An Automated Procedure for the Sensitive and Specific Determination of ATP

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An automated procedure is described for the measurement of ATP. This method, based on the linear luminescence response of firefly extracts added to ATP, is rapid, reproducible, and allows a relatively large number of samples to be run (up to 35/hr.). A procedure is also given for the preparation of the luciferin-luciferase system from dried firefly lanterns. Sample volumes required are small, and reading error is about ± 3%. Uniform timing of the addition of reagents and reading of light emission intensity for each sample are insured. Variables investigated include dilution of firefly extract, alterations in time after mixing sample and enzyme, linearity, cation, anion, and ADP and AMP interference.

Numerous procedures have been described for the quantitative measurement of adenosine triphosphate (ATP), however, most of these methods are rather time-consuming. The accuracy, reproducibility, and number of determinations which can be performed are limited by the many manipulations involved. Most of the earlier assays for ATP depended one one or more of its distinctive physical properties. LePage (1) used a procedure involving precipitation and acid hydrolysis. Methods based on ion exchange, paper chromatography (2, 3), or an enzymecatalyzed reaction between ATP and glucose (4-6) have also been described. Recently, modifications of the firefly-luminescence assay have received widest application since these methods appear to offer the advantages of increased specificity and sensitivity (7-11).

The present paper reports an automated determination of ATP based upon the measurement of the light emission produced during the oxidation of luciferin by molecular oxygen in the presence of ATP and magnesium ions. Variables investigated include dilution of firefly extract, linearity of standard curve, and cation, anion, and adenosine...
diphosphate (ADP) and adenosine monophosphate (AMP) interference.

**Methods**

The light-measuring equipment is depicted in Fig. 1 and 2. Figure 1 is a flow diagram of the detection system showing the major components, the tubing sizes, and the reagents used. The individual components and their source of purchase are as follows: Sampler Model II (Technicon Instruments Corporation, Chauncey, N. Y.; TIC); Pump (TIC); Photomultiplier tube housing from an Aminco-Bowman spectro-photofluorometer (American Instrument Corporation, Silver Spring, Md.; AIC); RCA-selected 931-A photomultiplier tube (AIC); mixing coil (13 gyres/coil; TIC); photomultiplier microphotometer (AIC); recorder adapter (AIC); two-pen recorder (TIC); and sample cups (TIC).

Figure 2 shows two views of the modified photomultiplier housing used. A small mixing coil is placed directly in front of the light-sensing

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**Fig. 1 (top)**. Flow diagram of automated system showing tube flow rates and location of equipment. The photomultiplier tube housing is described in more detail in Fig. 2. The recorder adapter is standard Aminco equipment, but may not as yet be in their catalogue. **Fig. 2 (bottom)**. Diagrammatic representation of photomultiplier tube housing. Left: side view. Right: front view of the housing. Distance between mixing coil and photomultiplier tube (P.M.) is 3 3/4 in.
portion of the photomultiplier tube so that the amount of light detectable will be a maximum. A front-surfaced mirror is placed behind the mixing coil and is positioned such that all emitted light will be reflected into the photomultiplier tube. The critical dimensions of the glass coil within the photomultiplier housing are shown in the diagram. The ends of the coil must be shortened about 3/8 in. to accommodate it inside the housing. Aluminum foil is wedged on both sides of the coil to hold it in front of the photomultiplier opening. The housing is sealed with black electrical tape and placed in a black rubber bag. The black bag and its contents are then placed in a small box (5 3/4 in. × 7 3/4 in. × 3 1/4 in.) with the appropriate inflow and outflow tubing emerging from one corner. It is important to note that the photomultiplier housing is placed on its side so that proper mixing can occur within the coil.

In actual practice, either a standard or an unknown solution containing ATP (labeled ATP in Fig. 1) is taken up from the individual sample cups and is mixed with air, then with the luciferin-luciferase system (labeled enzyme in Fig. 1). This latter mixing is done within the photomultiplier housing in front of the photomultiplier tube so that as much of the initial burst of light as possible can be detected. When time-delay coils were introduced between the point at which the sample and enzyme system were mixed and the photomultiplier tube, the light detected from a standard concentration of ATP gradually decreased as the time before reading the light emission increased. The output from the photomultiplier tube can be adjusted through use of the microphotometer so that the recorded deflection will remain on the recorder. The sampler is set to run at 70 samples per hour with a cup containing distilled water placed between each sample cup. Thus up to 35 samples per hour can be run. There is no detectable contamination from sample to sample going from high to low values of light emission when cups containing water are placed between samples. There is little or no decrease in sensitivity observed between standards run at the beginning or end of a 3-hr. period. The standard should be prepared daily and stored at ice bath temperature until used. The photomultiplier tube chosen appears to offer a good combination of high sensitivity and stable baseline.

**Preparation of Enzyme**

In our laboratory, we have used both commercially available lyophilized firefly lanterns (Sigma Chemical Co., St. Louis, Mo.; Worthington Biochemical Corp., Freehold, N. J.; or Calbiochem, Los Angeles, Calif.) or lanterns obtained from locally caught fireflies. We have found that the activity of the extracted luciferin-luciferase will vary
somewhat with the batch and the method of preparation. If locally
caught insects are used, the lanterns should be removed and freeze-
dried as soon as possible. All the studies reported in the present work
were performed with the firefly luminescent system obtained from
commercially available desiccated lanterns. Our extraction procedure is
somewhat similar to that described by Strehler and Totter (12).

As long as the proportion of firefly lanterns and buffer volume re-
 mains constant, any amount of enzyme solution may be prepared. In
our laboratory 500 mg. (approximately 100 luminous organs) of
desiccated firefly (Photinus pyralis) lanterns are homogenized for 2
min. in 60 ml. of 0.1 M arsenate buffer, pH 7.0, using an Ultra Turrax
homogenizer. This is prepared in a manner similar to that described
by Beutler and Baluder (9). The homogenate is centrifuged at 30,000 g
for 20 min. at 4° and the supernatant obtained decanted through two
layers of cheese cloth. This liquid has been designated as concentrated
enzyme solution. If the concentrated enzyme solution is allowed to
stand in the cold (2° for 7–8 hr. a white precipitate forms which can be
removed by centrifugation (30,000 g for 10 min.) or by Millipore
filtration. All luciferin-luciferase activity appears to reside in the
supernatant fraction. The concentrated enzyme solution is stored at
4° until used. We do not use enzyme prepared more than one week
prior to the experiment. Nine milligrams of MgSO₄·7H₂O/ml. are
added to the enzyme solution prior to use. Concentrated enzyme solu-
tions are stable for a few hours at room temperature, and dilute solu-
tions can be maintained in an ice bath for several hours without
precipitation or loss of enzymatic activity. Since the luciferin-luciferase
system exhibits a certain amount of light instability, the enzyme should
be stored in dark bottles. In general, we have found deterioration of
luciferin-luciferase solutions due to the instability of both substrate
and enzyme. Luciferin can be converted to an air-oxidized product
which competes for the luciferin substrate site (13). This is supported
by the observation that regeneration of oxyluciferin to luciferin will
take place after the addition of Coenzyme A (14).

Preparation of ATP Standards and Stability of Solutions

Our standard solutions are made from distilled-deionized water and
ATP-disodium 3.5 H₂O (Sigma Chemical Co.). These samples should
be prepared immediately before use and kept cold until needed. In
general, the stability of dilute solutions of ATP appears to depend on
the interaction of several factors. These include the concentration of

*Kinematics LMBH, Luzern, Switzerland.
ATP, temperature, pH, and concentration of metallic ions. Therefore, any extraction scheme from biologic material must take these factors into account. The reader is referred to the extensive work on extraction and stability of ATP by Kalbhen and Koch (15) and to the effect of pH and metal ions on ATP hydrolysis by Spiro et al. (16).

Results and Discussion

Linearity and Sensitivity

A unique feature of the automated system is its extremely wide range of sensitivity \((10^{-4} - 10^{-9} \text{ gm./ml.})\) with a reproducibility of \(\pm 3\%\) (see Fig. 3). This sensitivity range is obtained without the necessity of concentration or dilution of the ATP-containing sample. This is accomplished by electronic damping using the meter multiplier dial on the

![Fig. 3. Relation of light intensity to ATP concentration. Left, graph shows entire detectable concentration range. Right, lowest concentrations detectable at maximum sensitivity. Note change of scale. These are composite graphs. The peak heights shown do not coincide with the concentrations on the abscissa, but were positioned to show the height of the AutoAnalyzer peaks in relation to the actual data obtained. Numbers 1-10 show actual peak heights recorded for each of the following amounts of ATP: 1-5 indicate 0.4, 0.6, 0.8, 1.0, 2.0 \(\times 10^{-5}\) Six to ten indicate 2.0, 4.0, 6.0, 8.0, 10.0 \(\times 10^{-5}\) gm./ml.](image)
photomultiplier microphotometer. It is recommended, however, that a baseline adjustment be made for every three- to ten-fold increase in ATP concentration when measuring samples with wide differences in ATP content.

In general, there are two variations of measurement of ATP by the bioluminescent system currently employed. One method measures the initial burst of light produced when ATP and the enzymes are mixed in solution. The other procedure, although measuring the light derived from the initial burst, attempts to slow the decay of luminescence and measure the light at some point in time after the mixing of sample and enzyme. The method described in the current communication is based upon the latter of the two procedures. The light produced is picked up by our detection system approximately 30 sec. after the initial contact of sample and enzyme. This period allows a complete mixing of the two solutions within the mixing coil.

It should be noted (see Fig. 3) that although the relationship between light produced and amount of ATP present is linear in certain concentration ranges (at least over a ten-fold change), over the entire span of concentrations which are measurable \(10^{-4} - 10^{-9} \text{ gm./ml.}\), such a linear relationship does not exist. Such an effect using a nonburst system was first noted by Strehler and Totter (7) and has been confirmed by Addanki et al. (17). Initial burst measurement systems, however, do appear to exhibit linearity over a wide concentration range (18). Perhaps the chemical environment employed in the nonburst system (arsenate buffer, Mg\(^{++}\)) in some way favor a slower decay of light when larger amounts of ATP are present.

**Effect of Dilution of the Firefly Lantern Extract**

Firefly lantern extracts were prepared as described under Methods. The extract produced from the homogenization of 500 mg. of luminous organs in 60 ml. of 0.1 M arsenate buffer is referred to as concentrated enzyme solution. Various dilutions of this solution were prepared by the addition of appropriate quantities of arsenate buffer. Except where noted, 9 mg./ml. of MgSO\(_4\) \(\cdot\) 7H\(_2\)O were added to the enzyme solutions just prior to use in the automated system. The light intensity produced by several concentrations of ATP was then determined using each enzyme-containing solution (Fig. 4). At each dilution of firefly lantern extract, the light emission was directly proportional to the concentration of ATP, although the intensity of the light was diminished at higher dilutions. A full strength or concentrated extract had approximately twice the activity of a 1:1 dilution. For reasons of economy, if relatively large quantities of ATP are present, then dilutions of the
extract can be used and linearity will still be obtained. Beutler and Baluda (9) have also recommended the use of diluted extracts for routine analysis.

Effect of Mg$^{++}$ Ions on Luminescence

Divalent metal ions appear to be required in some enzymatic reactions involving ATP (19). It has long been known that an intimate relationship exists between ATPase activity and luminescence. This has been substantiated by the finding that when relatively purified firefly extracts are used—i.e., those with low ATPase activity—the luminescent system fails to respond to ATP (19). When any of a number of divalent ions including Mg$^{++}$, Co$^{++}$, or Mn$^{++}$ is added to the partially purified extract, luminescence as well as ATPase activity is restored (19). Since most recent methods for the measurement of ATP utilize Mg$^{++}$ to increase luminescence, we have attempted to determine the optimal amount of Mg$^{++}$ necessary to give us peak efficiency. The results of this study are shown in Fig. 5. Apparently the concentrated enzyme contains small amounts of Mg or some other divalent ion in quantities sufficient to stimulate light production without the addition of exogenous cations. This is supported by the observations that when EDTA was added to the enzyme extract no light was produced even when relatively large concentrations of ATP were added. Addition of various concentrations of MgSO$_4$·7H$_2$O to the con-

Fig. 4. Relation between light intensity and degree of dilution of the luciferin-luciferase enzyme solution. Ratios refer to the degree of dilution of the full strength extract (enzyme solution).
centrated enzyme solution resulted in increases in the amount of light produced by a given concentration of ATP. Since 15 mg./ml. of MgSO₄·7H₂O produced virtually no increase in light production over a concentration of 9 mg./ml., the latter concentration was chosen and used as an optimum in all future studies.


**Fig. 5.** Effect of magnesium ion on luminescence. Varying concentrations of MgSO₄·7H₂O were added to the concentrated luciferin-luciferase enzyme system immediately prior to use. EDTA was added to the enzyme without addition of magnesium.

**Effect of NaCl on Light Emission**

It has been demonstrated previously that the presence of NaCl can reduce the intensity of the light emitted from mixtures of ATP and firefly enzyme (7, 10). The latter authors felt that this was probably due to a general effect of inorganic salts, since the presence of KCl, NH₄Cl, LiCl, NaBr, MgCl₂, or NaHCO₃ all reduced the initial light flash to approximately the same degree. Since NaCl is the inorganic salt most likely to be encountered when working with biologic material, we have investigated the effect of this salt on the amount of light produced by the interaction of ATP with the luciferin-luciferase system. Various millimolar concentrations of NaCl were added to a standard solution of ATP (1 μg./ml.) in distilled water. The samples were then analyzed for their ATP content using the automated system described above. Figure 6 clearly demonstrates the inhibitory effect of Cl. Concentrations of from 20 to 200 mM markedly reduce the amount
of light detected by our system. This should not be a problem in determining the ATP content in biologic material, however, since we, like Holmsen et al. (10), have found that when the concentration of NaCl was kept constant, there still remained a direct proportionality between

Fig. 6. Intensity of light emitted from solutions of $2 \times 10^{-6}$ M ATP in the presence of varying millimolar amounts of NaCl.

intensity of the emitted light and the concentration of ATP in the sample. Thus, as long as the salt concentration was the same in each biologic sample, the amount of ATP could still be determined by light measurement. Alternatively, internal standards could be added after an initial luminescence determination was made on the unknown sample.

The presence of excess chloride ions will reduce the over-all sensitivity of the method, although this problem may be overcome to some extent as indicated above. However, in those instances where very small amounts of ATP are present and the chloride contamination is high, it may be advisable to remove the excess chloride. The chloride problem can be eliminated by separation of this ion from the ATP-containing solution by the use of a short Sephadex G-10 column. The results of using such a column system (9 mm. i.d. \times 216 mm. long) are shown in Fig. 7. Two hundred micrograms of ATP were dissolved in 2 ml.
physiologic saline, applied to the column, then eluted with distilled water. One-half-milliliter fractions were taken and analyzed for their ATP content. The detection of Cl− was made qualitatively using a concentrated AgNO3 solution. After an initial void volume of approximately 5 ml. ATP began to appear in the eluate, with the next 5.5 ml. containing most of the ATP (Fig. 7). Chloride ion did not start to appear in the eluate till all of the ATP had been eluted. Recovery of ATP based upon approximated flow rates was calculated to be about 86%. The column system took about 35 min. to run; considering the greatly increased sensitivity of detection, this separation procedure may prove useful when absolute determination of ATP is important. It is advisable that the column system be run at 4° so that stability of ATP is insured.

**Effect of AMP and ADP on the Determination of ATP**

The light emission from varying concentrations of ADP was measured both in the presence and absence of a fixed quantity of ATP; the results of such experiments are presented in Table 1. Only once, when concentrations of at least 2 μmol were reached, did ADP cause any significant contribution to light emission. Even at this concentration, the total deflection of the galvanometer was only about $\frac{1}{25}$ that of ATP. When both ADP and ATP were present in equimolar quantities, there was no significant contribution of ADP to the light emission. If, however, ADP is present in quantities approximately 10 times that of ATP, it will contribute a measurable amount of light. Holmsen et al. (1) have
Table 1. Effect of ADP on Determination of ATP

<table>
<thead>
<tr>
<th>ATP concentration (μmole)</th>
<th>ADP concentration (μmole)</th>
<th>Arbitrary light units</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.0</td>
<td>6.5</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>0.0</td>
<td>0.02</td>
<td>0.0</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0</td>
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</tr>
<tr>
<td>0.2</td>
<td>2.0</td>
<td>24.5</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2</td>
<td>18.0</td>
</tr>
<tr>
<td>0.2</td>
<td>0.02</td>
<td>17.0</td>
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</tbody>
</table>

The above light unit values were determined by measuring the light produced by mixing the concentrated luciferin-luciferase enzyme with ATP and ADP, either alone or in combination as indicated.

reported that the light emission from ADP itself influenced the initial flash caused by ATP only when the final concentration of ADP was greater than 0.2 μmole. Under our conditions, AMP had no effect on the light emission even when present in concentrations as high as 20 μmole.

General Comments

A discussion of the mechanism of firefly luminescence is beyond the scope and intention of the present communication, however certain methodologic considerations should be borne in mind which are based upon the present work and that of other authors.

The sensitivity and reproducibility of the automated system described is excellent. The reproducibility remains ± 3% for several hours, and the sensitivity of the detection system ranges from $10^{-4}$ to $10^{-9}$ gm./ml. without dilution of the samples. The Amieco microphotometer photomultiplier can adapt for increased amounts of light by electrical damping. The major interference in the system is the result of inhibition of the luciferase by Cl⁻, although color-quenching and turbidity will also diminish the amount of light detected. The inhibition by Cl⁻ can be compensated for by the addition of a standard amount of ATP and subsequent calculation of the percent-quenching. From their studies, McElroy and Strehler (19) have concluded that a pH of about 7.5 and a temperature of approximately 25° are optimal conditions for measuring luminescence.

The automated method for measuring ATP described, based on the linear luminescence response of firefly extracts to added ATP, is rapid, reproducible, and allows large numbers of samples to be run (up to 70/hr.). Sample volumes required are relatively small and the uniform timing of the addition of reagents and the reading of light emission intensity for each sample are insured. Preliminary examination of
biologic material containing ATP (e.g., adrenal gland perfusate, red blood cells) using the above automated procedure suggests the wide applicability of this system.

References