Use of Bioluminescence in the Field of Clinical Chemistry

WILLIAM D. McELROY and MARLENE DeLUCA

Department of Biology and Chemistry, M-001 University of California
La Jolla, CA 92039 U.S.A.

During the past few years significant progress has been made in the use of bioluminescence and chemiluminescence in the research and clinical laboratories. There are several reasons for these developments. In the first place highly purified enzymes and chemicals have become commercially available at a reasonable price. Second, industry has developed and placed on the market inexpensive and highly sensitive photometers. Other reasons for the increased interest in luminescent systems include high specificity, speed of analysis and sensitivity, as low as $10^{-18}$ moles in some cases.

In the present article we will review briefly the use of the firefly system for measuring ATP and the luminescent bacterial for NADH and NADPH. Through the use of other enzymes and coupling reagents it is possible to assay several hundred enzyme systems and their corresponding substrates, although most of these have not been completely researched for clinical applications.

In the present paper we will not attempt a comprehensive review of the literature. We refer the reader to a number of recent symposia and reviews on this subject.$^{1)*6)}$

Firefly Luminescence

Firefly luciferase catalyzes the oxidation of D-luciferin in the presence of ATP and Mg$^{++}$ with the production of light.

\[
\text{ATP} + \text{LH}_2 + \text{E} \overset{\text{Mg}^{++}}{\longrightarrow} \text{E-LH}_2-\text{AMP} + \text{PP}_i \\
\text{E-LH}_2-\text{AMP} + \text{O}_2 \longrightarrow \text{LIGHT} + \text{E} + \text{AMP} + \text{CO}_2
\]

Because of the extreme specificity of the enzyme for ATP, it has been widely used for the measurement of ATP in a variety of samples. Fig. 1 shows the kinetics of light production as a function of increasing ATP concentration. At low ATP concentrations the light rises to a maximum value and remains constant for a minute.

Fig. 1 Time course of light emission with increasing concentrations of ATP. Final concentration of ATP was, A=0.2 pmol; B=2 pmol; C=20 pmol; D=200 pmol; E=2 nmol; F=2 μmol. The reaction contained 0.2 μg luciferase. The increase in light intensity indicated on the ordinate is not quantitatively related to the ATP concentration and reflects only a qualitative presentation.

or longer. As the ATP is increased the peak light intensity increases and the enzyme becomes progressively more product inhibited. A plot of the peak height intensity against ATP concentration gives a straight line from 0.2 pmol to 2 μmol. The preparation of firefly luciferase and luciferin and the review of the mechanism of enzyme action and light emission can be found in references 2, 3 and 4.

Applications

It is not possible to review all the applications of the firefly system in detail; the reader is referred to the various reviews and symposia.

a. Determination of Bacteria

Chappelle, Picciolo and Deming² have written an excellent review on the use of the firefly system for detecting the bacterial contents in various fluids. Assaying over nineteen different species they found that the average ATP content is $2.5 \times 10^{-10}$ μg per organism. Under ideal conditions there is a straight line relationship between the ATP and the number of cells present. The method should prove to be useful for determining the bacterial count in urine, blood, water samples, oil and sludge. Industry is responding to this area of research by supplying small, portable photometers for field work.

b. Biomass

Holm–Hansen and Karl³ have reviewed the use of the firefly system for determining the total amount of living cellular material (Biomass). This has proven to be useful in many ecological studies particularly those in the aquatic environment.
c. Immunoassays

Radioimmunoassays have been used extensively to detect specific ligands. Typically competitive binding assays make use of an isotopically labelled ligand that competes with the unknown ligand for the antibody binding site. In this type of assay there is an inverse relationship of the amount of radioactivity bound and the unknown ligand concentration i.e. a competitive reaction for the active site.

Recently Wannlund et al.\textsuperscript{14} have used a bioluminescent immunoassay for determining methotrexate at the subpicomole level. Methotrexate was covalently linked to firefly luciferase. This derivatized luciferase retained over 60 percent of its catalytic activity. An antibody developed against methotrexate–hemocyanin readily bound to the luciferase–methotrexate. When the enzyme conjugates was incubated with varying concentrations of free methotrexate and the antibody, the amount of the enzyme–conjugate bound was inversely proportional to the concentration of free methotrexate. This relationship is shown in Fig. 2.

![](image)

**Fig. 2** Competitive binding curve of free methotrexate with luciferase–methotrexate conjugate.

d. Coupled Reactions

A large number of publications have been concerned with the assay of specific enzymes or substrates that either make or use ATP. The determination of creatine kinase has been studied extensively and is a typical example.

\[
\text{ADP} + \text{creatine phosphate} \xrightleftharpoons{\text{CK}} \text{ATP} + \text{creatine.}
\]

The rate of production of ATP is proportional to the amount of creatine kinase in the sample.

Another example is the determination of cyclic nucleotide phosphodiesterase or its substrate.

\[
\text{Cyclic AMP} \xrightarrow{\text{phosphodiesterase}} \text{5'-AMP}
\]
\[
\text{5'-AMP} + \text{ATP} \xrightarrow{\text{myokinase}} 2 \text{ADP}
\]
Using excess myokinase it is possible to assay for phospho-diesterase activity by measuring the rate of ATP disappearance. Fertel and Weiss\textsuperscript{2}) prefer to convert the two ADP to ATP and measure the increase in light.

\[ 2 \text{ADP} + 2 \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} 2 \text{ATP} + \text{pyruvate} \]

Cyclic GMP phosphodiesterase can be determined in a similar manner.

Using appropriate coupling enzymes it is possible to determine a large number of different compounds such as GTP, UTP, CTP and ADP.

Unfortunately most of these procedures have remained in the research laboratories. Specific kits have not been developed so they could be used routinely in the clinical laboratories. With the increased interest, the availability of cheap reagents, the speed of measurement, the availability of automated instruments and the concern about radioisotopes, this should lead to a rapid introduction of luminescence techniques into the clinical laboratories.

\textbf{Bacterial Luminescence}

Luminous bacteria are found mostly in the marine environment. Those isolated from seawater can usually be grown on very simple media using artificial seawater and carbon source.

Light emission is a system that competes for reducing power with the normal electron transport chain. Light emission occurs when reduced flavin (FMNH\textsubscript{2}) interacts with bacterial luciferase, a long chain aldehyde (RCHO) and oxygen.

\[ \text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightarrow{\text{LUCIFERASE}} \text{light} + \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} \]

The reaction is not specific for aldehyde although the light intensity (quantum yield) increases as the chain length is varied from eight to sixteen carbon atoms. In most applications dodecanal appears to be the preferred substrate.

Luminous bacteria also contain specific oxidoreductases that catalyze the reduction of FMN using reduced pyridine nucleotides.

\[ \text{NAD(P)}H + \text{FMN} + \text{H}^- \xrightarrow{\text{REDUCTASE}} \text{NAD(P)}^+ + \text{FMNH}_2 \]

If the assay conditions are such that the NAD (P) H is the limiting component, then the light intensity is directly proportional to the concentration of the reduced nucleotides. Any reaction that leads to the production or disappearance of NAD (P) H can be measured by the light system. Since many clinical assays can be coupled to the appearance or disappearance of reduced pyridine nucleotide this bioluminescent assay is of great potential importance. The bacterial system and its properties have been reviewed in a number of recent publications\textsuperscript{3)\textendash4}).
Use of Bioluminescence in the Field of Clinical Chemistry

Applications

Figure 3 shows the time course of light emission when FMNH$_2$ is injected into a luciferase-aldehyde mixture (A) or when NADH is injected into a solution containing oxidoreductase, luciferase and aldehyde (B).

![Fig. 3 Time course of light emission upon the addition of excess FMNH$_2$ to luciferase (A) and upon the addition of limiting NADH to a coupled reaction system of luciferase and NADH:FMN oxidoreductase (B).](image)

The use of the bacterial luminescent system for assays does not require expensive equipment. The assays are simple, rapid and very specific for reduced pyridine nucleotide. The light intensity is linear with NADH concentration in the range of $10^{-7}$ to $10^{-12}$ moles. This is much more sensitive than spectrophotometric or fluorometric procedures. Like the firefly system both crude and purified oxidoreductase and luciferase are available commercially at a reasonable price.

![Fig. 4 Initial light intensity as a function of (○) androsterone or testosterone (●) concentration. Either 3α-hydroxysteroid or 3β, 17β-hydroxysteroid dehydrogenase were co-immobilized onto Sepharose along with luciferase and NADH : FMN oxidoreductase.](image)
In most assay systems the dehydrogenase reaction is coupled directly to the luminescent system in the presence of excess NAD and FMN. The rate of production of NADH is measured directly by the light intensity. In Figure 4, for example, the initial light intensity is plotted as a function of androsterone (○) or testosterone (●) concentration. 3α-Hydroxysteroid dehydrogenase was coupled to the light system. This assay was linear down to 0.1 nanomoles of steroid. The light system has been used to assay several clinically important enzymes such as alcohol dehydrogenase, lactic dehydrogenase, malate dehydrogenase and glucose-6-phosphate dehydrogenase.

In Table I we list a few representative substrates and enzymes that have been assayed using the luminous bacteria system.

<table>
<thead>
<tr>
<th>Compound Assayed</th>
<th>Range of Detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>0.1 femtomoles</td>
<td>Lee et al. 1974(42)</td>
</tr>
<tr>
<td>NADPH</td>
<td>10 picomoles-200 nanomoles</td>
<td>Jablonski and DeLuca, 1976(41)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.5-6 picomoles</td>
<td>Brolin, et al. 1971(3)</td>
</tr>
<tr>
<td>3-Hydroxybutyrate</td>
<td>50-1,000 picomoles</td>
<td>Brolin, 1977(3)</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>5-60 picomoles</td>
<td>Stanley, 1978(13)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.003%-0.012%</td>
<td>Haggerty, et al. 1978(89)</td>
</tr>
<tr>
<td>Lactic Dehydrogenase</td>
<td>0.001-1 picomole</td>
<td>Haggerty, et al. 1978(89)</td>
</tr>
<tr>
<td>3-OH Butyrate Dehydrogenase</td>
<td></td>
<td>Berne, 1976(7)</td>
</tr>
</tbody>
</table>

Conclusion

Both the firefly and the bacterial light emitting system have been used for years in the research laboratories for determining a number of chemical compounds of biological interest. Both systems are extremely sensitive, highly specific and the assay is easy to perform. Unfortunately, until recently researchers have had to purify their own enzyme and chemical reagents in order to do highly quantitative measurements. The introduction of commercially available reagents of high purity and the supply of excellent instruments allows the researcher today to carry out hundreds of assays daily with relative ease.

Each experimental system that consists of a sequence of reactions must be tested critically and evaluated for errors. The researchers do this for their particular task but unfortunately adequate studies have not been done to make the light emitting systems a useful, routine clinical tool. But increasing use of these systems in clinical laboratories should correct this situation rapidly. The potential is enormous.
Use of Bioluminescence in the Field of Clinical Chemistry

References

8) Brolin, S. E. : (1977) Bioelectrochemistry and Bioenergetics, 4, 257—262.