed that specimens had 10,000 colonies per ml or more, an ATP assay of the same specimens showed positive results. About 20 percent of the urine specimens with a negative culture-colony count showed positive ATP results.

To assess how results from the ATP assay correlated to results from a culture-colony count, the average ATP content of a bacteria cell was determined. As shown in figure 4, in an ATP assay of the nine bacterial species commonly found in urine, the ATP content per cell ranged from 0.5 to 8.2 X 10^{-10} µgm. The value used for this conversion of ATP concentration into cells per ml was 3 X 10^{-10} µgm.

Results from selected urine specimens were used to determine a relationship between the

number of bacteria found by culture-colony count and the number of bacteria calculated from the ATP response and they are presented in figure 5. The ATP assay revealed bacteria in so many more specimens than the culture-colony count did that results from the culture-colony count were compared to results from the more accurate pour plate technique. Although the pour plate procedure revealed more bacterial cells than the culture-colony method did, the results were still much lower than those from ATP measurements.

Another potential clinical application of the ATP assay is the direct antimicrobial susceptibility testing of organisms found in urine. A typical result of this is shown in figure 6. A control containing organism but no antibiotic is represented by a solid line, and an organism grown in the presence of an antimicrobial agent is represented by the broken line. Ao is the ATP content of the growth control at zero time and A_t is the ATP content of the control culture after 3 hours incubation. Thus, the ATP index is a ratio of the change in ATP in the presence of an antibiotic to the change in ATP in the control. An empirical cut-off of 0.05 has been found to give good agreement with the results obtained by standard method (taking 72-96 hours). That is, an index of greater than 0.05 is indicative of resistance and one of less than or equal to 0.05 is indicative of susceptibility to the antimicrobial agent. From this one can really appreciate the importance of ATP assay.

This procedure has also been tried with blood and other body fluids in several pathological problems and the results have been equally successful.

What are the other practical applications of ATP assays? Three to five percent of swine on their way to market die of a condition called "porcine stress syndrome", which results in an annual loss to producers of $200 - $300 millions. One of 9,000 surgical patients under general anesthesia suddenly develop extremely high body temperature, severe metabolic acidosis, muscle rigidity, hyperalkemia, and cardiovascular collapse. The condition called "malignant hyperthermia", has been characterized as the "only" disease of the anesthesiologist, and is the major cause of unexpected death in anesthesia. With 15 million surgeries each year in the U.S. alone, one can expect at least 1,000 malignant hyperthermia victims with malpractice claims of $50,000 to $500,000 each, since malignant hyperthermia is preventable. Duchenne muscular dystrophy (DMD) occurs in about 1 of 3,000 male infants. No cure has been found, and the death usually occurs before age 25. These three seemingly different and unrelated problems have something in common: they are all associated with defective muscle ("myopathy") and resultant elevation of blood creatine phosphokinase (CPK). The methods currently available are either complicated or expensive for routine screening. However, ATP assay has given a new light. This assay can be used for routine screening even on newborn infants since it is simple, cheap, easy to perform, and oversensitive. The principle of this is described in figure 7.

Leprosy is a chronic infectious disease caused by an organism called Mycobacterium leprae, which resembles in morphology and staining, to tubercle bacillus causing tuberculosis. Leprosy affects approximately 15 million people in the world, and Asia, Africa and South America have the greatest number of reported cases. This number is increasing alarmingly and because of the rapidly developing resistance to drugs, it is predicted that if the present trend continues, the number of cases in the world will double within a quarter of a century. It is easy to understand the tremendous economic burden leprosy represents to developing countries, and indirectly to affluent societies. Only science and technology can solve the problem of leprosy.

Even though this organism was discovered in 1873, it has not yet been propogated in artificial media, and this has become a real bottleneck in leprosy research, especially in developing new drugs and vaccine. In the absence of in vitro growth the only method available to quantitate its biomass is microscopic; however, this method does not distinguish between living and dead organisms. We have been using Mycobacterium lepraemurium, causative agent of rat leprosy, as an interim model of M. leprae and we used ATP as a biochemical indicator to study the physiology and growth of this organism. Figure 8 demonstrates the utility of ATP data in such studies. M. lepraemurium were obtained from rat livers and transferred to in vitro environments with various nutrients. If the external environment is suitable for these organisms to grow then the traditional microscopic method is adequate to evaluate the growth (see curve B), and the same results will also be obtained using ATP assays (curve C). However, if the external environment is detrimental or neutral to the organisms, the mircroscopic method will not be able to distinguish between the two and data will be represented by curve A in either case. On the other hand, ATP assay technique can differentiate between the two processes. If it is harmless, it will be represented by curve A, while if the environment is detrimental to the organisms, the outcome will be curve D or E, depending on the degree of harmfulness of the environment. Thus, ATP is being used as a useful tool to study these organisms. But how is it directly helping the leprosy patients in endemic areas?

The only choice of drug presently available in endemic areas is 4,4'-diamino-diphenyl sulfone or Dapsone. But like any other disease-causing agents, the leprosy organisms are rapidly becoming resistant to dapsone, and thus, is becoming a serious problem in the eradication of this disease. Since children are more susceptible to contract the disease, the immediate danger is that a patient with dapsone-resistant leprosy organisms will transmit the disease to family contacts and there will be no readily available drug to treat these contacts. The only method presently available to detect the drug-resistant leprosy cases is the mouse foot pad technique. This is time consuming (takes 9-12 months to obtain valid information) and very expensive to use in endemic areas. Therefore, we have decided to use ATP assays for this purpose. Since, as mentioned above, this compound is ubiquitous in all living material, it will serve to determine the viability of leprosy organisms in patients under therapy and thus the susceptibility of these organisms to the given drug. The work in this direction is being initiated in collaboration with leprosy institutions from Argentina, Brazil, India and Surinam. The interpretation is simple and is demonstrated in figure 9. In short, intracellular ATP of M. leprae from a given specimen before initiating treatment will be determined, and simultaneously microscopic counts will be made on the same material. Next, ATP per one million microscopically counted M. leprae cells will be calculated. This procedure will be repeated at intervals during the course of treatment. If ATP per aliquot (per one million M. leprae cells) decreases during treatment, it will be interpreted as a positive response to the treatment since the drug therapy has inhibited biosynthetic capability of M. leprae cells and their ability to grow and multiply, and thus rendered them non-viable. On the other hand, if ATP per aliquot remains steady or increases over original level, the conclusion will be that the treatment had no effect in inhibiting the matabolism of M. leprae and thus are still able to grow and multiply in the host.

Thus, you can now appreciate well that the investigations undertaken by NASA has given a hope of a major breakthrough to control this most dreadful disease on the earth.

Besides these clinical applications of ATP assay, this technique is being used in other fields also that are equally important in our day-to-day life and these are described here briefly.

First is the industrial application for biocide control in oil production. Large volumes of water are used to aid secondary recovery of oil. Natural water, water recovered from produced oil, and well waters are the primary subjects for this use. Growth of microorganisms in these systems through such deleterious effects as corrosion and by-products can quickly create conditions whereby the water can no longer be injected into the ground, which will result in a complete shutdown of an entire oil field. Most of these systems are pressurized (1,000 - 3,000 lb/in^2) and the troublesome organisms are difficult to culture under laboratory conditions and require at least 28 days incubation. The ATP assay technique is now widely used in this area to detect the microorganisms and develop the microbiocides for use in oil field waters.

This assay is also used as a rapid method for measuring the efficiency of disinfection of wastewater for purposes of regulating the addition of disinfectants. It is shown that decreases in ATP concentration in combined sewer overflows treated with chlorine and chlorine dioxide parallel decreases in the traditional indicators of disinfection, total and fecal coliform bacteria.

The ATP assay is also used to monitor the quality of potable water supply by way of enumerating planktonic biomass before and after treatment in a direct filtration water treatment plant. The routine microscopic counting of plankton is very tedious and involves large operator errors.

Similar applications of ATP assay technique are being used in environmental studies where the determinations of microbial levels are involved.

FIGURE 1. Firefly bioluminescent reaction for ATP.

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

$$E \cdot LH_2 \cdot AMP + O_2 \longrightarrow E + AMP + CO_2 + hv + T$$

E = firefly luciferase;

LH_2 = reduced luciferin;

AMP = adenosine monophosphate;

PP = pyrophosphate;

T = thiazolinone (dehydroluciferin);

hv = light (550 nm)

Figure 2. A Schematic Drawing of the Instrumentation Used for the Light Measurements.

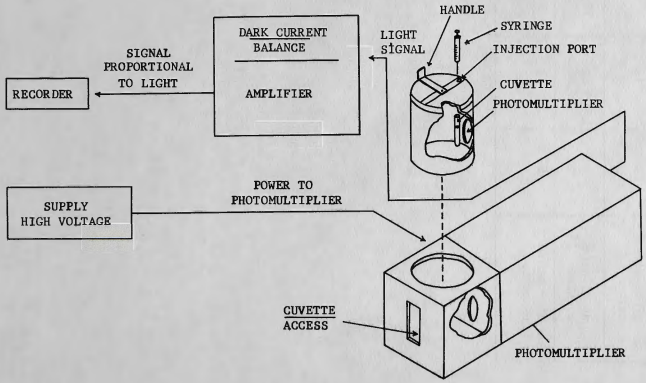


FIGURE 3. Comparison between culture-colony counting and ATP assay.

No. of specimens tested	282	78	39	57
No. of colonies counted	0	1 to 10,000	10,000 to 100,000	over 100,000
No. of + ve ATP responses	60	42	39	57

FIGURE 4. ATP content in bacteria.

Species	µgm ATP/cell ($\times 10^{-10}$)
Escherichia coli	1.2
Streptococcus faecalis	1.1
Staphylococcus aureus	1.8
Staphylococcus epidermidis	8.2
Proteus mirabilis	4.3
Klebsiella	2.8
Pseudomonas	0.5
Proteus rettgeri	1.4
β streptococcus	1.5

FIGURE 5. Number of bacteria per ml urine sample determined by ATP assay, culture-colony plate, and pour plate.

Sample No.	ATP method	Culture-colony plate	Pour plate
1	7×10^8	0	1×10^4
2	4×10^7	1×10^4	1×10^7
3	3×10^7	1×10^4	2×10^7
4	3×10^7	0	0
5	5×10^9	2×10^4	3×10^5
6	3×10^7	0	0
7	8×10^7	0	0

FIGURE 6.

ATP ASSAY IN ANTIMICROBIAL SUSCEPTIBILITY TESTING

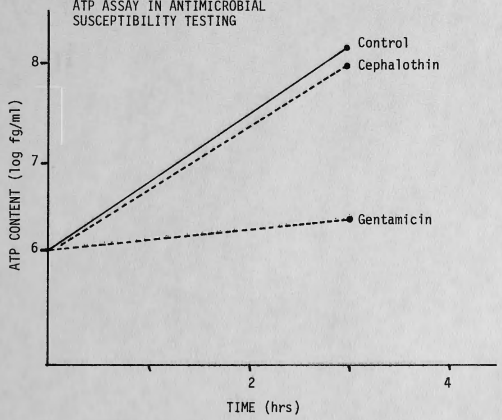


FIGURE 7. Creatine Phosphokinase (CPK) reaction.

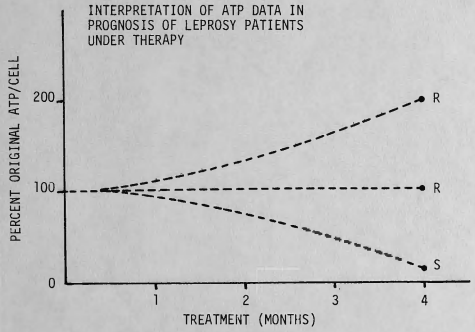
$$\text{Creatine phosphate} + \text{ADP} \longrightarrow \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{++}]{\text{Luciferase}} \text{AMP} + \text{PP} + \text{Dehydroluciferin} + \text{Light.}$$

FIGURE 8

SUPERIORITY OF ATP ASSAYS
OVER MICROSCOPIC METHOD
IN LEPROSY RESEARCH

FIGURE 9


INTERPRETATION OF ATP DATA IN
PROGNOSIS OF LEPROSY PATIENTS
UNDER THERAPY
PERCENT ORIGINAL ATP/CELL
200
100
0
1
2
3
4
TREATMENT (MONTHS)
R
R
S