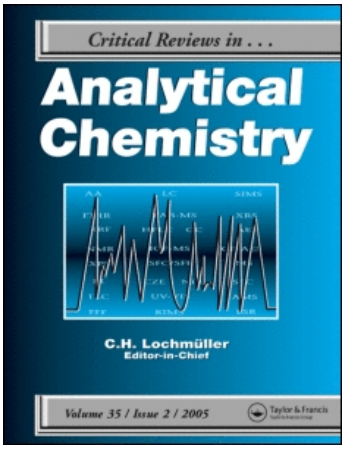

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CHEMILUMINESCENCE AND BIOLUMINESCENCE ANALYSIS: FUNDAMENTALS AND BIOMEDICAL APPLICATIONS

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I. INTRODUCTION

A. Current Status

Analytical methods based on chemiluminescence (CL) and bioluminescence (BL) are not widely used in clinical laboratories at present. However, they are receiving close attention as possible methods in the future. Interest is particularly focused on four systems: the firefly reaction which is widely used to determine adenosine triphosphate (ATP), bacterial BL for measuring the reduced form of nicotinamide adenine dinucleotide (NADH), the luminol reaction, and chemiluminescence accompanying phagocytosis. Together these reactions offer a wide range of analytical possibilities. They can be coupled to other analyses via enzymes that generate or consume ATP, NADH, or hydrogen peroxide. In addition, they can be used for immunological assays by labeling antigen or antibody with one of the reactants required for light production. Several other reactions also offer possible clinical application. Examples include peroxyoxalate CL for peroxide analysis and aequorin BL for measuring free calcium. Once CL and BL methods become established in the clinical laboratory, these other reactions are more likely to gain wide-spread acceptance.

There are several reasons for the recent surge of interest in CL and BL analysis. One is the need for alternatives to the widespread use of radioisotopes as labels in immunological assays. This requires a label that can be measured at extremely low concentrations. CL and BL are among the few methods that have sufficiently low detection limits to compete with radioisotopes in a significant number of assays. CL and BL methods can also potentially be used to increase sample throughout for high volume analyses in clinical labs and to minimize required sample volumes for certain assays. Another important reason for increased interest in CL and BL is the recent commercial availability of improved instrumentation and reagents. Several companies including Packard, LKB, LUMAC, Analytical Luminescence Laboratories, and Jobin Yvon have all introduced new instruments in the last couple years. Some of these new instruments offer features such as automatic injection and temperature control which make it possible to achieve improved precision relative to the older instruments. Also, it is now possible to purchase completed automated systems for CL and BL analysis. The improved instrumentation has been accompanied by improved reagents. Purified firefly and bacterial reagents are now available from several companies. The purified reagents are stable for a day or more at room temperature and lead to lower detection limits if sufficiently sensitive light detection is used. They modify reaction kinetics so that the light-intensity decays more slowly with time which simplifies coupled analyses in which ATP or NADH is consumed or generated by a second reaction.

B. Other Sources of Information

The field of chemiluminescence and bioluminescence has generated an unusually large number of reviews. The specific topic of applications of CL and BL in clinical analysis has been reviewed three times within the past two years.[1-3] A number of other reviews have covered general analytical applications of CL and BL.[4-15] The review by Strehler,[16] while dated in some respects, contains the most complete information on analytical aspects of the firefly ATP assay. Proceedings of symposia on applications of the firefly ATP assay[17] and on analytical applications of bioluminescence and chemiluminescence[18] are also available. The latter proceedings contain a large number of articles on clinical applications and will be extensively cited in this review. In addition, papers presented on CL and BL at the 11th symposium on Advanced Concepts in Clinical Chemistry at Oak Ridge, Tenn. are published in Clinical Chemistry.[19]

A particularly useful source of information is Vol. 57 of Methods of Enzymology.[20]

This contains articles on both fundamental and applied aspects of CL and BL written by experts in the field. Many of the articles summarize the various subfields in CL and BL providing an excellent introduction to these fields as well as directing readers to original references.

Other valuable references on fundamental aspects of CL and BL include Gundermann's book on chemiluminescence[21] and in-depth reviews of the firefly reaction[22] and bacterial BL.[23,24] The proceedings of a 1973 symposium on CL and BL have been published[25] as are the proceedings of a more recent symposium.[26] Several reviews of CL and BL have appeared in Photochemistry and Photobiology[27-31] as well as elsewhere.[32] Other reviews of interest deal with luciferin structure[33] and a new proposed mechanism for many light-producing reactions.[34]

C. Objective of This Review

This review will concentrate on analytical aspects of CL and BL, i.e., factors affecting precision, accuracy, convenience, detection limits, sensitivity, etc. The principles and instrumentation for CL analyses will be considered in some detail, as well as the analytical characteristics of important reactions. In reviewing applications, I will attempt to point out situations in which various analytical considerations become important.

The principles of CL analysis apply equally to both gas and liquid phase analyses. The instrumentation and applications sections deal only with liquid phase reactions. It should be recognized that there are also many important applications of gas phase CL.

II. PRINCIPLES

A. Origin of Chemiluminescence and Bioluminescence

Chemiluminescence is observed when a chemical reaction yields an electronically excited product which either luminesces or transfers its energy to another molecule which then luminesces. This may be represented:

$$A + B \longrightarrow C^* \qquad \text{(Reaction 1)}$$

$$C^* \longrightarrow C + h\nu$$

where A and B are reactants and C is the excited state product. Three conditions are generally cited as requirements for a reaction to produce CL. These are: (1) sufficient energy to produce an excited state product, (2) a reaction pathway favorable to excited state production, and (3) the electronically excited product must either itself luminesce or transfer its energy to a luminescent molecule.

The chemiluminescence efficiency, ϕ_{CL}, depends on the efficiency of excited state production, ϕ_{ex}, and the luminescence efficiency, ϕ_L, which should include the efficiency of any energy transfer process:

$$\phi_{CL}\left(\frac{\#\text{ photons emitted}}{\#\text{ molecule reacted}}\right) = \phi_{ex}\left(\frac{\#\text{ molecules electronically excited product formed}}{\#\text{ molecules reacted}}\right) \cdot \phi_L\left(\frac{\#\text{ photons emitted}}{\#\text{ electronically excited molecules formed}}\right) \qquad (1)$$

The intensity of CL at any time depends both on ϕ_{CL} and the number of molecules reacting per unit time.

$$I_{CL}\left(\frac{\text{photons}}{\text{second}}\right) = \phi_{CL}\left(\frac{\text{photons}}{\text{molecule reacted}}\right) \times \frac{dC}{dt}\left(\frac{\text{molecules reacted}}{\text{second}}\right) \quad (2)$$

In general, I_{CL} decreases with time as reactant is consumed.

The term bioluminescence is generally used for light accompanying a reaction derived from nature. It may be considered a special case of chemiluminescence. In this review, "chemiluminescence" will often be used in its broadest sense covering both chemiluminescence and bioluminescence. From the point of view of the analyst, the two phenomena are the same.

B. Chemical Analysis Based on Chemiluminescence and Bioluminescence

To use CL or BL for chemical analysis, the reaction is performed under conditions such that the light intensity is a function of the level of reactant to be determined. Most frequently, this is accomplished by adjusting concentrations so that the species to be analyzed (the analyte) is the limiting reactant, i.e., all other reactants are present in excess.

Because CL intensity varies with time as reactants are consumed, it is necessary to initiate CL reactions in a controlled manner to achieve repeatable results. Two basic approaches may be distinguished. The most common method is to rapidly mix the CL reactants and then to measure the resulting CL intensity as a function of time after mixing. We will call this the *batch method.* The batch method is used in most commercial systems for solution phase reactions. The main reason for this is that the batch method minimizes reagent consumption. The other method is to mix CL reactants continuously and measure steady state light output. This approach will be designated the *continuous method.* The continuous method has been used in several automated and semiautomated instruments reported in the literature. It has been the method of choice in commercial instruments for gas phase CL analysis.

The distinction between the batch method and the continuous method for CL analysis is analogous to the distinction between batch and continuous reactors in chemical engineering. The vessel in which a CL reaction is performed is, in fact, a tiny reactor. The distinction between the two is that conventional reactors are designed to maximize product formation while CL reactors should be designed for precise, rapid, and sensitive measurement of light output.

1. Batch Method

In the batch method CL reactants are rapidly mixed and the resulting intensity measured as a function of time. Figure 1 illustrates a hypothetical intensity-time curve. The shape of this curve depends on the kinetics of the reaction as well as any variation in ϕ_{CL} as the reaction proceeds.

$$I_{CL}(t) = \phi_{CL}(t) \cdot \frac{dC^*}{dt}(t) \quad (3)$$

The initial part of the intensity-time curve may also be affected by the mixing process if the mixing process is not fast relative to the rate of the reaction.

The fact that ϕ_{CL} may be time dependent has often been overlooked. In fact, ϕ_{CL} will vary with time if either the reactant or product quenches light emission. As the reaction proceeds the concentration of quencher will change thus affecting the luminescence efficiency. CL efficiency will also change with time if the light emission process involves energy transfer to a reactant or product since the efficiency of energy transfer will vary

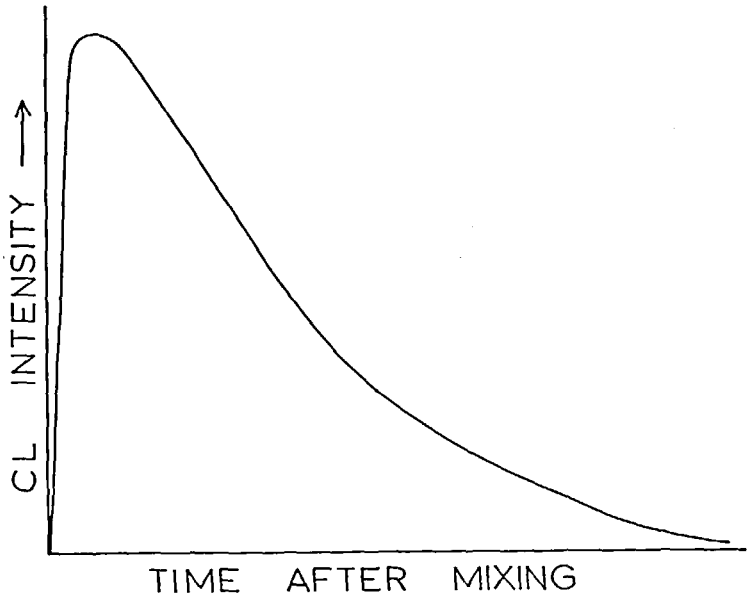


FIGURE 1. Hypothetical curve showing CL intensity as a function of time after the reagents are mixed to initiate the reaction. This is characteristic response for the batch method of CL analysis.

with concentration. Examples of CL reactions known to involve quenchers and/or energy transfer to a reactant include the oxidation of lucigenin[35,36] and reaction of tetrakis-n-alkylamino-ethylenes with oxygen.[37,38]

The variation in $\frac{dC^*}{dt}$ with time depends on the rate law for the CL reaction. If the reaction is first order in analyte, then Equation 3 reduces to the following:

$$I_{CL}(t) = \phi_{CL}(t) \cdot K \cdot [A(t)] \quad (4)$$

where [A(t)] is analyte concentration as a function of time. While the first order case is simple to analyze, most important analytical CL reactions have considerably more complex kinetics.

The intensity-time curve can be analyzed either by measuring intensity at a fixed time after mixing or by integrating intensity over time for part or all of the reaction. One common practice has been to measure the maximum intensity and relate this to concentration. This has the advantages of speed, convenience, and high sensitivity. Also, it minimizes systematic error from unwanted side reactions that generate or consume analyte. This has been a problem for the firefly ATP assay when the luciferase is not highly purified. On the other hand, the maximum intensity may be subject to poor precision due to variations in mixing, particularly if the reaction is fast. Also, since maximum intensity reflects reaction rate, this is a form of kinetic analysis and is subject to the limitations of kinetic analysis. Any factor affecting the reaction rate will affect the maximum intensity. For example, using the published activation energy data,[39] it is possible to estimate that the peak height observed in the firefly ATP assay will vary by several percent/°C. For a detailed discussion of errors in kinetics analysis, a recent article by Carr should be consulted.[40]

Alternatively, the intensity-time curve may be completely or partially integrated. If the

reaction is initiated in the presence of the light detector, the intensity-time curve can be completely integrated. However, some instruments, notably liquid scintillation counters, do not permit *in situ* mixing. There must be an interval between mixing and the start of integration to allow the sample to be placed in the measuring device. The general expression for integrated intensity is:

$$I_{CL\ integrated} = \int_{t_1}^{t_2} \phi_{CL}(t) \cdot \frac{dC^*}{dt}\, dt \qquad (5)$$

where t_1 to t_2 represents the time interval over which intensity is integrated. This expression has been solved for first-order kinetics assuming ϕ_{CL} is constant.[13] It has been used to calculate hypothetical detection limits using assumed values for the rate constant, ϕ_{CL}, detection efficiency and background signal.

If the complete intensity-time curve is integrated, the resulting integral will be proportional to concentration and independent of reaction rate. This is a form of "equilibrium" or "endpoint" analysis and is not subject to error due to factors causing variability in reaction rate. It should, however, be pointed out that the value for ϕ_{CL} may vary with changes in conditions.

If the intensity-time curve is only partially integrated, the resulting analysis is somewhere between pure endpoint and pure kinetic analysis. The effect of reaction rate variations on integrated intensity can only be determined if the rate parameters and the integration interval are known.

2. Continuous Method

In the continuous method of CL analysis, reactants are mixed on a continuous basis using some sort of flow cell. The CL value will reach a steady state when the rate at which reactant enters the cell is balanced by the rate of reactant consumption plus the rate at which reactant exits from the cell. A typical signal from the continuous method is shown in Figure 2. The signal climbs to a steady state value after mixing starts. Variations in the rate of mixing may cause deviations in CL response around the steady state value. This flow cell is, in fact, a tiny chemical reactor. Reactor theory is a well-developed subject,[41,42] although the fact that it is applicable to CL flow cells does not seem to be generally recognized.

The behavior of real chemical reactors is a complex subject well beyond the scope of this review. Real reactors fall somewhere between two idealized extremes, which are amenable to simple mathematical treatments. These are the continuous stirred tank and the plug flow reactor, illustrated in Figure 3. In the continuous stirred tank, it is assumed that mixing is so efficient that the reactor solution is homogeneous. In the plug flow reactor it is assumed that mixing occurs at the base of the cell and the mixed solution proceeds through the cell with no lateral mixing. The characteristics of these two ideal types of CL flow cells have been compared.[43] It was concluded that the plug flow reactor offers somewhat better sensitivity and faster response than the continuous stirred tank type of cell. To approximate a plug flow reactor, one should use narrow diameter tubing for the flow cell. To be compatible with detector geometry, it generally needs to be coiled in the form of a spiral.

The continuous flow method can be either kinetic or equilibrium based. If the reaction is fast relative to residence time in the flow cell, it is possible to observe all the light emitted. In effect, the entire intensity-time curve is integrated. This corresponds to an equilibrium analysis. On the other hand, if the reaction is slow relative to flow cell residence time, then much of the light occurs after the sample has left the cell. In this case,

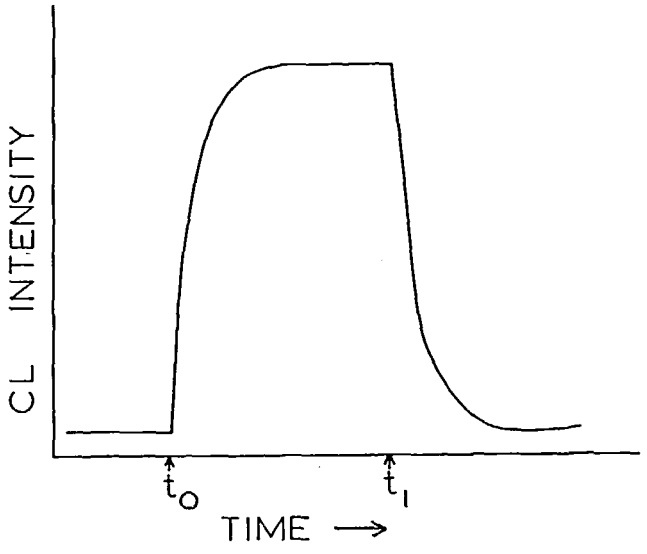


FIGURE 2. Hypothetical signal show CL intensity vs. time using the continuous method of CL analysis; t_o is the point at which reactants and analyte start to mix on a continuous basis; t_L is the point where analyte is no longer mixed with the other reactants.

the measured signal depends on reaction rate and the method is kinetic. This subject has been considered in more detail.[43]

Since continuous measurements involve a steady-state, they do not require any timing considerations. On the other hand, the shape of the intensity-time curve obtained in batch mode measurements may provide information on the light-producing process that is not available in the continuous measurement.

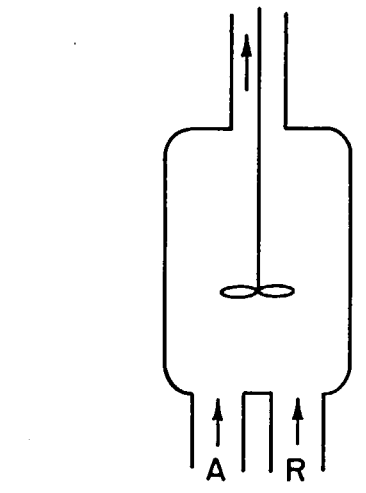
3. Heterogeneous Reaction Systems

There are a number of CL analyses reported in the literature which involve contact between two distinct phases. Examples include the use of immobilized enzymes as solid phase catalysts for solution reactions[44,45] and the measurement of concentrations in the gas phase by contacting the gas with a solid or liquid reactant phase.[46,47] In these systems, the CL intensity depends not only on the kinetics of the light-producing reaction but also on the efficiency with which the mass transfer processes bring the necessary reactants to the interface between the two phases.

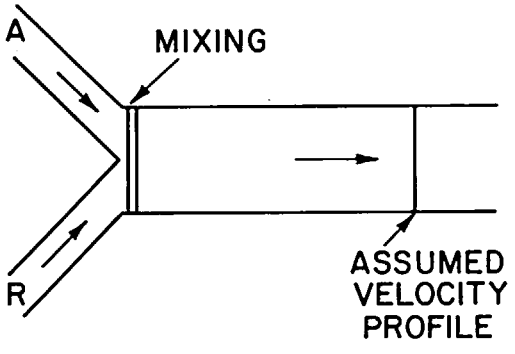
Theory relating chemiluminescence intensity to reaction rate and mass transfer efficiency has been developed for the system where convection is the primary means of mass transfer in the analyte phase, reaction kinetics are first-order in analyte, and the analyte can diffuse from solution into the interior of the reactant phase.[48] In this system steady-state intensity will be established when the rate of analyte reaching the reactant phase equals the rate at which analyte reacts. The intensity is directly proportional to analyte concentration.

$$I_{CL} = \phi_{CL} \frac{\sqrt{KD_c}\ A\ (D_c/\delta)\ C_{bulk}}{\sqrt{KD_c} + D_c/\delta} \qquad (6)$$

where I_{CL} is steady state CL intensity, K is the first order rate constant for the light generating reaction, A is the interface areas, D_c is the diffusion coefficient of the analyte



A



B

FIGURE 3. (A) *Continuous stirred tank.* It is assumed that stirring is so efficient that the reactor solution is completely mixed at all locations. In practice, there will always be an excess of reagent R, near the reagent inlet and an excess of analyte A, near the analyte inlet. (B) *Plug flow reactor.* It is assumed that mixing occurs at the base of the reactor, and solution moves through the reactor as a "plug" with no lateral mixing. In practice there will always be some lateral mixing since solution near the walls moves more slowly than solution in the center of the reactor.

in the solution phase, $\overline{D}_c$ is the diffusion coefficient for the analyte in the reactant phase, C_{bulk} is the bulk concentration of analyte and δ is the thickness of the Nernst diffusion layer, i.e., the thin layer of solution at the phase boundary that is assumed to be unstirred. This equation does not account for the depletion of analyte in the bulk of the solution as the reaction proceeds. This would be a significant consideration if the reaction kinetics are fast, stirring efficient and the interface area is large. Equation 6 is easily modified to account for the depletion of bulk concentration.

$$I_{CL}(t) = \phi_{CL} \frac{\sqrt{K\overline{D}_c}A(D_c/\delta)}{\sqrt{K\overline{D}_c} + D_c/\delta} C_{bulk} \exp\left(\frac{-\sqrt{K\overline{D}_c}A(D_c/\delta)}{\sqrt{K\overline{D}_c} + D_c/\delta)V} t\right) \quad (7)$$

The only new term is V, the volume of the analyte solution.

Equations 6 and 7 can be reduced to simpler expressions under certain conditions. If the reaction rate is fast relative to mass transfer, then $\sqrt{K\bar{D}_c} >> D_c/\delta$ and the $\sqrt{K\bar{D}_c}$ terms cancel out. Under these conditions the CL intensity is controlled only by mass transfer. Even though the measured intensity reflects a reaction rate, it will be independent of small changes in rate. The opposite situation can also occur in which mass transfer is fast relative to reaction kinetics, i.e., $D_c/\delta >> \sqrt{K\bar{D}_c}$. In this case, the D_c/δ terms cancel and steady state intensity depends only on $\sqrt{K\bar{D}_c}$. In this situation, the steady state signal varies with K, i.e., even though the signal is kinetically controlled, it is less subject to fluctuation in reaction rate than a normal homogeneous kinetic assay in which the signal depends directly on the rate constant.

The system in which analyte solution is stirred but the analyte cannot diffuse into the reactant phase has not been considered in the literature. It is similar to above system, however. The following expression for steady state CL intensity is readily derived.

$$I_{CL} = \phi_{CL} \frac{K_h A (D_s/\delta)}{K_h + D_s/\delta} C_{bulk} \quad (8)$$

In this equation, K_h is a heterogeneous rate constant (units are cm/sec). As above, if mass transfer or kinetic control predominates, then the equation is simplified by neglecting the appropriate term in the denominator.

Unstirred analyte phases in which diffusion is the primary means of mass transfer are also possible. In these systems, CL intensity does not reach a steady-state. Instead intensity decreases with time as reactants are consumed at the interface between the two phases. It is the reviewer's opinion that convection is the preferred means of mass transfer in a two-phase system. It is more efficient than diffusion, yielding a larger signal. Also, it reaches a steady-state which is readily measured.

Aside from Reference 48, the theory of two-phase CL systems has not been considered. However, the system is similar in many respects to electrochemical measurements and to chemical reactors that are packed with a solid catalyst. Many of the problems that can be posed with regard to two-phase CL systems can be solved easily from solutions to related problems in electrochemical and/or reactor theory.

4. Coupled Reactions

Many applications of CL and BL involve coupled reactions. The analyte is generally an enzyme or substrate in a reaction or a series of reactions that either generates or consumes a reactant required for CL and BL. Nonenzymatic coupling reactions are also possible.

There are two distinct approaches to coupling reactions. The most common approach is to perform the coupling and analytical reactions suquentially. The reaction generating or consuming CL reactant is allowed to proceed under controlled conditions. This reaction mixture is then analyzed for the CL reactant. It may also be necessary to analyze the reaction mixture to determine the amount of reactant initially present before the coupling reactions are initiated. The sequential approach can be performed using either the batch or the continuous method. Either method can be automated. Using the sequential approach, it is possible to manipulate solution variables such as pH so that the coupling and analytical reactions occur under different conditions. If the analytical reactions and the coupling reaction(s) do not proceed under the same set of conditions, then the reactions must be performed sequentially. For example, the luminol reaction can be used to determine hydrogen peroxide which in turn can be generated by various coupling reactions.[49] The sequential approach is necessary in these systems

because the luminol reaction requires high pH for efficient light production while the coupling reactions proceed most rapidly at neutral pHs.

The other approach is the continuous monitoring method. The coupling reaction and the analytical reaction are performed simultaneously. Originally, it was developed to follow phosphorylation upon irradiation of chloroplasts with light.[50] However, it has been used with a variety of other systems. Schematically, the continuous monitoring method may be represented:

$$A + B \longrightarrow C \qquad \text{(Coupling Reaction)} \qquad \text{(Reaction 2)}$$

$$C + \text{Excess Reactants} \longrightarrow \text{light} \qquad \text{(Analytical Reaction)} \qquad \text{(Reaction 3)}$$

The kinetics of coupled reactions have been considered elsewhere.[51] These equations can be easily modified to calculate intensity-time curves, knowing the rate equations and rate constants for both reactions. The case of two first-order coupled reactions yielding CL has been specifically considered including effects of side reactions.[52]

The kinetics of the analytical reaction relative to the coupling reaction are critical in determining the intensity-time response. Two extreme situations may be distinguished. If the analytical reaction is very slow relative to the coupling reaction, then the concentration of intermediate will not be depleted by the analytical reaction and the CL signal will reflect the *concentration* of intermediate as a function of time. This situation can be approached in coupled analyses involving ATP by running the assay under conditions such that ATP consumption is negligible.[53]

The other extreme situation is when the analytical reaction is fast relative to the coupling reaction. In this case, reactant C is consumed as soon as it is formed by the coupling reaction. Therefore, the CL signal reflects the *rate* at which the coupling reaction produces reactant C. As will be discussed in Section IV-B-1, NADH analysis using bacterial BL approximates this situation.

Continuous monitoring is most easily implemented if the coupling reaction generates CL analyte. It is also possible if the coupling reaction consumes CL analyte, provided the analytical reaction is slow relative to the coupling reaction. In this case, CL intensity decreases more rapidly with time than in the absence of coupling reaction.

The response of a coupled reaction system may change with time as the CL reaction proceeds due to reagent depletion, catalyst inhibition, generation of a quencher or other effects. One approach to this problem is to correct for it by developing an expression for the change in CL intensity with time and then using a standard addition of CL reactant to determine response at some point in time after the coupled reaction scheme is initiated.[54] However, it is better, if possible, to choose reaction conditions where these effects are avoided or at least minimized. Coupled analyses employing the continuous monitoring approach have been most often implemented involving the firefly ATP assay as the analytical reaction.

Where feasible, the continuous monitoring approach offers several significant advantages over the sequential approach. The continuous monitoring approach requires fewer operations and a single reagent (since all necessary reactants are combined in one reagent). It generates information continuously rather yielding a single data point. As microprocessor-based CL instruments become available, it may be possible to routinely employ continuous coupling schemes involving complex kinetics (See Section III. B. 5.).

5. Competitive Binding Assays

Any component of a CL reaction can serve as a label in assays based on competitive binding. The procedures are analogous to those for more common types of labeling such

as fluorescence and radioisotope. The final determination of the amount of bound or free label is performed by adding the remaining required reactants for CL and measuring the resulting intensity. Because of its low detection limits, CL labeling is a possible alternative to radioisotopes in a large number of assays.

CL labeling is more difficult and complex than other types of labeling. Radioisotope labels are readily incorporated into a variety of chemical structures. The radioactivity is, of course, totally unaffected by the chemical state of the isotope. Similarly, fluorescent and enzymatic labels can generally be coupled to various analytes with relatively little change in their properties. With CL labels, the problem is by no means so simple. First, the number of available reactions yielding efficient CL is relatively small, limiting the number of potential labels. More importantly, covalent coupling to another molecule necessarily requires a change in chemical structure. This can affect the CL reaction in three ways: it can modify θ_L, the luminescence efficiency, θ_{ex}, the excitation efficiency, and/or it can modify the reaction rate. Bioluminescence reactions in particular are usually highly specific for certain structures, making it difficult to design labels that yield efficient light. Because of this plus the high expense of most BL reagents, generally useful CL labeling schemes are more likely to be derived from non-BL light-yielding reactions. In work to date this has proven to be the case. Labeling schemes based on luminol have proven to be more successful than schemes based on firefly or bacterial BL.

Excitation and luminescence efficiencies as well as reaction rate are also likely to be sensitive to the environment of the labeled reactant. Thus, there are a number of possible effects that may make it possible to distinguish bound and free labeled analyte without a separation. It has, in fact, been possible to design homogeneous competitive binding assays using luminol as a label (See Section IV.C.4.a.). The possibility of a change in reaction rate is particularly interesting, since this would make it possible to measure both free and bound label from a single intensity time curve. The feasability of such a system remains to be demonstrated although it is possible to kinetically resolve two processes to analyse two components from a single intensity time curve.[55]

If CL labeling is to be used on a large-scale basis, it will be necessary to improve the precision routinely attainable in typical CL measurements. This is illustrated in Figure 4 which shows a standard curve for a hypothetical competitive binding assay in which bound label is measured by CL. As the figure shows a small uncertainty in the CL measurement leads to a large relative uncertainty in analyte concentration, particularly at either end of the operating range. At present, high precision is not routinely attainable in many CL measurements.

It is also feasible to use CL to measure the activity of an enzyme label. For example, glucose oxidase could be a label which would catalyze the formation of hydrogen peroxide to be determined by the luminol reaction. The sensitivity of CL would permit a significant reduction in time and/or reagent required. However, precision again is a problem given current practices. This is particularly true for homogeneous competitive binding assays using enzyme labels since the activity of free label usually does not differ that much from the activity of bound label.

6. Optimizing CL Analyses — General Comments

The optimum way to do a particular analysis is a complex function of the characteristics of the particular method and the demands of the application with respect to accuracy, precision, sensitivity, speed, convenience, cost, etc. It is, however, possible to make a number of generalizations about ways in which the optimization of CL analyses differs from the optimization of other types of analyses.

- The reaction kinetics are a crucial variable influencing precision, sensitivity, speed, and cost. They also determine the form of the output in coupled assays using continuous

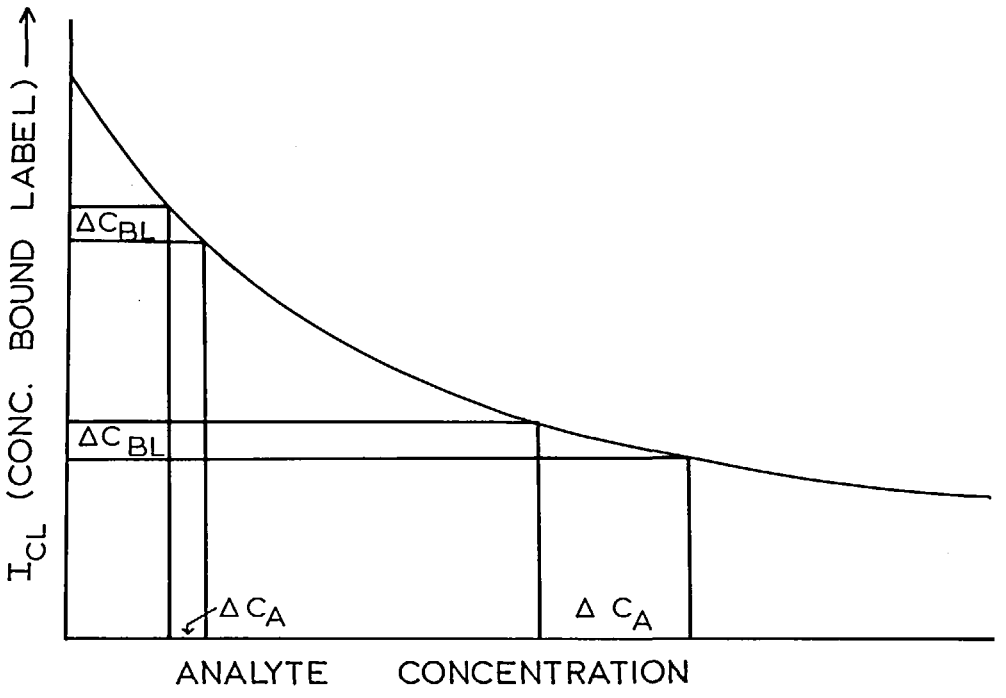


FIGURE 4. Hypothetical standard curve for a competitive binding assay with CL detection of bound label. ΔC_{BL} represents ± 3% uncertainty in the CL measurement. ΔC_A is the corresponding uncertainty in analyte concentration.

monitoring. Thus, a significant part of an optimization procedure would be to find reaction conditions yielding kinetics most suited for a particular application. This has been largely overlooked although there are examples in the literature in which the kinetics have been manipulated for analytical purposes. The paper describing a study of the best conditions for continuous monitoring of ATP is an example of a situation where the kinetics of the analytical reaction were a crucial variable.[53] Another interesting example of kinetic manipulation is the use of low temperature to slow down the firefly reaction so that the decay in intensity is very slow.[56] This makes it possible to measure CL intensity using a liquid scintillation counter without having to rigorously control the time interval between initiation of the reaction and performing the measurement.

• Instrumentation is often a relatively unimportant factor in optimizing CL assays. It is, of course, necessary to have adequate sensitivity to precisely measure CL intensity, but this is rarely a problem for most of the analyses covered in this article. In fact, if photon counting is employed, too much sensitivity may be a problem since dead time loss becomes a serious source of error at high count rates. The most significant instrumental variable is the method of mixing the reactants, which affects the repeatability of analytical results. In most practical situations, the mixing method is established by the available instrumentation. Given a particular mixing method, the kinetics of the reaction can then be modified to assure that variations in mixing will not cause unacceptable losses in precision. Temperature control is also an important instrumental consideration if the measured response is kinetic.

• Detection limits for important CL and BL assays are much lower than required for many applications. Thus, it is often possible to modify the assay to reduce sensitivity in return for some other benefit such as very slow kinetics.

III. INSTRUMENTATION

Modest instrumentation requirements are one of the principle advantages of CL and BL methods. The required components for a CL photometer are a light detector, a cell, provisions for mixing the reactants, and electronics to read out and process data. Spectral resolution is rarely required. Commercial instruments designed for CL and BL measurements are available at reasonable costs. Many instruments designed for other purposes include the necessary components for CL and BL measurements. Liquid scintillation counters, in particular, have been widely used for CL measurements. Because of the simplicity of CL instrumentation, construction of home-made instrumentation is possible without requiring highly sophisticated support services.

A. Nomenclature

Most instruments designed for measuring CL and BL are correctly called photometers since they use photoelectric detection but don't have a monochromator for wavelength discrimination. Recently, the term *luminometer* has been used. This suggests an instrument designed to measure luminescence. Since luminescence includes fluorescence, phosphorescence, and other types of light emission in addition to CL and BL, *luminometer* is somewhat misleading. Commercial instruments have generally been designated by trade names such as Biocounter® and Chem-Glo® Photometer.

B. Components

1. Detectors

Photoelectric detectors are widely used for measuring CL. They are based on the photoelectric effect, i.e., the fact that incident photons can eject electrons from certain materials. In a simple phototube, one measures the current associated with electrons ejected from a photocathode. A photomultiplier incorporates several internal amplification stages so that one ejected electron produces a cascade of electrons, i.e., a short burst of current.

For many applications, CL is sufficiently intense so that any photomultiplier tube responding to visible light will provide satisfactory response. In fact, it is often possible to use less expensive and less sensitive semiconductor detectors. However, when dealing with very low light levels, photomultiplier selection can become an important consideration affecting measurement precision. To maximize signal-to-noise, one should select a photomultiplier with a high sensitivity-to-dark current ratio and with adequate spectral response at the CL wavelengths.

Two detection modes are possible. If the CL signal is intense, one can measure the analog current from the detector. For small signals, the signal-to-noise ratio can be improved by photon counting, i.e., counting the number of current spikes that occur when photons eject electrons from the photocathode.[56] Photon counting is illustrated in Figure 5. To be counted, a current pulse must exceed the level set by the discriminator. Since some of the processes producing dark current yield smaller current pulses than incident photons, this portion of the dark current can be selectively rejected by appropriate setting of the discriminator level. The number of dark counts can be further reduced by operating the tube at reduced temperature. While photon counting makes it possible to measure lower light levels, it does cost more to implement than analog detection. Also, photon counting is limited to relative low intensities. If count rates become too high, separate pulses cannot be distinguished by the detection electronics and counting errors result. Typically, counting rates can go up to about 10^6/sec before errors become significant. Photon counting is inherently an integrating method since one measures the number of counts over a preset time interval. However, if the

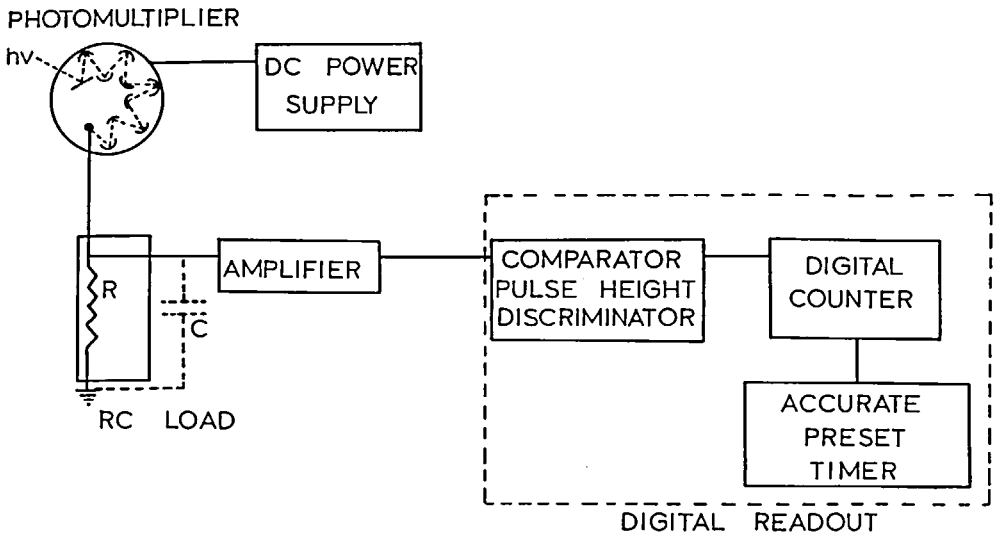


FIGURE 5. Block diagram of photon counting equipment.

count rate is short relative to intensity decay, then the number of counts is effectively a measure of instantaneous intensity. It is possible to construct an intensity time curve by measuring the number of counts for successive time intervals after initiating the reaction.

If CL intensity is low, then the principle source of variation in the CL signal will be statistical fluctuations in the rate of arrival of photons at the photocathode. In this situation, an expression for the signal-to-noise (S/N) ratio can be derived (57)

$$\frac{S}{N} = \frac{R_S^{1/2}\, T^{1/2}}{(1 + 2\, R_B/R_S)^{1/2}} \qquad (9)$$

where R_S = signal count rate, R_B = background count rate and T = observation time. The signal-to-noise ratio increases with the square root of the observation time if intensity is constant. The detection limit may be defined as the concentration at which S/N = 3. In practice, however, for many reactions the detection limit is established by variations in background light emission in the absence of added analyte. If the background emission is fairly intense, then statistical fluctuations in photon flux are not significant sources of noise. Photon counting is available in at least one commercial CL photometer (Biocounter from LUMAC, Inc.)

2. Cells

The only requirement for CL cells is that they be reproducible optically and that they be reproducibly positioned with respect to the detector. Processes such as scattering and reflection are of no consequence as long as they occur to the same extent for the measurements of sample and standard light intensity. If one uses the same cell in the same position for all measurements, then there will be no cell variations from measurement to measurement. However, many instruments designed around the batch mixing approach use cells on a disposable basis. In this case, variations in the optical properties of cells may be a significant source of imprecision. The reviewer is not aware of any data on this effect for CL. However, for fluorescence analysis using a centrifugal fast analyzer

it was found that cell deviations were responsible for a variation of 6% relative standard deviation.[58] The reviewer has taken a careful look at the glass culture tubes that serve as cells for one of the commercial instruments. Optical imperfections are clearly seen as the cell is turned in front of a light. Almost certainly cell variations are sufficient to make a significant contribution to imprecision. Plastic cuvettes are more reproducible than glass.

3. Geometry Considerations

The detection efficiency of a CL photometer may be defined as the ratio of the light striking the detector to the total light emitted. The relationship between detection efficiency and the relative positioning of CL cell and detector has been considered in detail.[59,60] In practice, high detection efficiency is achieved by placing the cell very close to the detector. If the CL intensity is low, detection efficiency can be further increased by placing a focusing element behind the cell to direct light onto the detector as illustrated in Figure 6. The primitive technique of coating the back of the cell white is quite effective. Placing a reflective surface on the interior of the cell is even better. The effect of cell depth and reflectance on CL signal has been considered theoretically and experimentally.[61] In addition, an expression has been derived to account for the effect of sample absorbance in this paper.

4. Mixing Methods

Many different approaches to mixing have been employed. For batch analyses, the sample can be added gently to reagents and swirled or mechanically stirred to achieve complete mixing. Alternatively, sample and/or reagents can be added to reagents with sufficient force to induce mixing without further stirring.

In continuous analysis, streams of reagent and sample flow together. Mixing can be accomplished by natural hydronamic processes or by using special devices to accelerate mixing.

A given mixing method may not work equally well for all reactions. Mixing becomes particularly difficult for reactions involving mixed solvent systems.

5. Electronics

The electronics involved in transducing small photomultiplier currents will not be covered here except to point out references describing photometer electronics in some detail.[62,63] The topic of interest is the impact of rapid developments in integrated circuit technology which have reduced the cost of microprocessors so that they can be economically incorporated into CL photometers. Some of the newer commercial photometers include microprocessors or computer interfaces and offer considerably more versatile data analysis than earlier instruments. This is a development which is sure to continue.

Photometers equipped with microprocessors digitize and store the intensity-time curve as a series of data points. From these points it is a simple matter to calculate the following:

1. Integrated intensity over any time interval designated by user.
2. Maximum intensity.
3. Intensity at any fixed time after mixing.
4. Slope or rate of intensity change at any time after mixing.

Several intensity-time curves can be analyzed and the data stored. These can then be manipulated to do the following kinds of calculations:

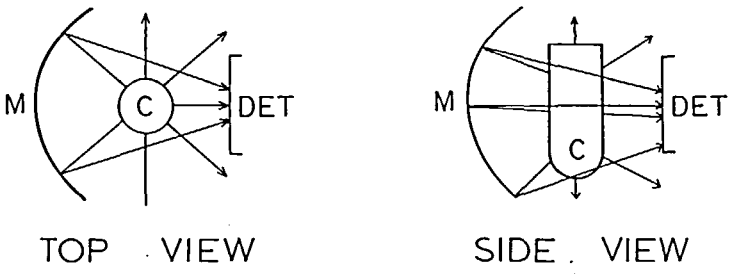


FIGURE 6. Diagram of optics for improving detection efficiency of CL measurements. C = cell containing sample; M = mirror serving as focusing element; and DET = active detector surface.

1. Subtract out blanks.
2. Calculate standard deviations for replicate measurements.
3. Calculate concentration in the unknown from CL signals for the unknown and standards.

The above examples are obvious applications of microprocessor technology to the analysis of CL data. Other possibilities may not be as obvious. For example, using earthworm bioluminescence for hydrogen peroxide analysis, the shape of the intensity-time curve has been analyzed to determine if interferences are present.[64] This may also be applicable for other reactions performed on a batch basis. Another possible application of microprocessors would be to calculate the total integrated intensity from the initial part of the intensity-time curve, providing the rate law for the reaction is known. This is analogous to the approach to kinetic analysis recently demonstrated by Pardue and co-workers.[65,66] It has the advantage that the total integrated intensity is independent of reaction rate even though the individual measurements do vary with rate.

Microprocessor calculations may also potentially be used to interpret curves involving coupled reactions run in the continuous monitoring mode when the intensity is a complex function of both the relative rates of the two reactions and the depletion of CL reagents.

C. Instrumental Standards

It is not common practice to determine absolute response of a CL photometer to a known intensity. However, it can be readily done and would provide a basis for comparing instruments. From the absolute value of the signal with a known light intensity and the background signal in the absence of incident light, the minimum detectable light intensity can be calculated. This would be of considerable value in evaluating the suitability of various instruments for low-level measurements.

The luminol reaction performed under a specific set of conditions can serve as secondary light standard.[67] This is a convenient system for standardizing photometers. Seliger has discussed in detail procedures involved in absolute calibration of CL measurements.[68]

Instead of luminol, it is possible to use a scintillating solution as a standard light source. A scintillating solution includes a radioactive isotope in the presence of fluorophors which effectively convert the energy of radioactive decay to a pulse of photons. The absolute flux of a carbon-14 scintillating solution has been determined.[69] Commercially available C-14 scintillators have been used in a comparison of commercial CL photometers.[70]

These solutions are not suitable as standards for photon counters because they yield

multiphoton pulses. However, by adding a quencher to reduce fluorescence efficiency it is possible to reduce emission intensity to a single photon per radioactive decay. This has been done using tritium in toluene with chloroform as the quencher.[71]

Scintillation standards emit essentially constant light intensity with time. Thus a single solution can be used to standardize several instruments or to check day-to-day stability of a single instrument. It could also potentially be used to determine how much cell-to-cell variability affects precision.

D. Instruments For Measuring Chemiluminescence

1. CL Measurements on Instruments Designed for Other Purposes

a. Liquid Scintillation Counters

A liquid scintillation counter (LSC) is, in essence, a two-channel photon counter with a variable discriminator. The sample is placed immediately between two detectors such that optical efficiency is high. As a radiation counter, the LSC is operated in the coincidence mode. A count is only registered when photon simultaneously strike both photocathodes. Thus, radiation decay processes that generate a burst of photons will cause a signal to occur at both detectors essentially simultaneously and will be counted while dark and background pulses will not generate a count except in the relatively improbable case that they occur simultaneously.

When the LSC is used to measure CL, the coincidence feature is normally disabled and the LSC is operated as a straight photon counter. The discriminator level is adjusted to transmit single photon events and reject small dark pulses. In this mode, normally only one photomultiplier is used although some LSCs allow the use of both photomultipliers in the noncoincidence mode, thus doubling the effective count rate. It should be noted that for CL detection the photomultipliers in the LSC must have appropriate response characteristics for the reaction understudy.

The use of LSCs does place several constraints on the CL measurement. The LSC has no provisions for *in situ* mixing. Instead, the reaction must be initiated externally and then placed in the counter. This usually means that there must be some provision for controlling the interval between mixing and making the measurement. Alternatively, the reaction can be performed under conditions where the reaction rate is so slow that intensity remains effectively constant over a long interval such that counting can be completed before intensity changes to a significant degree. For the firefly reaction this has been accomplished by cooling to 4° C.[56] Although slowing down the reaction reduces intensity, this is acceptable. Because the LSC is extremely sensitive, count rates are still high enought not to limit precision.

The other limitation of the LSC is that as a photon counter it is limited to relatively low intensities. If the intensity is too high, the phenomena of pulse pile-up and deadtime loss occur causing errors in counting rate and a nonlinear relationship between intensity and total counts. Reducing intensities into a satisfactory range is rarely difficult. It can be done by reducing volumes and/or reactant concentrations, slowing the reaction down, or reducing the fraction of transmitted light. Switching to the coincidence mode reduces the apparent count rate, but the count rate will still be a complex function of intensity if count rates are high. At low count rates, the signal in the coincidence mode is proportional to intensity squared.

Many LSCs are designed for automated counting of large numbers of samples. The scintillation vials are placed in a conveyor belt and are sequentially placed in the counting chamber. Thus, LSCs can be used for automated BL and CL measurements. The mixing still must be done manually. However, a large number of samples can be mixed with reagent in a short period of time and then be placed in the LSC for automated counting.

When using LSCs for CL measurements, phosphorescence from the caps of the

scintillation vials or the vials themselves can contribute to the background count rate causing errors. To minimize this effect, the caps and vials should be dark adapted and kept in dim or red light. Plastic vials are less subject to background than glass. The use of LSCs for BL and CL measurements has been considered in more detail in the LSC literature.[72-75]

b. Spectrophotometers and Spectrofluorometers

Spectrophotometers and spectrofluorometers can be used for CL measurements if the source can be turned off while the detector remains active. However, the detection efficiencies are much lower than with LSCs. Like the LSC, spectrophotometers and spectrofluorometers do not provide for *in situ* mixing so the reaction must be initiated externally unless the instrument is modified.

2. Instruments Designed for CL Measurements — Batch Mode Measurements

a. Instruments Based on the Liquid Scintillation Counter Design

The LSC has been so successful as an instrument for CL measurements that a commercial CL photometer, the ATP-Photometer manufactured by SAI Technology, has been developed based on the LSC design. It has been described in detail in an article on commercial instruments for CL measurements.[70] This instrument uses scintillation vials as cells. Unless specially adapted for *in situ* mixing, mixing must be done externally. However, the problem of controlling the time interval between mixing and measuring has been simplified. A foot pedal is pressed when sample and reagents are mixed. The photometer starts integrating light intensity vs. time after a preselected delay interval allowing the operator to place the scintillation vial in the photometer. The shortest delay interval is 6 sec. This instrument is convenient to use and is capable of high sensitivity and good repeatability. Its principle limitations are that it does not generate a complete intensity-time curve, and it is not suitable for fast reactions. Also, it is not readily amenable to automation. Unlike the LSC, the ATP-Photometer is not a photon counter although output is displayed in digital form.

b. Instruments Providing for In Situ Mixing

Most instruments designed for CL measurements provide for *in situ* mixing so that the reaction is initiated immediately in front of the detector and a complete intensity-time curve can be recorded. The simplest version of this type of instrument is illustrated in Figure 7. A syringe is used to inject reagents into a small vial containing the sample positioned in front of a photo-multiplier. The syringe has to be pushed through a rubber septum which serves to exclude ambient light. The force of injection serves to induce mixing. Most frequently the reagent is injected into the sample although the reverse is also possible. Commercial instruments based on this design have been described in detail elsewhere.[70]

Manual injection of reagents with a syringe is convenient but suffers from several limitations. The force of injection and the exact positioning of the syringe tip will vary from measurement-to-measurement. This will adversely affect precision if the analytical reaction is fast enough so that a significant fraction of the analyte reacts before the solution is homogeneous. Bubble formation upon injection causes scattering of the light which is not reproducible. Also, the force of injection may not be sufficient to inducing mixing if reagents and sample differ in density. For example, the reviewer has found that mixing would not occur when trying to do some measurements involving reagents in organic solvents. The ability to mix different solvents will depend on several factors including viscosities, densities, injection velocity and container shape.

The manual syringe injection system is readily improved. Figure 8 shows two designs.

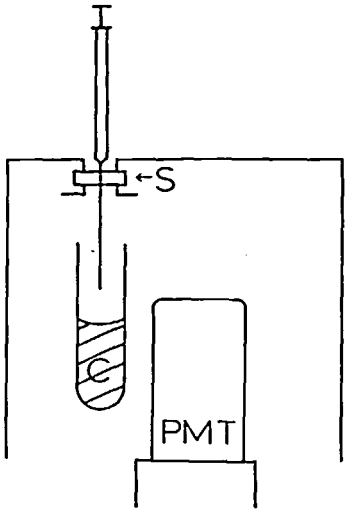


FIGURE 7. Diagram of simple CL photometer with manual reagent injection through septum S into cell C. The cell is roughly the same size as the photocathode of the photomultiplier detector, PMT.

Design A provides for reproducible positioning and force of injection.[76] However, it is not convenient and the entire assembly must be protected from ambient light. Design B is considerably more flexible. It provides for variable volume and injection force by controlling the rate of the motor driven syringe and length of time it runs.[77] Since there is a large reagent reservoir, a large number of consecutive measurements can be made without needing to refill the reagent container. Thus, this type of injection is well suited for automated batch systems. This type of injection can also be adapted to sequentially inject two or more different solutions. This is accomplished by adding extra valves and flow lines so that the different solutions are sequentially drawn into the injector. A system similar to Design B has been compared to manual injection for the determination of luminol by adding microperoxidase and hydrogen peroxide, a reaction with fairly rapid kinetics.[78] The relative standard deviation observed for manual injection was 18% while for automatic injection it was 8%. In this study, the relative standard deviation for light standard measurements was 0.9% indicating that mixing variations were primarily responsible for lack of precision. While neither 18% nor 8% R.S.D. can be characterized as excellent precision, these data do show the improvements obtained from automatic injection. For a slower reaction, the firefly ATP assay, a R.S.D. of 1.5% was obtained for replicate measurements using an injector of Design B.[77] In another study, several automatic injection syringes were compared to manual injection for the firefly ATP assay.[79] Automatic injection gave significantly improved precision. At 5×10^{-7} μg ATP/mℓ, the relative standard deviation for manual injection was 21% while it was 2% for the best automatic injector.

Reproducible *in situ* mixing of reagents and sample can be accomplished in several ways other than the injection systems considered above. Tangential mixing provides for mixing times on the order of a few milliseconds and is routinely employed in the study of fast reaction kinetics by stopped flow techniques. This type of measurement has been employed in fundamental studies of BL and CL reaction kinetics.[39,80] but has been rejected for routine analytical use on the basis of convenience. In fact, however, automated stopped flow instruments designed for analytical purposes have been

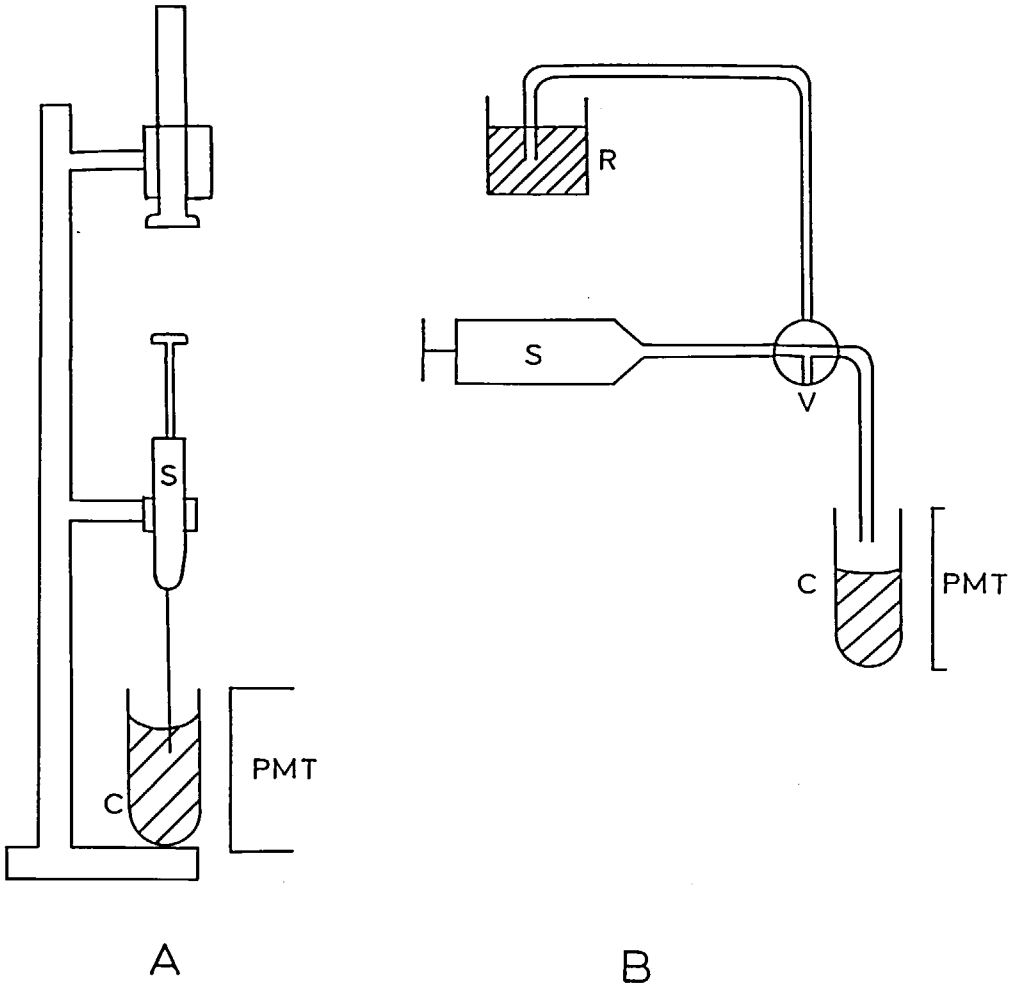


FIGURE 8. (A) Automatic injector design providing for reproducible position and injection force. The rod on top is preumatically released to fall on the syringe S causing injection into cell C. The entire assembly is in a light tight housing with a door to facilitate access. (B) Automatic injector design providing for multiple injections. The syringe, S, is driven by a motor. It is refilled by pulling the plunger out with three-way valve, V, turned so that reagent R is drawn into the syringe.

developed.[81] The precision on these instruments is less than 1% R.S.D. for fast reactions suggesting that use of tangential mixing could improve the precision of analytical CL and BL. Figure 9 illustrates a stopped flow instrument designed for rapid measurement of CL.[82] When the syringes are pulled back, the check valves open allowing reagents and sample to fill the syringes. When the syringes are pushed forward sample and reagents mix and enter a cell from which CL is measured. Carryover from sample to sample can be eliminated by running through one or more rinse cycles in between measurements. Using this type of instrument, it is possible to make either continuous or batch measurements depending on whether the flow is stopped or not.

Another instrument that is well suited for CL measurements is the centrifugal fast analyser.[83] Sample and reagents are placed in separate compartments on a rotor. Figure 10 shows a cross-sectional view of the compartments. As the rotor is turned, centrifugal force drives both reagents and sample into the outermost compartment where

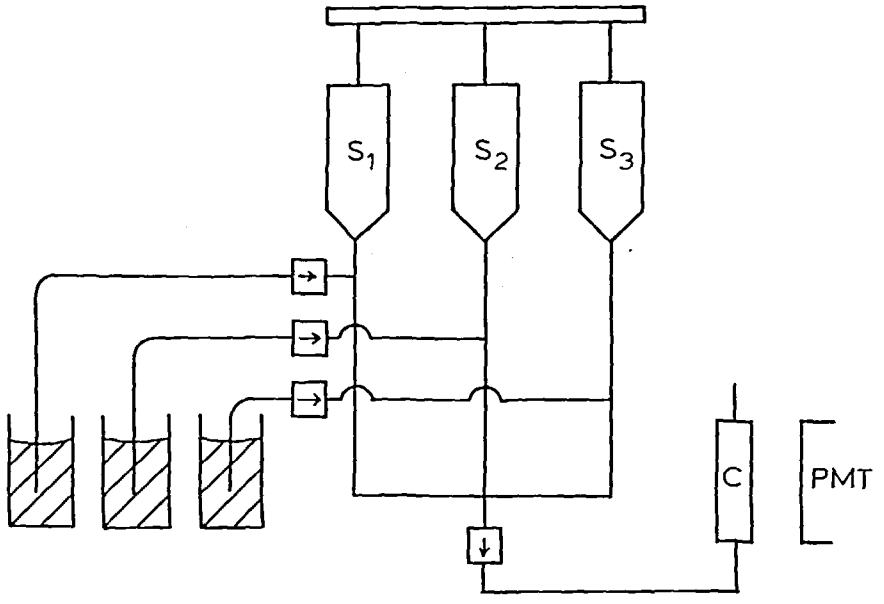


FIGURE 9. Diagram of stopped flow photometer for CL measurements. Check valves (designated ⊣) cause solution to be forced into the flow cell C, when the syringe plungers are pushed forward. When they are pulled back the syringes S_1, S_2, and S_3 are filled with reagent and/or sample. This particular instrument did not employ tangential mixing since it was designed for relatively slow reactions. However, tangential mixing could readily be incorporated. This instrument makes it possible to simultaneously mix three solutions, a useful capability for some reactions in which it isn't possible to prepare a single stable reagent. (Adapted from Steig, S. and Nieman, T. A., *Anal. Chem.*, 49, 1322, 1977. With permission.)

FIGURE 10. Cross section of centrifugal fast analyzer rotor showing compartments for reagents, R_1 and R_2, and sample S. When the rotor is turned, reagents and sample are forced into the outermost compartment where the measurement is made.

they are mixed. Mixing is highly reproducible. As the rotor turns, all outermost channels pass over a measurement port. Each sample spends only a small fraction of its time in the measurement port. Nevertheless, once the rotor reaches full speed, the interval between measurements is quite short and a complete intensity-time curve can be constructed. Centrifugal fast analyzers are available commercially with spectrophotometric detection. For CL measurements, it is necessary to increase detection efficiency and use a more sensitive detector. The centrifugal fast analyzer has been used for peroxide detection using the luminol reaction. [84] Cell-to-cell variability appears to be a factor affecting precision. Samples can be loaded on a standard rotor. The mixing time using the rotor of Figure 10 is fairly long (on the order of seconds). More advanced rotor designs shorten the mixing time.[85]

Flow injection analysis provides still another method for doing CL analyses.[86,87] Figure 11 diagrams the simplest type of flow injection system. A slug of sample is

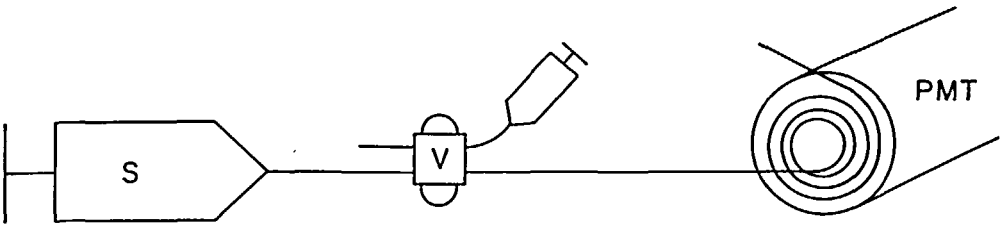


FIGURE 11. Diagram of system for flow injection analysis with CL detection. Syringe, S_1 is filled with reagent. The valve, V, is used to place a slug of sample in the reagent flow line.

interposed in a flowing stream of reagents. Reagents and samples mix as they move down the tubes. The mixing process is a complex function depending on diffusion and the hydrodynamics of fluid flow.[88,89] It occurs in a coil of tubing positioned in front of a detector. The flow injection approach is inexpensive to implement and provides for rapid analysis with relative standard deviations of 2 to 3%. It effectively measures CL intensity only in the first few seconds after mixing, so it is best suited for fast reactions. Reagent consumption can be minimized by using two valves in separate flow lines, one valve for sample and one for reagent. The slugs of sample and reagent then flow together and mix, producing CL. Coupled chemistries are feasible by adding further flow lines provided the coupling reactions are kinetically fast.

The various batch mode CL measurements can be automated. The batch measurement is compatible with the discrete analyzers, i.e., machines that dispense reagents in sequence and move the sample in a conveyor belt or reaction wheel. Instruments of this sort are widely used in clinical laboratories. Their major disadvantage is mechanical complexity which can lead to breakdowns.

Batch mode CL measurements have also been coupled to continuous flow analysis for the automated analysis of bacteria in urine by extracting and measuring bacterial ATP.[90,91] This combination offers the advantages of continuous flow analysis while consuming minimal reagent. However, it presents some difficulties since the reagent must be injected into a cell through which sample is continuously flowing and the injections need to be synchronized with the arrival of sample. Unfortunately, details of the cell and injection method are not provided.

On other automated instrument that should be mentioned is a system designed specifically for measuring the ATP content of cells.[92] All the reactions required to extract and measure ATP are performed on a moving filter tape. The sample is applied by squirting it into the tape which retains the cells but transmits solution. The precision attainable with this system was not specified but is almost certainly more than adequate for the intended application.

c. Instrumentation for Continuous CL Measurements

Continuous CL measurements require a flow cell positioned in front of the detector, a pump to drive the flow and some means of mixing reactants and sample. Figure 12 shows a collection of flow cells used for continuous CL measurements. Cell A is simply a helix manufactured by Aminco for use in continuous flow analysis. The size and shape of the helix matches the photocathode of side window photomultipliers of the popular 1P21 variety. A cell of this design has been used for automated continuous flow ATP analysis.[93,94] In continuous flow analysis, mixing of sample and reagents is brought about by the natural flow patterns in each segment of solution.[95] Thus, there is no need to use any other means to induce mixing. The weakness of this approach is that mixing is

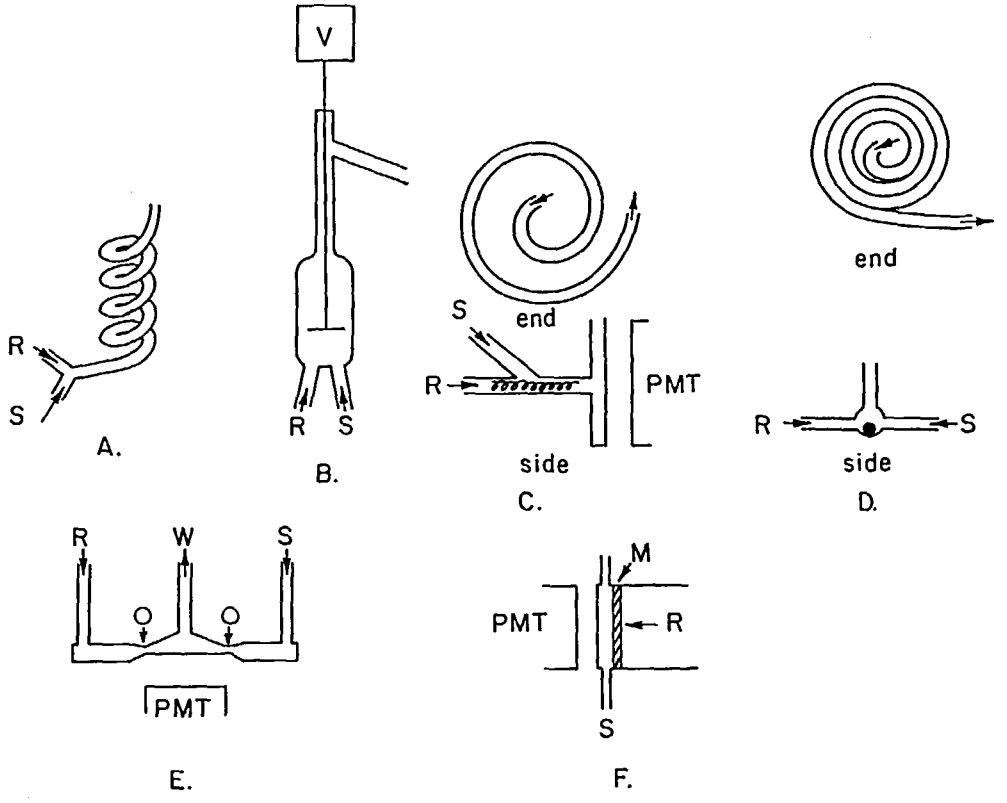


FIGURE 12. Flow cells for continuously mixing reagent, R and sample, S and measuring CL. (A) helical flow cell; (B) flow cell using vibrational mixing (V = Vibrator); The overflow leaves through the sidearm; (C) Spiral flow cell using coiled spring for mixing; (D) spiral flow cell using magnetically activated ball for mixing; (E) Microcell. The points, designated O, are very small orifices so that reagent and sample enter flow cell at high velocity; (F) flow cell in which reagent is slowly pushed through membrane M into a flowing stream of sample.

relatively slow. If one were to remove the air bubbles before detection, there would be a significant interval between mixing and measuring CL. Instead, it has been more common to pump the bubbles directly through the flow cell, causing some discontinuity in the CL signal. Fluctuations in flow accompanying peristaltic pumping may also cause the signal to vary. These problems are of little consequence if the analytical reaction has slow kinetics but are more serious if the reaction rate is fast. This is another example of the influence of reaction kinetics on BL and CL analysis. Precision using these cells has been reported to be about 3% R.S.D. This is rather poor for automated analysis possibly reflecting some of these problems.

Cell B in Figure 12 has also been used in conjunction with continuous flow analysis.[96] The sample and reagent enter the cell separately and are mixed in the cell by a vibrator. Air bubbles are pumped through the cell. As with cell A, this cell will be subject to noise if the analytical reaction is fast.

Cells C and D were both used in conjunction with syringe-driven flow systems in which the sample was separated from surrounding solutions by air bubbles but was otherwise unsegmented. As a consequence, both cells require provisions for mixing sample and reagents. In Cell C, mixing is promoted by a coil of wire which causes tangential flow.[97] In cell D, a small ball is moved magnetically to cause mixing.[98] In both cells, the flat spiral geometry is used in conjunction with the circular photocathode of an end-on photomultipler tube.

Cell E was designed for the measurement of low levels of isoluminol.[78] The sample and reactants enter opposite sides of a small volume (8 μℓ) flow cell. Both solutions are forced through narrow orifices causing them to enter the cell with high velocity so that they mix quickly. Excess solution leaves through the top of the cell. A syringe pump is used to drive sample and reactants through the cell at a constant rate. Alternatively, the flow could be stopped to get an intensity-time curve. An R.S.D. of 2% was achieved for the measurement of isoluminol with flow cell E. This compares with an R.S.D. of 8% for the same chemical system and an automatic injector of the type shown in Figure 8B.

In cell F, a slow flow of reagent is forced through a porous membrane into a flowing stream of sample.[99] This arrangement makes it possible to use reagent very slowly. However, it would probably be difficult to couple it with segmented flow. General aspects of flow cell design have been considered in a book dealing with sampling and mixing techniques.[99]

IV. APPLICATIONS

A. Firefly Reaction

1. Analytical Characteristics

The chemistry of the firefly reaction is summarized below.

$$LH_2 + E + ATP \xrightarrow{Mg^{+2}} E{:}LH_2{:}AMP + PP \qquad \text{(Reaction 4)}$$

$$E{:}LH_2{:}AMP + O_2 \longrightarrow E + L{=}O + CO_2 + AMP + \text{light } (\lambda_{max} = 562 \text{ nm}) \qquad \text{(Reaction 5)}$$

where LH_2 is firefly luciferin, L = O is oxyluciferin, E is firefly luciferase, PP is pyrophosphate and AMP is adenosine monophosphate. Although the firefly reaction can be used to determine Mg^{+2} (100), the analyte of major importance is ATP.

The detection limit for ATP varies for different luciferase preparations. Typical values fall in the range of 10^{-11} to 10^{-14} moles of ATP. The more highly purified the luciferase, the lower the detection limit. With crude luciferase, there is background emission in the absence of added ATP which establishes the detection limit. Purification procedures have been reported by Nielson and Rasmussen[101,102] as well as Green and McElroy.[102a] Response is linearly proportional to ATP concentration over several orders of magnitude.

Crude firefly luciferase preparations contain luciferin. However, addition of further luciferin yields a substantial increase in light output, resulting in a lower detection limit.[103,104] Purification removes luciferin which must be readded before performing the reaction. The synthesis of firefly luciferin and related compounds has been discussed by Bowie.[105] Luciferin is available commercially although it is quite expensive.

Stability is also a function of preparation method. The purified luciferase described by Lundin and Thore[77] is stable for several days at room temperature. Luciferase with similar properties is available commercially now from LKB and Lumac. This material is undoubtedly the reagent of choice for clinical applications, although it is rather expensive. Characteristics of other luciferase preparations are considered in the comprehensive review by Strehler.[16] These preparations are less stable.

The kinetics of the firefly reaction have been studied in depth.[39] After a lag time of about 25 msec, there is a rapid increase in light intensity to a maximum followed by a gradual decrease. The rising part of the curve can be used for ATP analysis,[77] but this will make it more difficult to obtain high precision. The decrease in light intensity with time is caused by inhibition of the luciferase by pyrophosphate and oxyluciferin produced in the

reaction. ATP consumption is minimal. By avoiding high ATP concentrations, it is possible to minimize product inhibition and achieve almost constant light intensity with respect to time over several minutes.[77] Product inhibition can also be minimized by including arsenate in the reaction medium. Arsenate effectively "preinhibits" luciferase so that the pyrophosphate product does not lead to further inhibition. However, since it is an inhibitor, arsenate causes a significant decrease in intensity. Crude luciferase contains contaminating enzymes which may catalyze ATP consumption or production affecting the intensity-time curves and leading to apparent kinetic differences between crude and purified luciferases. A recent summary of firefly reaction kinetics elaborates on the above information and discusses some of its analytical implications.[106]

For all practical purposes, the firefly reaction is specific for ATP. Deoxy-ATP induces weak BL but is not present in practical situations. However, the firefly ATP assay is subject to two kinds of interferences. One type of interference involves unwanted ATP produced by reactions catalyzed by contaminated enzymes in the luciferase preparation. To minimize this type of interference, it has been recommended that the intensity maximum be related to ATP concentration. However, a thorough study of this type of interference has shown that it is not normally a problem with purified luciferase,[77] although it may become a problem in coupled assays requiring the addition of a substrate that can be reacted to ATP. This freedom from interference is an additional reason why commercial purified luciferase is most suitable for clinical application.

The other type of interference affecting the firefly ATP assay is anion and ionic strength effects on the intensity-time curve.[107] Anions such as citrate that bind Mg^{+2} are particularly strong inhibitors of BL. Anion effects can be a source of error in variable matrices such as urine. Anion effects are also an important consideration in choosing a buffer to control the pH of the reaction. Since response remains linearly proportional to ATP concentration, interferences of this sort can be adequately accounted for by using the method of standard additions.

Because of the widespread use of CL instruments with provisions for *in situ* mixing, it is possible to make a standard addition of ATP directly to the reacting sample. This will yield an intensity-time similar to the one shown in Figure 13. The increase in CL intensity upon adding standard ATP is used to calculate the concentration of ATP initially present in the sample. The volume of added standard should be small relative to sample volume to minimize dilution effects on the sample matrice. The affect of dilution on ATP concentration and the effect of decreasing intensity with time can be accounted for in the calculations. This procedure has several benefits. It is convenient since it involves fewer operations than conventional standard additions in which sample is divided into separate portions. Since it is a standard addition method, it automatically corrects for any interference affecting the "slope" of the analytical response. It also achieves some of the benefits of internal standardization. For example, since both measurements are made in the same cell in the same position, variations in cell optical properties will not be a source of error. This procedure is usually referred to as internal standardization in the CL literature. In fact, however, it is more a standard addition method than internal standardization although it provides the benefits of both. It might appropriately be called an *internal standard addition* method.

The internal standard addition method is practical only if the intensity change with time is slow enough to be neglected or adequately accounted for in the calculations. Since it is a one-point standardization it will be subject to relatively large random error affecting precision. The magnitude of random error will be at a minimum if the amount of added ATP standard equals the amount of ATP in the original sample.

The firefly ATP assay has been critically compared to other methods for determining ATP.[108] The limitations of the firefly assay cited in this comparison apply mainly when

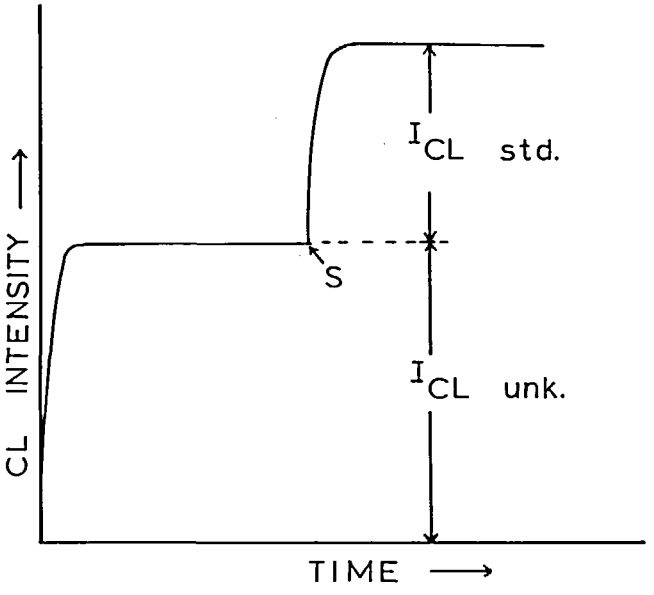


FIGURE 13. Typical intensity-time curve for ATP determination using the method of standard additions. S is point when ATP standard is added. I_{CL} unk is intensity due to ATP in unknown and I_{CL} std is intensity due to standard ATP. Moles ATP in unknown = moles ATP in standard x I_{CL} unk/I_{CL} std.

crude luciferase and preparations are used. Preparations similar to that characterized by Lundin and Thore[77] are not subject to these limitations.

a. Immobilized Luciferase

Because firefly luciferase is expensive, it would be highly beneficial if it could be successfully immobilized on a solid support so that it could be reused. Firefly luciferase has been immobilized on glass beads glued to a glass rod.[109] Using these rods, linear response to ATP was demonstrated over a concentration range from 1×10^{-8} to 1×10^{-5} M. The immobilized luciferase was reported to have about 0.1% of its original activity. However, the measurement was made by immersing the rod in an unstirred ATP solution. In arriving at the 0.1% figure, the authors do not appear to have accounted for the slow rate of mass transfer in unstirred solution. The reviewer suspects that convection to enhance the mass transfer of ATP to the enzyme phase might yield a significantly higher apparent activity. A single rod with immobilized luciferage could be used for up to 50 measurements.

2. Applications Based on "Biomass" Determination

The majority of applications of the firefly ATP assay involve relating ATP concentrations to biomass, i.e., the total weight of living organisms, usually bacteria, in various types of samples. The assumption and requirements for assaying bacterial content of fluids have been discussed in general terms.[10] A few of these applications are of value in clinical chemistry. In particular, ATP levels can be used to screen for bacteriuria or elevated bacteria levels in urine, which correlate with the presence of infection of the urinary tract. The ATP measurement is far more rapid than the alternative method of culturing a sample and counting the number of colonies that develop.

Table 1
REAGENTS FOR EXTRACTING ATP FROM BACTERIA

Tris-EDTA
KOH
Tris-EDTA, arsenate, n-butanol
Ethanol
Butanol
Chloroform
Formic acid
Sulfuric acid
Trichloroacetic acid
Perchloric acid

Adapted from Lundin, A. and Thore, A., *Appl. Microbiol.*, 30, 713, 1975.

To accurately measure bacterial ATP, it is first necessary to destroy nonbacterial ATP including free ATP and ATP in mammalian cells. The addition of detergent selectively lyses mammalian cells to release ATP. The ATP hydrolyzing enzyme apyrase is added along with the detergent to destroy free ATP. After inactivating apyrase by heat or acid, the sample must be treated to release bacterial ATP. Table 1 lists reagents employed for release of ATP. These methods have been critically compared with the conclusion that trichloroacetic acid extraction yielded the most accurate ATP measurement.[111] The trichloroacetic acid method is tedious, however, and other methods are often suitable. Tris-buffer plus EDTA has been a popular choice for bacteriuria because it does not require neutralization prior to the analytical step and does not significantly interfere with the ATP analysis.[112,113] More recently a commercial reagent from LUMAC has been shown to extract nonbacterial ATP more efficiently than Tris-EDTA.[114] The method has been automated.[91,113]

Clinical studies show a useful correlation between high ATP level and high bacteria counts determined by culture yields.[113] Sources of error include incomplete destruction of nonbacterial ATP and variation in the ATP levels per bacterium. Because of these uncertainties, there is nothing to be gained by doing the analytical step with high precision. However, because urine is a highly variable matrix, a standard addition analysis is recommended to insure accuracy.

Another clinical application of biomass measurement involves the determination of antibiotics. A sample containing an antibiotic is incubated for 1 to 2 hr with a culture of a chosen strain of bacteria. At the end of the incubation period, the bacterial ATP is extracted by one of the methods mentioned above and measured. A standard curve is prepared by exposing known antibiotic levels to the bacterial culture. Figure 14 shows a typical standard curve. Increased antibiotic levels causes a decrease in measured ATP. This decrease reflects not only fewer cells but the fact that growing cells have a higher level of ATP per bacterium. Antibiotics assayed include gentamicin, ampicillin, doxycyclive, trobramycin, nitrofurantoin cephaloridine, and folate.[115-123] This method has been successfully applied to serum samples.[117,118,123] BL methods have correlated well with accepted standard methods.

The determination of antibiotics by the above method may be considered a coupled assay in which the bacterial strain serves a reagent. The sensitivity of the assay to antibiotic concentrations depends on how susceptible the bacterial strain is to the antibiotic analyzed. The choice of bacterial strain varies according to the antibiotic to be

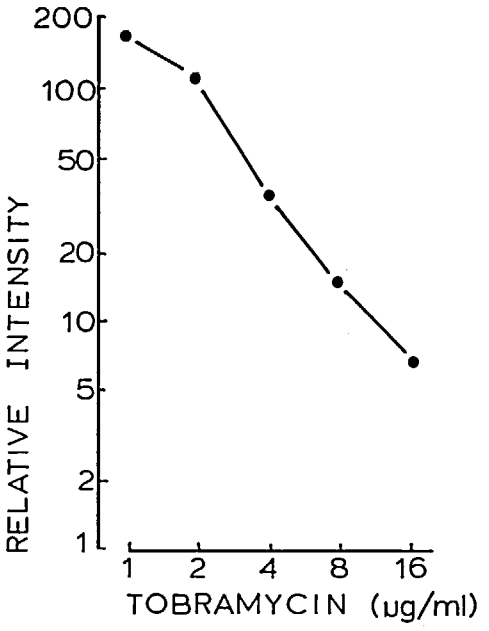


FIGURE 14. Standard curve of measured intensity due to ATP vs. tobramycin. Note that both scales are logarithmic. (From Harber, M. J. and Asscher, A. W., in *Proc. Int. Symp. Anal. Appl. Bioluminescence Chemiluminescence*, 1979, 531. With permission.)

assayed. Alternatively, the assay can be used to determine the susceptibility of various bacterial strains to known antibiotic concentrations. In fact, this general approach is applicable to any species modifying bacterial growth. This type of assay does not require high precision in the measurement of ATP nor is accuracy a problem.

Normally, biomass determinations are made on a large number of cells. It is, however, possible to measure the ATP in single isolated cells.[124] This requires very low detection limits since the amount of ATP in single cells is on the order of a few femptomoles. The addition of extra luciferin helps to make subfemptomole detection limits possible.

3. Other Applications of ATP Measurements

ATP has been measured in other types of samples of clinical interest including erythrocytes, platelets, and pancreatic islets. The ATP-level in erythrocytes is a critical factor affecting red blood cell activity. Low ATP-levels appear to be associated with certain disease states.[125,126] Studies of erythrocyte properties such as aggregation, lysis, viability, etc. have utilized ATP measured by the firefly assay.[127-130] The determination of ATP is readily accomplished by adding a reagent to release the ATP from the cells followed by addition of firefly reagent.

An apparatus has been developed for measurement of ATP in individual red blood cells.[131] The procedure involves positioning a cell under a microscope so that it can be irradiated with a laser to release ATP into a medium containing the firefly reagents. Since analysis of a single cell requires very high sensitivity to get measureable signals, extra luciferin was added to get satisfactory intensities. Using this technique, it was shown that the content of ATP varies considerably from cell to cell. The average value for many single cells agreed with the bulk value for multiple cells as usually measured. This apparatus can look at ATP levels for abnormal red cells which can be identified

microscopically. Using this apparatus, it has been shown that aged red blood cells contain normal ATP levels.[132]

Platelet ATP can be measured by treating isolated platelets with a reagent to cause ATP release followed by addition of the firefly reagents.[133,134] ATP in isolated Islets of Langerhans has also been measured by the firefly assay.[135]

4. Coupled Reactions Involving ATP

Since many enzymatic processes involve ATP as a reactant or product, a very large number of analyses based on ATP measurement are feasible through coupled reactions. Clinical interest has centered on the measurement of creatine kinase (CK) activity. Creatine kinase catalyzes the following reaction:

$$\text{ADP + creatine phosphate} \xrightleftharpoons{\text{CK}} \text{ATP + creatine} \qquad \text{(Reaction 6)}$$

where ADP is adenosine diphosphate. The activity of creatine kinase can be determined on a sequential basis by incubating the sample with ADP and creatine phosphate and measuring the ATP generated after a known time period. However, the continuous monitoring method has been more widely employed to measure the increase in ATP concentration with time.[136-141]

Creatine kinase consists of two subunits which are of two different types, M (for muscle) and B (for brain). Increases in total creatine kinase activity usually reflect an increased level of the MM isoenzyme, which is indicative of muscle disorders. A simplified test based on total creatine kinase activity has been developed to screen for Duchenne muscular dystrophy.[142-144] Increases in the MB isoenzyme level accompany myocardial infarction. It is possible to use anti-M antibody to inhibit subunit M and selectively measure the B subunit.[136-140] A complete experiment illustrating all aspects of the continuous monitoring assay is illustrated in figure 15. In the absence of added creatine phosphate, ATP is formed due to adenylate kinase activity in serum.

$$\text{ADP + ADP} \xrightarrow[\text{adenylate kinase}]{} \text{ATP + AMD} \qquad \text{(Reaction 7)}$$

This can be eliminated by adding a specific adenylate kinase inhibitor, diadenosine pentaphosphate (DAPP). The addition of creatine phosphate causes an increase in ATP associated with total creatine kinase activity. Addition of anti-M antibody inhibits the M subunit. The remaining increase in ATP is a measure of B subunit activity. Finally, standard ATP is added to the reaction mixture. The above experiment, containing considerable information, is only possible when the analytical reaction is slow enough so that BL intensity is not affected by product formation or reagent consumption.

It is also possible to distinguish M and B subunits based on kinetic differences but this is more involved than the use of an antibody to specifically inhibit the M subunit.[145] A variety of other coupled assays involving ATP has been reported. Table 2 lists several of them.

While this list looks impressive, it should be realized that many of these assays have not been developed to the point of being applicable in real life situations. In particular, for the coupled reactions described in Reference 16 all that was done was to show that the intensity-time curve was modified by addition of the coupling reagents in a manner suggesting possible analytical application.

One method of particular interest for clinical purposes is the analysis of glycerol using glycerol kinase.[152] This method has been successfully applied to serum. It serves as an

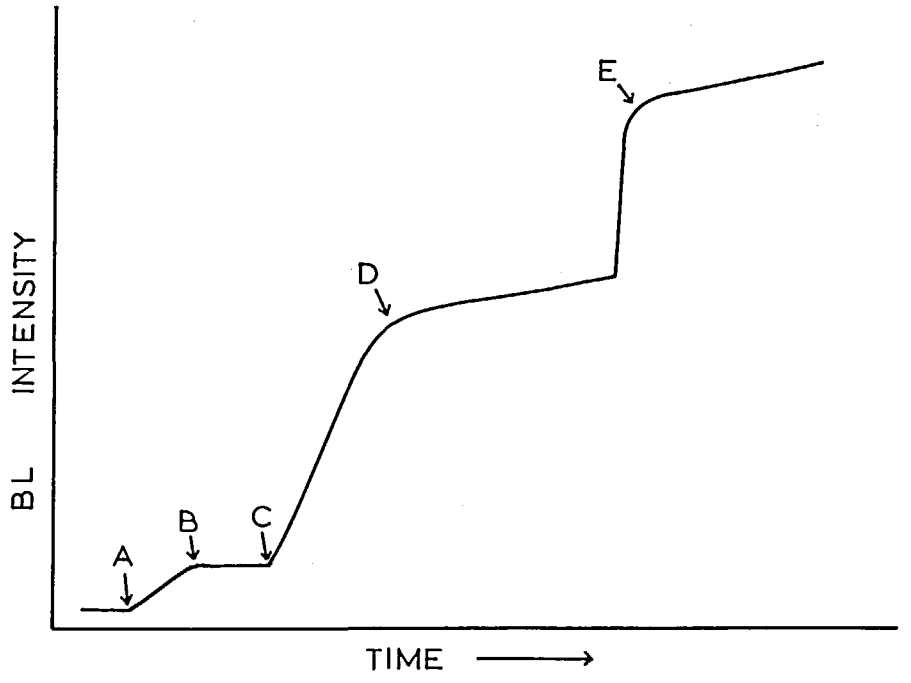


FIGURE 15. Intensity-time curve illustrating basis of coupled assay for creatine kinase MB subunit. A. Addition of ADP results in ATP formation due to adenylate kinase activity. B. Addition of diadenosine pentaphosphate inhibits adenylate kinase activity preventing further ATP formation. C. Addition of creatine phosphate initiates ATP formation catalyzed by creatine kinase. D. Addition of antibody specifically inhibits M subunit so that remaining creatine kinase activity only reflects B subunit. E. Addition of ATP to standardize activity measurement. Throughout this sequence, ATP consumption is negligible. (From Lundin, A., in *Methods in Enzymology*, 1979, 56. With permission.)

example of the tradeoffs required when designing an analysis based on ATP consumption. This analysis can be done on a continuous monitoring basis or sequentially by measuring residual ATP after incubation with glycerol kinase. In the reported glycerol method the reaction was allowed to go to completion before analyzing the remaining ATP. The initial ATP concentration is a critical variable in designing this type of assay. If the ATP concentration is much higher than glycerol, then the decrease in ATP concentration will be small, relative to the initial amount. In this case small uncertainties in measured amounts of ATP will cause relatively large uncertainties in the measured amount of glycerol. This problem can be reduced by choosing an ATP concentration that is only slightly larger than the glycerol concentration. However, this causes the coupling reaction to proceed more slowly. The reaction rate will decrease in a complex manner with time since both ATP and glycerol are consumed. For example, in the reported work it was found that the required incubation time for glycerol to completely react with ATP was greater at high glycerol concentrations (corresponding to smaller residual ATP levels). These tradeoffs affect any similar analysis involving ATP consumption. This is an example of a system in which the precision of the analytical measurement is a critical variable affecting the practicality of the method.

5. Immunological Assays

The specificity of the firefly reaction complicates its use in competitive binding assays.

Table 2
COUPLED ASSAYS INVOLVING ATP

I. Substrate Analysis

Analyte	Coupling reaction(s)	Mode	Ref.
ADP	$2\ ADP \xleftrightarrows[\text{adenylate kinase}]{} AMP + ATP$	Continuous	16
	$ADP + PEP \xrightarrow[\text{pyruvate kinase}]{} PP + ATP$	Sequential	146,147
AMP	$AMP + PEP \xrightarrow[\text{adenylate kinase}]{\text{pyruvate kinase}} 2\ PP + ATP$	Sequential	148
GMP, GDP, and GTP	$GMP + ATP \xleftrightarrows[\text{guanosine-5'-monophosphate kinase}]{} GDP + ADP$	Sequential	16,148
	$GDP + PEP \xrightarrow[\text{pyruvate kinase}]{} pyruvate + GTP$		
	$GTP + ADP \xleftrightarrows[\text{Nucleoside-5'-diphosphate kinase}]{} GDP + ATP$		
	Analogous methods are possible for other nucleotides		
Phosphocreatine	$Cr{-}P + ADP \xleftrightarrows[\text{Cr-kinase}]{} Cr + ATP$	Continuous	16
		Sequential	149
PEP	$PEP + ADP \xleftrightarrows[\text{pyruvate kinase}]{} pyruvate + ATP$	Continuous	16
		Sequential	56
Creatine	$Cr + ATP \xleftrightarrows[\text{creatine kinase}]{\text{heat}} Cr - P + ADP$	Sequential	16
	$ADP \xrightarrow[\text{myokinase, ATPase}]{\text{heat}} AMP$		
	$Cr - P + AMP \xleftrightarrows[\text{creatine kinase}]{} ATP + Cr$		
Glucose	$Glucose + ATP \xleftrightarrows[\text{hexokinase}]{} G6P + AMP$	Continuous	16

Cyclic AMP	Cyclic AMP $\xrightarrow[\text{3',5'-cyclic nucleotide phospho diesterase}]{}$ AMP AMP + ATP $\xrightarrow[\text{adenylate kinase}]{}$ ADP ADP + PEP $\xrightleftharpoons[\text{pyruvate kinase}]{}$ ATP	Sequential	150
Adenosine tetraphosphate	$AT_4 \xrightarrow[\text{hydrolysis}]{}$ ATP (?)	Continuous	151
Glycerol (Triglycerides)	Glycerol + ATP $\xrightarrow[\text{glycerol kinase}]{}$ ADP + glycerol-3-phosphate	Sequential	152
Aminoglycoside Antibiotics	e.g. Gentiamicin + ATP $\rightleftharpoons$ AMP-Gentamicin + PP	Sequential	153
Digoxin	ATP + $H_2O \xrightleftharpoons[\text{ATPase (inhibited by digoxin)}]{}$ ADP + H_3PO_4	Sequential	154

II. Enzyme Analysis

Adenyl kinase	2 ADP $\rightleftharpoons$ AMP − ATP	Continuous	16
Apyrase	ATP + $2H_2O \rightleftharpoons$ AMP + 2 H_3PO_4	Continuous	16
ATPase	ATP + $H_2O \rightleftharpoons$ ADP + H_3PO_4	Continuous	16
Pyruvate kinase	PEP + AMP $\rightleftharpoons$ ATP + pyruvate (ADP)	Continuous	16
Nucleotide phosphokinases	XTP + AMP $\xrightleftharpoons[\text{(X = any nucleotide)}]{}$ ATP + XMP	Continuous	16
Hexokinase	ATP + hexose $\rightleftharpoons$ ADP + hexose-B-phosphate	Continuous	16
ATP-sylfurylase	SO_4^{2-} + ATP $\rightleftharpoons$ APS + PP	Continuous	155
Cyclic nucleotide phosphodiesterase	Cyclic 3',5'XMP $\longrightarrow$ 5'XMP 5'XMP + ATP $\xrightleftharpoons[\text{guanylate kinase}]{}$ XDP + ADP	Sequential	156,157

Note: ADP = adenosine diphosphate, AMP = adenosine monophosphate, PEP = phosphoenol pyruvate, GMP = guanosine monophosphate, GDP = guanosine diphosphate, GTP = guanosine triphosphate, Cr−P = Creatine phosphate, Cr = creatine, G6P = glucose-6-phosphate, AT_4 = adenosine tetraphosphate, APS = adenosine phosphosulfate.

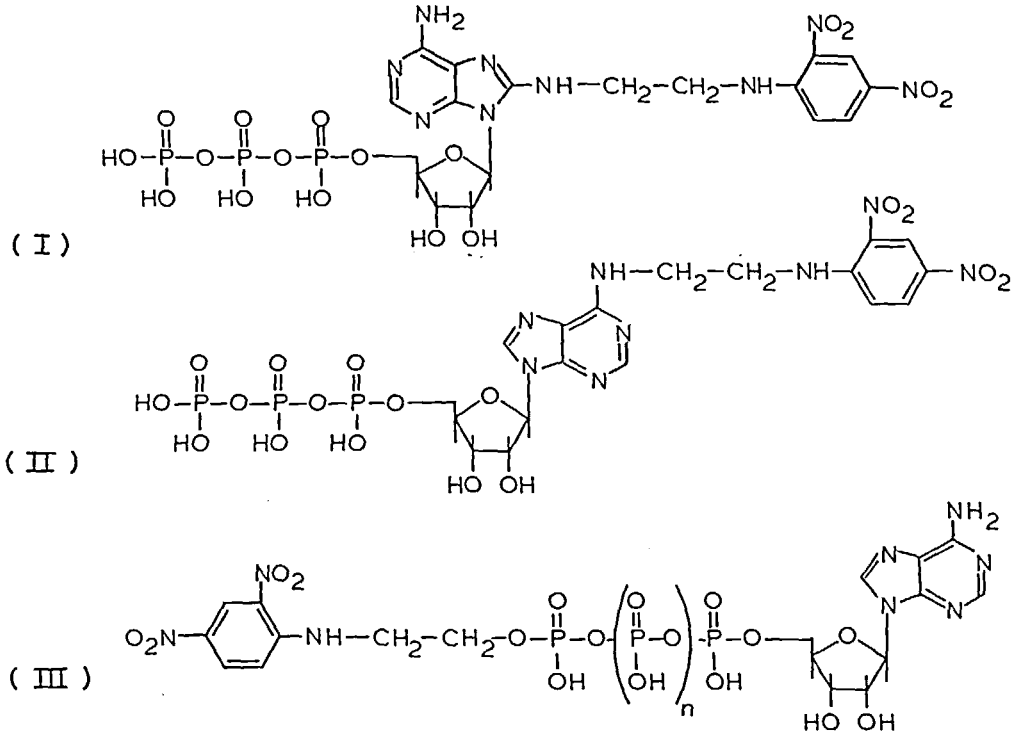


FIGURE 16. Structures of dinitrophenyl-ATP conjugates. (From Carrico, R. J., et al., in *Methods in Enzymology*, 1978, 113. With permission.)

Nevertheless, procedures have been developed using ATP-derivatives as labels.[158,159] Figure 16 shows three structures of three dinitrophenyl-ATP conjugates. Structures I and II react to produce light but for both the slope of the analytical curve is about 20 times less than for ATP. Sensitivity for III is almost as great as for ATP by itself. This apparently is due to the presence of a contaminating enzyme in the luciferase which hydrolyzes III yielding ATP. In the presence of excess antibody to dinitophenyl, maximum BL intensity is 10% of its value as compared to that in the absence of antibody. Thus, this system is suitable for homogeneous competitive binding analysis. If this method were to be applied to serum, it would be necessary to first degrade endogenous ATP. Ligands can be measured at concentrations as low as 10 nM with this system.

Luciferase has been coupled to immunoglobulins with the intent of developing immunoassays in which luciferase serves as the label.[160] The yields of active conjugate have not been great enough to proceed with an analytical evaluation. Luciferase stability may be a problem which prevents practical application of an otherwise promising label.

The analytical characteristics of the firefly reaction used for assaying small quantities of luciferase have not been established. The usual advantage of using an enzyme as a label is that product can be accumulated. If firefly luciferase is a label, total light can be integrated. However, the fact that the reaction is subject to product inhibition will limit the light output.

B. Bacterial Bioluminescence

1. Analytical Characteristics

Several strains of marine bacteria bioluminesce. This reaction has been extensively

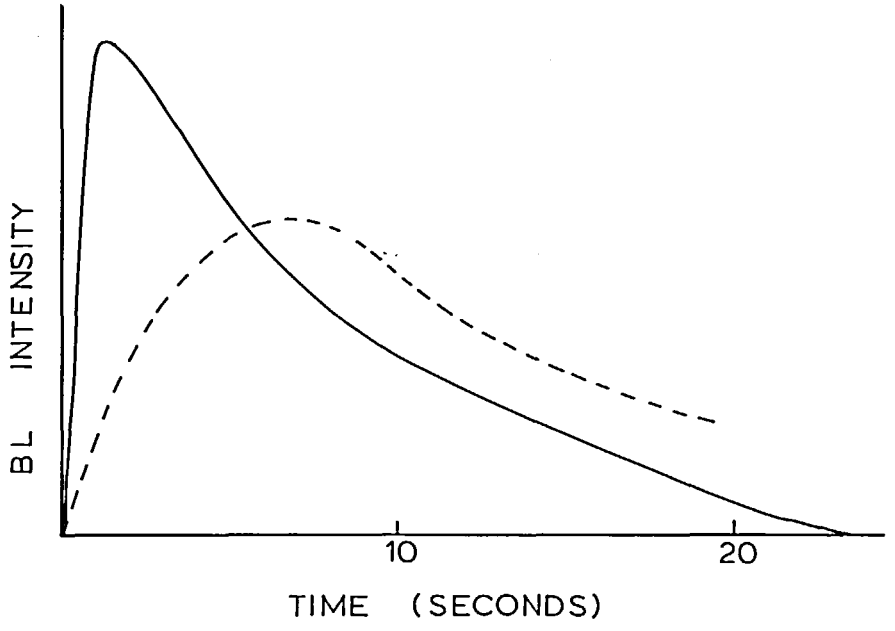


FIGURE 17. Typical intensity-time curve for bacterial bioluminescence: after addition of $FMNH_2$ (——), and after addition of NADH using the coupled reaction system (----).

studied and current knowledge has been summarized.[23,24,161] While some differences exist between strains, the basic reaction is the same for all of them. The reaction responsible for light emission is

$$FMNH_2 + O_2 + R\text{-}CHO \xrightarrow[\text{luciferase}]{} FMN + RCOOH + H_2O + light \qquad \text{(Reaction 8)}$$

where $FMNH_2$ is the reduced form of flavin mononucleotide and RCHO is a long chain aldehyde. Differences between the various bacterial strains are of significance to the analyst only in so far as they might affect luciferase stability. Of greater interest is the occurrence of mutant strains which involve alternate chemistries.[162,163] In particular, a mutant strain which responds to myristic acid as well as well long-chain aldehydes has been reported and evaluated for analytical purposes.[164]

While crude bacterial extracts will yield efficient light upon addition of $FMNH_2$ and R-CHO, detection limits and luciferase stability are improved by employing purified enzyme. Several purification procedures have been reported[165-167] and one study has critically compared four different procedures.[168] Several companies currently market a purified preparation which is stable at room temperature for over 24 hr. The luciferase is stabilized by the presence of phosphate ion and bovine serum albumin.

Figure 17 shows typical kinetics for bacterial BL. There is a rapid rise in intensity upon addition of $FMNH_2$ followed by a decrease in intensity. The rapid rise is associated with rapid binding of $FMNH_2$ to bacterial luciferase. The intensity decay is associated with the relatively slow reaction of the $FMNH_2$-luciferase complex to yield light. This reaction competes with the rapid nonenzymatic oxidation of $FMNH_2$ by oxygen

$$FMNH_2 + O_2 \longrightarrow FMN + H_2O_2 \qquad \text{(Reaction 9)}$$

Any $FMNH_2$ not immediately bound to luciferase will be oxidized nonenzymatically without resulting in light. If the luciferase concentration is lowered, the intensity decreases because a greater fraction of added $FMNH_2$ is nonenzymatically oxidized. However, the relative intensity as a function of time is unchanged. From an analytical point of view, this is unfortunate since it precludes the possibility of modifying kinetics to optimize a particular analytical measurement. The effects of pH, aldehyde chain length and concentration, ionic strength, etc. on intensity may be taken from other publications.[169]

The majority of analytical applications of bacterial BL involve coupling the oxidation of pyridine nucleotides to the production of $FMNH_2$ as shown below.

$$NADH + FMN \xrightarrow[\text{NADH:FMN oxidoreductase}]{} NAD^+ + FMNH_2 \qquad \text{(Reaction 10)}$$

NADPH:FMN oxidoreductase can also be employed for response to NADPH. The oxidoreductases occur together with luciferase in luminous bacteria, and it is believed that in vivo BL involves coupled reactions 8 and 10. However, the oxidoreductases are separated from luciferase during purification. The NADH and NADPH oxidoreductases can then be separated from each other by affinity chromatography.[170] This makes it possible to achieve specific response to either NADH or NADPH with the coupled reaction system. Dithiothreitol is generally added to stabilize the oxidoreductase. Determination of reduced pyridine nucleotides by bacterial BL is an example of a coupled reaction system in which the second reaction is relatively fast. The kinetics of the coupled system can, however, be controlled by varying the oxidoreductase activity. This is illustrated in Figure 17. Controlled intensity-time curves by adding appropriate amounts of oxidoreductase may simplify coupled assays involving NADH production as well as permitting the internal standard addition method of standardization that has been successful in the firefly ATP assay.

Detection limits below 10^{-15} moles of NADH have been obtained using purified luciferase. Response is linearly proportional to NADH concentration over several orders of magnitude.

The bacterial BL reaction does not seem to suffer from serious interferences. Specificity is not a problem. Production or consumption of $NADH/FMNH_2$ due to unwanted side reactions catalyzed by contaminating enzymes in the luciferase-oxidoreductase preparation has not been reported as a problem. Since most measurements have been made at short time intervals after mixing, this type of interference would not show up unless the interfering process were rapid. It should, however, be recognized that many of the reported applications have not been verified on actual samples or compared to accepted methods. Thus, it is possible that problems of this sort occur and have not yet been recognized. Various matrix effects on BL, such as ionic strength, turbidity, absorption, and modification of reaction rates by diverse ions can be accounted for by standard additions.

a. Immobilization

Bacterial luciferase has been immobilized on polyacrylamide.[171] The intensity observed when $FMNH_2$ was added to a stirred suspension of immobilized enzyme suggested that most of the luciferase activity was retained upon coupling. The thermal stability was affected by coupling but the immobilized luciferases did not have a long half-life at room temperature. The stirred suspension provides for a high surface area of immobilized enzyme plus efficient mass transfer. However, it is not convenient for rapid analytical measurements.

Table 3
COUPLED ASSAYS INVOLVING IMMOBILIZED LUCIFERASE AND OXIDOREDUCTASE

Analyte	Coupling Reactions
Ethanol[a] Alcohol dehydrogenase	Ethanol + NAD^+ $\xrightarrow[\text{alcohol dehydrogenase}]{}$ acetaldehyde + NADH
	Ethanol + NAD^+ $\longrightarrow$ acetaldehyde + NAD
Glucose[a], hexokinase	D-glucose + ATP $\underset{\text{hexokinase}}{\rightleftharpoons}$ D-glucose-6-phosphate + ADP
G6P[a], G6PDH	D-glucose-6-phosphate + $NADP^+$ $\underset{G6PDH}{\rightleftharpoons}$ D-6-phosphogluconolactone + NADPH
LDH	L-lactate + NAD^+ $\rightleftharpoons$ pyruvate + NADH
MDH	L-malate + NAD^+ $\rightleftharpoons$ oxaloacetate + NADH

Note: G6P = glucose-6-phosphate, G6PDH = glucose-6-phosphate dehydrogenase, LDH = lactate dehydrogenase, MDH = malate dehydrogenase.

[a] These analytes have been determined using coimmobilized enzymes in addition to luciferase and oxidoreductase. Alcohol dehydrogenase, hexokinase, and G6PDH have been successfully coimmobilized.

More recently, bacterial luciferase and the oxidoreductase have been coimmobilized onto arylamine coated glass beads glued onto a glass rod.[172,173] The glass rod is placed in a small tube positioned in front of the photomultiplier. This system responds linearly to NADH from 1 picomole to 50 nanomoles. Upon injection of NADH, the signal goes to a maximum and gradually decays. Since the reaction vessel is unstirred, the decrease in intensity is presumably associated with the depletion of reactants at the surface of enzyme phase and/or by slow diffusion of product away from the enzyme phase. Immobilized systems responding specifically to both NADH and NADPH have been prepared by using the appropriate purified oxidoreductase.[174] The immobilized systems responding to NADH have been employed in a variety of coupled assays.[45,175] These are listed in Table 3. In some cases, additional enzymes have been coimmobilized along with the luciferase and oxidoreductase.

Immobilized luciferase and oxidoreductase offer some substantial advantages for clinical applications. However, the reported method of manually injecting analyte into a vial containing a glass rod lacks both precision and convenience. In addition, the sensitivity is reduced because the vessel is unstirred. A packed column of immobilized enzyme on a rigid support may prove more viable for applications. It should also be noted that the immobilized luciferase-oxidoreductase has not been applied to any serum samples. Thus it remains to be demonstrated that the immobilized enzymes can be used on a long term basis with serum samples without interference. For many potential applications, this should not be a problem since the serum can be diluted by a large factor prior to analysis.

2. Applications Involving Living Bacteria

While most analytical applications of bacterial BL involve extracted enzymes, several applications have employed suspensions of live bacteria. A mutant strain of bacteria yielding BL with myristic acid as well as aldehyde has been used for myristic acid analysis.[162,164] This reaction has been coupled to the measurement of lipase and phospholipase activities by using as the lipase substrate a fat that hydrolyzes to myristic acid.[176-178]

The intensity of BL from a bacterial suspension is a measure of the rate of metabolism

of the bacteria. As a consequence, these bacteria are uniquely suited for the bioassay of species affecting their metabolic rate. This concept has been developed for the general detection of toxic materials in water samples.[179] It can also be used to detect anesthetics.[180] Conceivably, it could be used to measure antibiotics, although no work of this sort has been reported.

Bacterial suspensions have also been employed for oxygen detection.[181] The detection limit is on the order of 10^{-10} M. This assay was specifically developed to determine the oxygen requirements of the cytochrome oxidase system. Response to oxygen in other samples can be accomplished by separating the sample from the bacterial suspension by a membrane permeable to oxygen.[16] However, this reduces sensitivity and does not offer any advantages relative to oxygen measurement with an oxygen electrode.

Assays involving bacterial suspensions are relatively slow and are restricted to applications in which high precision is not critical. Nonetheless, they are simple and convenient.

3. FMN Analysis

Bacterial luciferase can be used to determine FMN. The FMN must be reduced to $FMNH_2$ in the absence of oxygen and then injected into a luciferase solution. The reduction can be performed photochemically using EDTA as an electron donor or chemically with dithionite or H_2 in the presence of a Pt catalyst.[165,169,182,183] $FMNH_2$ has been suggested as an alternative to ATP for biomass measurement,[184,185] however, this would require a great deal of work to establish FMN levels in various organisms and does not offer any particular advantages. The $FMNH_2$ assay does serve to measure luciferase activity when a known level of $FMNH_2$ is used.

4. Measurement of Protease Activity

Bacterial luciferase can serve as a substrate for the determination of protease activities.[186] The procedure involves measuring the extent to which proteolytic attack reduces luciferase activity as measured by the $FMNH_2$ assay. The assay is rapid and simple requiring only picomole amounts of luciferase. It has been applied to a number of proteolytic enzymes and shown to agree with other methods.[187]

5. Pyridine Nucleotide Analysis: Coupled Assays

Procedures for using bacterial BL to analyze for NADH and NADPH have been reported by several investigators.[166,169,182,183] NADH and NADPH are important analytes because many enzymatic processes are conveniently coupled to the formation or consumption of either NADH or NADPH. Table 4 lists some enzyme and substrate assays that have been coupled to bacterial BL. As the table indicates, these assays can be performed either sequentially by analyzing NAD(P)H after a suitable incubation period or continuously. By adding appropriate amounts of oxidoreductase and luciferase it should be possible to work under conditions where NAD(P)H consumption is relatively slow, and the BL intensity can be considered proportional to NAD(P)H concentration as the reaction proceeds without having to make any corrections. The internal standard addition method can be used to calibrate sensitivity to NAD(P)H.

Assays involving both production and consumption of NAD(P)H are listed in Table 4. Unfortunately, equilibrium usually favors $NAD(P)^+$ formation unless a large excess of appropriate substrate is added to promote NAD(P)H formation. Assays in which NAD(P)H is oxidized to $NAD(P)^+$ are subject to some serious constraints. If the initial NAD(P)H is high then, the decrease in intensity due NAD(P)H consumption is small and difficult to measure with adequate precision. However, a lower initial NAD(P)H level slows down the coupling reaction, requiring a larger amount of coupling enzyme or a longer amount of time. If an assay involving NAD(P)H

consumption is performed on a continuous basis, it may be necessary to correct for NAD(P)H consumption by the BL process. In general, it has been recommended that coupled assays be standardized using known amounts of analyte. As pointed out in Section III-B-5, the use of microprocessors may be of value in interpreting complex intensity time curves observed in continuous coupled assays, such as those observed with bacterial BL.

An alternative approach to coupling has been demonstrated for assays that involve the oxidation of NAD(P)H to $NAD(P)^+$[197]. The excess NAD(P)H can be destroyed by treatment with acid. Following neutralization, the $NAD(P)^+$ can then be reduced to NAD(P)H and analyzed. If the coupling of $NAD(P)^+$ reduction to the bacterial BL assay is done on a continuous basis, one has an enzymatic cycling system. After NAD(P)H is oxidized by the oxidoreductase, it can again be reduced and once more react to yield light. This increases total light output and yields a readily measureable steady state intensity. The same cycling approach can be applied to NAD(P)H analysis provided no $NAD(P)^+$ is present. The operations can be automated although it would require considerably more complex instrumentation than usually used.

The high sensitivity of bacterial BL has been used to advantage in the analysis of microtissue samples.[192,196-199] Although bacterial BL can be used to analyze for several serum components of clinical interest, none of these possible applications has been investigated in depth with analyses of actual samples comparing the BL method to accepted methods. This is at least partially due to instability and lack of reproducibility of commercial luciferase preparations. This appears to be becoming less of a problem.

6. Competitive Binding Assays

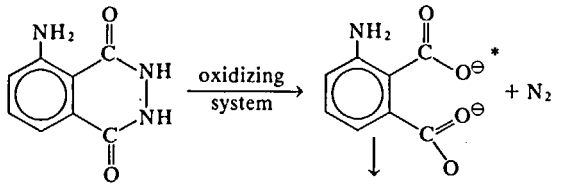
It is possible to couple pyridine nucleotide analogs to various analytes for competitive binding assays.[200] This has been employed in competitive binding assays for biotin and dinitrofluorobenzene using the bacterial BL reagents plus alcohol dehydrogenase to reduce the label.[201] It was possible to do these assays homogeneously since the free label reacted to give considerably more BL than the bound label. However, this system has not been pursued further, because of luciferase stability problems as well as precision problems.

An attempt has been made to employ bacterial luciferase as a label but stability problems prevented satisfactory results.[202]

Another possible application would be to use bacterial BL to determine NADH generated by an enzyme label. This would allow considerable savings of time and/or reagent because of the low BL detection limit. This application may be developed as more stable reagents become commercially available. This assay will also require that the analytical step be performed with high precision, particularly if the assay is performed homogeneously, since there is generally a relatively small change in the activity of the enzyme label upon binding.

C. Luminol

The reaction of luminol (5-amino-2,3-dihydro-1,4-phthalazine dione) with an oxidizing agent is summarized below.



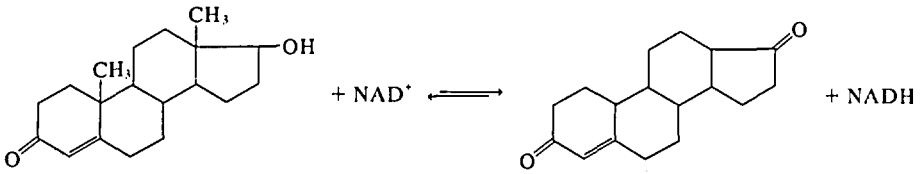
luminol light

Table 4
COUPLED ASSAYS INVOLVING NAD(P)H

I. Substrate Analysis

Analyte	Coupling Reaction(s)	Mode	Ref.
Ammonia	NH_3 + α-Ketoglutoric acid + NADH $\xrightarrow[GDH]{}$ L (+)-glutamic acid + NAD^+	Continuous	188
Ethanol	Ethanol + NAD^+ $\xrightarrow[ADH]{}$ Acetaldehyde + NADH		189
Glucose	Glucose + ATP $\xrightarrow[hexokinase]{}$ glucose-6-phosphate + ADP		
	glucose-6-phosphate + NAD^+ $\xrightarrow[G6PDH]{}$ 6-phosphogluconolactone + NADH	Sequential	190
Glycerol-3-phosphate	Glycerol-3-phosphate + NAD^+ $\longrightarrow$ NADH + L-glycerol-3-phosphate dehydrogenase	Continuous	191
3-hydroxy butyrate	3-hydroxybutyrate + NAD^+ $\xrightarrow[HBD]{}$ NADH + acetoacetate	Sequential	191
Malate	Malate + NAD^+ $\underset{MDH}{\rightleftharpoons}$ NADH + Oxaloacetate	Continuous	191,192,193
NAD^+	NAD^+ + ethanol $\xrightarrow[ADH]{}$ NADH + acetaldehyde	Sequential	190,194
$NADP^+$	$NADP^+$ + glucose-6-phosphate $\underset{G6PH}{\rightleftharpoons}$ NADPH + 6-phosphogluconolactone	Continuous	195
Oxaloacetate	Oxaloacetate + NADH $\underset{MDH}{\rightleftharpoons}$ Malate + NAD^+	Continuous	5,193,194
Pyruvate	NADH + pyruvate $\underset{LDH}{\rightleftharpoons}$ NAD^+ + lactate	Sequential	196
	NADH (excess) + acid $\longrightarrow$ decomposition NAD^+ + 3-hydroxybutyrate $\underset{HBD}{\rightleftharpoons}$ NADH + aceto acetate		

Testosterone, androsterone



45

Hydroxysteroid dehydrogenase

II. Enzyme

ATP:NMN transferase

$$ATP + NMN + H^+ \xleftrightarrow[\text{adenylyltransferase}]{} NAD^+ + PP:$$

$$NAD^+ + \text{ethanol} \rightleftharpoons NADH + \text{acetaldehyde}$$

194

Also dehydrogenases used in substrate analyses can all be assayed. (See Table 3 for other examples of coupled assays.)

Note: GDH = glutamate dehydrogenase, ADH = alcohol dehydrogenase, G6PDH = glucose-6-phosphate dehydrogenase, HBD = hydroxybutyrate dehydrogenase, MDH = malate dehydrogenase, LDH lactate dehydrogenase, NMN = nicotinamide mononucleotide.

Table 5
OXIDIZING SYSTEMS REACTING WITH LUMINOL TO GENERATE LIGHT

Oxidant	Catalyst/cooxidant	Ref.
H_2O_2	$H_2S_2O_8$	203
	Peroxidase	80
	Ferricyanide	204
	Heme compounds	205
	Transition metal ions (CO^{+2}, Cu^{+2}, Cr^{+3}, Ni^{+2}, Fe^{+2}, VO^{+2})	206,207
	Hypochlorite	208
O_2	Ferricyanide	209
	Fe^{+2}	210
OCl^-		211
I_2		212
MnO_4^-		206
NO_2		213

Table 5 lists oxidizing systems that react with luminol to yield light. Oxidizing systems involving hydrogen peroxide require a third component which serves as a catalyst and/or cooxidant. As Table 5 shows, a wide variety of catalyst/cooxidants are possible with peroxide. Because of the large number of oxidizing systems yielding light, there are a correspondingly large number of possible analyses based on luminol CL. Most of the species listed in Table 5 have served as analytes in luminol-based determinations. Other analyses have been developed using coupling reactions to generate or consume one of the analytes listed in the table. Luminol itself is an important analyte in competitive binding assays involving luminol or related compounds as labels.

It is difficult to generalize concerning the analytical characteristics of luminol CL. The kinetics and mechanism of the reaction vary for different oxidizing systems. For most of the systems in Table 5, the kinetics and mechanism have not been determined. The reaction is typically performed in aqueous solution at a pH of 10-11. However, it is possible to observe light at pHs as low as 7, although the intensity is generally reduced. Because luminol is relatively inexpensive, it is not necessary to design luminol-based analyses to conserve reagent. Also, the reaction is not unique for luminol. A large number of cyclic hydrazides react similarly to luminol to yield CL. For most of them, however, the CL efficiency is lower than for luminol itself.

1. Peroxide Analysis

Several important clinical assays are conveniently coupled to the formation of hydrogen peroxide via oxidase enzymes. The hydrogen peroxide can then be reacted with luminol to yield CL. This application has been reviewed.[49] A variety of catalyst/cooxidants can potentially be used for hydrogen peroxide analysis with luminol. The two that seem to be most suitable are copper(II)[86,214] and ferricyanide.[204,215,216] With ferricyanide CL is proportional to hydrogen peroxide concentration from a detection limit of less than 10^{-8} M to 10^{-4} M. To measure peroxide using luminol/ferricyanide, however, requires that a relatively large background CL signal from the reaction of luminol, ferricyanide, and oxygen be subtracted out. With copper(II) as a catalyst, background CL is reduced, but CL is not linearly proportional to peroxide.[214] The kinetics of the ferricyanide/luminol/peroxide seem to fall in a suitable range for analytical measurements as evidenced by high precision. However, kinetic information is not available. Likewise, the kinetics of copper(II) catalyzed luminol CL have not been studied, although it is known that the

reaction can be either very fast or relatively slow depending on copper(II) concentration and the presence or absence of ligands that complex copper(II).

The luminol-based analysis for peroxide has been coupled to the analysis of glucose[204,215-217] uric acid,[218] amino acids[219] and cholesterol[58] via appropriate oxidase enzymes. In all cases, the coupling was done sequentially, rather than by continuous monitoring because of the need to go from neutral pHs for the coupling reaction to more basic pHs to get efficient luminol CL. The glucose method was applied to serum samples and found to correlate well with standard method.[215] However, uric acid interferes in the determination of glucose in urine.[217] More recently, we have found that glucose interferes in the determination of uric acid in serum using copper(II) catalyzed luminol CL.[220] The nature of the interferences is unknown. Because of these interferences, it is the reviewer's belief that other reactions such as peroxyoxalate CL (Section IV.E.) will ultimately prove more useful for peroxide analysis.

Peroxidase can be used as the catalyst for peroxide analysis based on luminol.[80] This extends the pH range of luminol CL to lower values. However, CL is proportional to the square of the peroxide concentration; complicating possible continuous monitoring assays. A system in which peroxidase was immobilized on the end of a fiber optic has been developed as a flexible analytical probe for hydrogen peroxide.[48] This system was developed with the idea of developing probes for glucose and other species via immobilized coupling enzymes. This is feasible, but the project was not pursued because of the nonlinear response to peroxide. The idea of immobilizing light-catalyzing enzymes on the fiber optic was treated theoretically.

2. Analysis for Catalyst/Cooxidants

As pointed out above, a catalyst/cooxidant is required for efficient CL from the reaction of luminol with hydrogen peroxide. The catalyst/cooxidant can be the analyte if luminol and peroxide are in excess. Catalyst/cooxidants that can be determined include low concentrations of certain metal ions and hematin compounds including peroxidase. Peroxidase is primarily of interest as a label in competitive binding assays and will be considered in Section IV-C-4.

It is possible to measure very low concentrations of metal ions using luminol CL, however, specificity and matrix effects are problems. A specific method for chromium(III) has been developed and successfully applied to ashed biological samples.[221,222] In this method, EDTA is added to complex otherwise interfering ions and render them inactive as catalysts. The chromium(III)-EDTA complex is kinetically slow to form so chromium(III) remains active as a catalyst.

The luminol reaction responds more sensitively to cobalt(II) than to other metal ions. In fact, the detection limit for cobalt(II) has been estimated to be 10^{-11} M. A method for vitamin B12 has been reported based on cobalt catalysis of luminol CL.[223] Unfortunately, cobalt in the B12 complex is considerably less active as a catalyst than free cobalt(II), and the reported method is only suitable for determining B12 in vitamin tablets after separation from interfering compounds.

Low levels of hematin compounds can be determined by reaction with luminol and hydrogen peroxide or perborate.[205,224-228] This assay has been considered as a means of estimating biomass, assuming that total heme-catalyzed CL is a measure of total biomass. This is analogous to the widespread use of ATP as a biomass indicator and offers a considerable advantage to ATP-based methods with respect to reagent costs. The detection limit for bacteria is on the order of 10^5 cells; not as low as the detection limit for ATP methods. There has been some work done to compare CL response to various microorganisms.[224,225] It should be recognized that this comparison may depend on oxidizing conditions as well as on the conditions of the CL measurement (i.e., peak height

vs. integrated intensity measurement as well as time of integration). This assay has been applied to the rapid screening of urine samples for bacteria.[226] It is potentially applicable to other clinical bioassays, e.g., antibiotic monitoring.

3. Analysis for Other Oxidizing Agents

A number of assays have been developed using oxidizing systems which do not involve hydrogen peroxide. Examples include halogen determinations[212] and a sensitive and selective method for ferrous iron.[210] The determination of iodine as I_2 can potentially be coupled to enzyme-catalyzed oxidation-reduction reactions that either generate or consume iodine. Since I_2 can be measured at concentrations as low as 10^{-9} M, such methods would be capable of high sensitivity. The design of such methods is complicated by the fact that CL intensity varies in a complex higher order manner as a function of iodine concentration.[212] Nevertheless, iodine-luminol CL has been successfully coupled to nonenzymatic iodine-generating and consuming reactions.[229]

4. Competitive Binding Assays

Because of reagent stability and cost considerations, luminol has been the preferred reaction for competitive binding assays using chemiluminescence. Both luminol, itself, and peroxidase have been employed as labels.

a. Luminol as a Label

Because the luminol reaction is general for aryl hydrazides, it is relatively easy to couple luminol-related compounds to an analyte without losing CL activity. Figure 18 shows some of the strategies employed to couple a chemiluminescent hydrazide moiety to an analyte of interest. In the first approach, the amino group of luminol was diazotized, and the diazonium salt was then reacted with sheep antirabbit antibodies. The CL efficiency of diazoluminol is on the order of 1% of the CL efficiency for luminol itself so this strategy while simple involves a considerable loss in sensitivity. The decrease in CL efficiency probably reflects the decrease in fluorescence efficiency of the phthalate product. The labeled antibody was then used in a two-site heterogenous method for rabbit IgG antibody. The CL reaction was performed in 0.1 M sodium hydroxide using hydrogen peroxide and hypochlorite as oxidizing agents. Detection limits were on the order of 1 to 5 ng/mℓ.

The second coupling strategy in Figure 18 involves more chemical steps, but does not destroy the amino group of luminol. As a result, the coupled luminol derivative should have a CL efficiency higher than the diazoluminol prepared by the first approach. This was not evaluated, however. This derivatization strategy was used to prepare a testosterone protein-luminol conjugate to analyze for testosterone. The method for testosterone was done on a heterogeneous basis using cupric acetate and hydrogen peroxide as reagents to measure the amount of bound-labeled testosterone. The analytical curve ranged from about 0.1 to 10 ng. However, the slope of the analytical curve is low indicating a large uncertainty in measured testosterone concentration unless the CL measurement could be made with a high degree of precision.

The third strategy involves isoluminol, i.e., (6-amino,-2,3- dihydro 1,4 phthalazine-dione). The CL efficiency for isoluminol is only about one third that of luminol. However, with isoluminol, coupling can be accomplished without steric interference from the carbonyl group. Also alkylation of the amino group in isoluminol does not cause a decrease in CL efficiency. Isoluminol has been employed as the label in homogeneous competitive binding assays for biotin[232] and progesterone.[233] In both cases, the bound isoluminol-labeled material yielded more efficient CL than the free material. To do the assay homogeneously, it is necessary to perform the luminol reaction at near neutral pHs since strongly alkaline pHs will interfere with the binding process on which

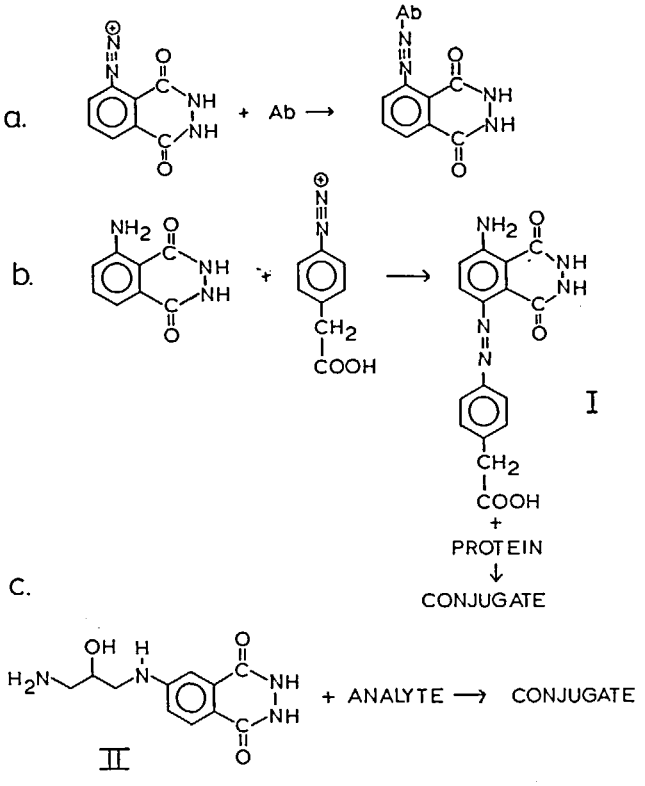


FIGURE 18. Approaches to coupling chemiluminescent aryl hydrazides to potential analytes. A. Coupling to diazonium salt of luminol (230). B. Coupling via azo carboxy methyl phenyl derivative (231). Structure I was not confirmed experimentally. C. Coupling to an isoluminol derivative (232). Structure II was prepared in a multistep sequence not shown here. It was coupled to analyte via an amide linkage involving the free amino group of II.

the assay is based. The sensitivity of the biotin method was relatively poor because of this. To improve sensitivity, a comprehensive study of various oxidizing systems was undertaken.[234] It was found that isoluminol was most sensitively determined when oxidized by hydrogen peroxide with microperoxidase as a catalyst. However, because this is a fast reaction kinetically, it was subject to poor precision with conventional injection methods. An improved mixing system was required to achieve high precision. The improved system was used to determine progesterone at the 25 pg level.[233] Isoluminol as a label has also been used for thyroxine analysis.[235] Unfortunately, this method could not be done homogeneously. The bound and free thyroxine had to be separated prior to analysis.

b. Peroxidase as a Label

Antigens and antibodies can be readily coupled to peroxidase. Luminol CL can then be used to measure the activity of the peroxidase label.[236] This has been used in conjunction with a heterogeneous immunoassay for cortisol. This assay had a detection limit of 10 pg. The CL measurement was performed at pH 7. This is, of course, a branch of enzyme immunoassay since the resulting CL is a measure of peroxidase activity.

Peroxidase labeling with luminol-based analysis has also been performed in conjunction with a solid-phase immunoassay of human serum albumin.[237,238] In this case,

the peroxidase was determined by reaction with perborate and luminol at pH 13. The peroxidase is not acting as an enzyme in this case but merely as a heme-containing catalyst. The advantage of working at higher pH is the greater CL intensity.

It should be noted that luminol is not the only substrate that yields CL with peroxidase. Others include pyrogallol and Pholad Luciferin (See Sections IV.F.6. and IV.G.4.).

c. Glucose Oxidase Labeling

Glucose oxidase can serve as a label for competitive binding reactions. The generated hydrogen peroxide has been detected either continuously by reaction with luminol or by reaction with luminol after an incubation period to build up peroxide concentration.[202] Using this approach it is possible to lower detection limits simply by extending the incubation period.

D. Chemiluminescence Accompanying Phagocytosis

Phagocytic cells recognize, surround, and destroy foreign particles. This process is an important component of the body's defense against bacterial infection. The final phase of phagocytosis, i.e., the destruction of the invading particle, is accompanied by chemiluminescence. A recent review serves as an excellent introduction to phagocytic CL.[239] This review summarizes the various lines of evidence that link CL emission to the destruction of invading particles as well as describing experimental methods for isolating phagocytic cells and measuring CL. Since this above-mentioned review, there have been a large number of papers dealing with phagocytic CL reflecting considerable current interest in this topic. Further evidence linking bactericidal activity to CL has been presented.[240,241]

Phagocytic CL is observed by adding a particle (or soluble stimulant) eliciting membrane perturbation phagocytosis to a suspension of phagocytic cells and measuring CL intensity as a function of time. The CL climbs to a maximum and then gradually decreases as illustrated by a typical intensity-time curve shown in Figure 19.

Determining the chemical processes responsible for phagocytic CL is difficult. Intrinsic CL intensity is very low requiring sensitive photon counting equipment, usually a liquid scintillation counter for observation. It is not possible to identify an emitter or even get a satisfactory emission spectrum. The main approach used to date has been to add agents that selectively interact with suspected CL reactants, superoxide, hydrogen peroxide, and hydroxyl radical, and to observe their effect on CL response. In interpreting such experiments, it must be kept in mind that the added agents may affect cell behavior in other ways than just interacting with the suspected chemical intermediate. Evidence suggests that all three species, superoxide, hydrogen peroxide and hydroxyl radical, are involved in the CL processes.[239,242,243]

The clinical value of phagocytic CL is evident. The absence of CL is a direct indication of a defect in a patient's phagocytic system. These can arise from a variety of disorders summarized elsewhere.[228,229,239,244,245] In particular, the absence of CL has been useful in diagnosing chronic granulomatous disease.[246] CL characteristics can be combined with other measurements to help narrow down the source of the effect.

The clinical measurement is facilitated by adding small amounts of luminol to the reaction mixture. This significantly enhances CL intensity making it possible to perform measurements on ordinary CL photometers rather than requiring high sensitivity photon counting.

Many studies have utilized CL as a measure of the effect of various drugs or toxins on phagocytosis. These studies are summarized in the review of phagocytic CL.[239] Several of the more recent publications involve this type of application.[247-251]

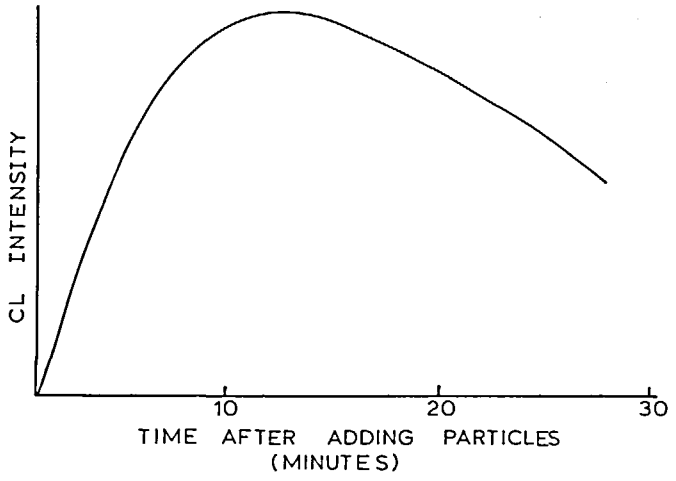
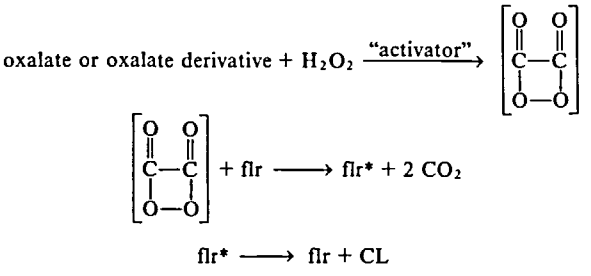


FIGURE 19. Typical intensity-time curve for chemiluminescence accompany phagocytosis. Because photon counting is normally employed for this measurement, the intensity-time curve is constructed from a series of counts at various times after particles are added to initiate phagocytosis.

E. Peroxyoxalate Chemiluminescence

Peroxyoxalate chemiluminescence refers to a large class of reactions summarized below.



The characteristics of peroxyoxalate CL have been reviewed.[252,253] The mechanism is thought to involve the cyclic C_2O_4 intermediate shown above but this species has never been directly observed. Peroxyoxalate CL is the most efficient nonbiological CL reaction known and is used in a commercial CL light source. Several studies have utilized peroxyoxalate CL to determine hydrogen peroxide. This application is considered in a review of CL detection of peroxide.[49]

Peroxyoxalate CL has a number attractive features for peroxide analysis. The emission wavelengths can be varied by changing the fluorophor and the kinetics can be modified by choosing appropriate levels of "activator", usually a weak base. The major limitation of peroxyoxalate CL is the requirement of an organic solvent. Although the reaction is compatible with the presence of water, oxalates yielding efficient CL are not water-soluble. Ethyl acetate/methanol/water,[254] t-butanol/water,[97] and purified dioxane/water[255,256] systems have all been used. The lowest detection limit for hydrogen peroxide, 1×10^{-9} M, was observed with purified dioxane. Detection limits reflect background CL rather than detector sensitivity. In all cases, CL was linearly proportional to hydrogen peroxide concentration over several orders of magnitude. All reported work has involved flow systems since mixed solvent systems do not mix well

upon direct injection. Also, the reactants are not particularly expensive so significant rates of reagent consumption can be tolerated.

Peroxyoxalate CL analysis for peroxide has been coupled to glucose, uric acid, cholesterol, lactose, and maltose analysis via appropriate oxidase enzymes.[97,254-256] All these analyses have been done on a sequential basis in a flow system. However, it should be possible to perform coupled assays on a continuous monitoring basis, if the proportion of organic solvent is maintained low enough so that it does not interfere with the coupling reaction. A more ambitious reaction scheme was developed to couple peroxyoxalate CL to the automated measurement of lactate dehydrogenase (LDH) activities.[96] It is based on the following reactions:

$$NAD^+ + \text{Lactate} \xrightarrow[LDH]{} NADH + \text{pyruvate}$$

$$NADH + MB_{ox} \longrightarrow NAD^+ + MB_r$$

$$MB_r + O_2 \longrightarrow MB_{ox} + H_2O_2$$

$$H_2O_2 + \text{Peroxyoxalate reagents} \longrightarrow \text{light}$$

where MB_{ox} and MB_r refer to oxidized and reduced forms of methylene blue.

Unlike luminol, peroxyoxalate CL used for glucose analysis in urine was not subject to interference from uric acid.[238] It is the reviewer's opinion that peroxyoxalate CL will ultimately prove more useful for peroxide analysis than luminol because of its freedom from interference.

Peroxyoxalate CL can also be used to determine fluorophors. The initial study involved detection of dansylated amino acids separated on a TLC plate.[257] More recently, peroxyoxalate CL has been used to detect dansylated amino acids separated by liquid chromatography.[258] The detection limit was 10 femptomoles, lower than detection limits achieved with conventional fluorescence. In addition to low detection limits, the use of peroxyoxalate CL for fluorophor detection simplifies instrumental requirements. A limitation is that the fluorophor must be in a medium compatible with the peroxyoxalate reaction.

F. Other Chemiluminescence Reactions

There are many reactions that yield CL besides the above. Some of them that have been evaluated for analytical applications are listed below. Some of these reactions are quite promising, e.g., acridinium phenyl carboxylates, oxygen-detecting reactions and polyphenols as CL substrates for peroxidase. In other cases, however, lack of specificity is a distinct problem. In general, any potential application should be carefully evaluated.

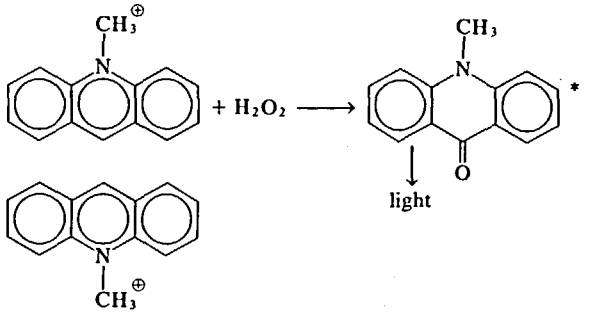
1. Acridinium Phenyl Carboxylates

CL from the oxidation of phenyl carboxylates has been used to determine hydrogen peroxide.[259] This reaction has been coupled to glucose analysis using glucose oxidase to generate hydrogen peroxide.

The analytical characteristics of this reaction are similar to those of peroxyoxalate CL. The reaction is compatible with aqueous solutions at neutral pHs. The kinetics can be modified by varying constituents. The acridinium phenyl carboxylate has to be in an organic solvent so the reaction must be performed in a mixed solvent system.

2. Lucigenin

Lucigenin (bis-N-methyl acridinium nitrate) reacts with hydrogen peroxide in basic solution to produce CL:



Recent studies have helped to pin down the mechanism of this reaction.[35,36]

Analytically, the lucigenin reaction has been evaluated for the determination of metals that enhance CL[260] and for the analysis of reducing agents including glucose and ascorbic acid.[261] In these applications, lucigenin reaction is limited by its lack of specificity. While hydrogen peroxide analysis based on lucigenin would seem feasible, the assay would have to be carried out at high pH for efficient CL.

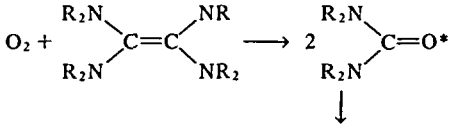
When lucigenin is incubated with hypoxanthine and xanthine oxidase, enhanced CL is observed.[262]

3. Reactions for Determining Oxygen

The determination of oxygen using suspensions of luminous bacteria has already been mentioned (Section IV-B-2). This method was developed for a particular application requiring high sensitivity. It is not generally practical.

Methods for small concentrations of oxygen in the gas phase have been reported based on the reaction of oxygen with luminol in strongly alkaline DMSO[263] and with molten white phosphorus.[46] Both of these methods have very low detection limits, but they are subject to interferences from other gas species and are not suitable for solution measurements.

Recently we have developed a new approach to oxygen measurement based on the reaction of oxygen with tetrakis N-alkyl aminoethylenes.[264]



The analytical configuration is illustrated in Figure 20. A concentrated solution of the aminoethylene is separated from the analyte by a hydrophobic oxygen permeable membrane. As oxygen diffuses through the membrane, steady state CL is established. Intensity is proportional to the partial pressure of oxygen. The detection limit is estimated to be 1 part per million (in the gas phase). Steady state CL remains constant for hours or days depending on the amount of oxygen crossing the membrane and the amount of aminoethylene originally present in the analytical solution. This configuration for oxygen is suitable for both solution and gas phase analysis as well as being free from most interferences because of the membrane. It may become a superior alternative to the oxygen electrode for some applications.

4. Reduction of Ru(III)-tris-bipyridyl

The reduction of ruthenium(III)-tris-bipyridyl by hydrazine is accompanied by orange CL. This reaction has been evaluated for the determination of hydralazine, a hydrazine

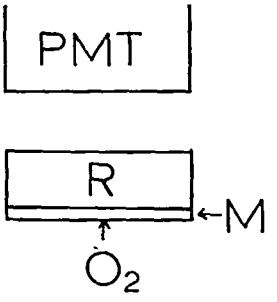


FIGURE 20. Configuration of oxygen probe based on CL of tetrakis N-alkyl aminoethylenes O_2 diffuses through Teflon membrane M to react with reagent R yielding steady-state CL detected by the photomultiplier, PMT.

derivative.[265] A check for interferences, however, revealed that many other reducing agents also lead to CL.

5. Reaction of Polyphenols, Formaldehyde, and Hydrogen Peroxide

Polyphenols react with formaldehyde and hydrogen peroxide in basic solution to yield CL. This reaction has been used to measure low levels of formaldehyde with good selectivity[266] and to determine tannins in plants.[267] These studies have considerable data on mechanistic aspects of the reaction as well as analytical characteristics. This information would facilitate possible applications for other analyses.

6. Pyrogallol as a Peroxidase Substrate

Pyrogallol and other polyphenols can serve as chemiluminescent substrates for measuring peroxidase activity.[268,269] The kinetics are accelerated by adding o-phenylenediamine. This reaction has been successfully used to measure the activity of labeled peroxidase for immunochemical measurement of bacteria and viruses.[270-272] The peroxidase label was detected by adding appropriate amounts of peroxide and polyphenol after the bound and free label had been separated. Sensitivity is reported comparable to spectrohotometric methods.[272] However, when measuring peroxidase activity spectrophotometrically it is possible to reduce detection limits by allowing more time for product to build up.

In one study, detection limits for peroxidase were compared using purpurogallin and luminol as chemiluminescent substrates.[268] The detection limit was about two orders of magnitude lower with purpurogallin. The detection limit for luminol was poorer due to background emission in the absence of added peroxidase.

G. Other Bioluminescence Reactions

Besides firefly and marine bacterial BL, several other BL reactions have been evaluated for possible analytical application. Several of these methods have attractive analytical characteristics but widespread application is unlikely in the foreseeable future because the reagents are not available.

1. Earthworm Bioluminescence

Earthworm bioluminescence can be used to determine hydrogen peroxide.[273] This assay has several desireable analytical features.

The detection limit is comparable to CL methods for peroxide. Intensity is directly proportional to peroxide concentration over several orders of magnitude. The assay is

performed at a neutral pH in a completely aqueous solvent system. The kinetics are slow; intensity reaches a plateau which lasts for several minutes. Thus, this reaction is better suited for continuous monitoring of peroxide formation (or consumption) than any of the CL methods. The weaknesses of this assay are reagent availability and stability. It may someday be the method of choice for hydrogen peroxide.

2. Aequorin

Aequorin is a "photoprotein" isolated from aequoria, a kind of bioluminescent jelly fish. BL is observed upon addition of calcium. Because of its sensitivity, the aequorin assay for calcium has been developed as a means of measuring intracellular ionic calcium.[274-276] Availability considerations preclude other possible applications, at least for the present.

3. Renilla

Renilla BL may be used to assay for PAP (3',5'-diphosphoadenosine) and PAPS (3'-phosphoadenyl sulfate).[277,278] The method is sensitive with linear response to PAP concentration. It has been compared to other methods.

4. Pholas Dactylus

Pholas Dactylus is a luminescent mollusk, once found in abundance in the Mediterranean. The luciferin can serve as a peroxidase substrate, improving the detection limit for peroxidase detection by several orders of magnitude relative to luminol.[202] This system has been used for immunoassay using peroxidase as a label and for coupled assays for glucose and glucose oxidase.[279] Despite this promising potential, it is unlikely that practical applications will result because the species appears to be near extinction.[279]

ADDENDUM

In August, 1980, the reviewer attended a symposium on chemiluminescence and bioluminescence in San Diego, Calif. The proceedings of this symposium will be published by Academic Press under the title, *Bioluminescence and Chemiluminescence,* M. A. DeLuca and W. D. McElroy, editors. Analytical applications of CL and BL comprised a significant part of this meeting. Some of the more significant developments are cited below.

E. Schram and M. Ahmad have made a detailed study of the kinetics of the firefly reaction accounting for product inhibition by both pyrophosphate and oxyluciferin. They have developed a mathematical expression that successfully describes intensity-time curves under a variety of conditions. This could be of considerable value for continuous monitoring assays involving ATP. J. J. Webster, J. C. Chang, J. L. Howard, and F. R. Leach have compared several commercial firefly luciferase preparations, with respect to sensitivity, shape of intensity-time curves, and other parameters. A. Lundin and A. Myhrman reported a simplified method for purifying firefly luciferase by ammonium sulfate precipitation and isoelectric focussing. W. W. Nichols, G. D. W. Curtis, and H. H. Johnston used dilution to demonstrate that clinical urine specimens inhibit firefly luciferase. BL intensity in diluted urine specimens is greater than expected on the basis of dilution factor because inhibitors are diluted out. This emphasizes the need for internal standard additions when using the firefly ATP assay to measure bacteria in urine.

Two very interesting applications of bacterial BL were reported at the San Diego meeting. E. A. Meighen, K. S. Slessor, and G. G. Grant measured trace amounts of

certain insect pheromones that can serve as the aldehyde required for bacterial BL. S. Ulitzur, I. Weiser, and S. Yannai showed that mutagenic and carcinogenic agents induce BL in dark mutant strains of bioluminescent bacteria. Thus, these dark mutants can potentially be used to test for mutagenicity.

Several presentations dealt with CL immunoassays. J. Wannlund and M. A. DeLuca have successfully employed both firefly luciferase and bacterial luciferase as labels for heterogeneous BL immunoassay of methotrexate and dinitrophenol. Detection limits were on the order of two picomoles.

D. W. Reichard and R. J. Miller, Jr. have evaluated pyruvate kinase as a label using the firefly assay to determine pyruvate kinase activity by measuring the rate of ATP consumption. F. Kohen et al. have developed several heterogeneous immunoassays all using alkylated isoluminol as a label. R. D. Lippman has also employed isoluminol as a label for a thiol assay of serum cholinesterase. J. S. A. Simpson et al. have used luminol as a label for heterogeneous immunoassay. The amino group of luminol was coupled to antigen via an amide linkage. D. W. Reichard and R. J. Miller, Jr., have used peroxidase as a label to detect virus/antibody aggregates. The peroxidase was detected by reaction with luminol at high pH.

Two new CL labels were reported. J. S. A. Simpson et al. have evaluated acridines as labels while H. Wynberg et al. are developing chemistry to couple 1,2 dioxetanes to antigens.

J. R. Blinks presented an excellent critical review of applications of aequorin BL to intracellular calcium determinations.

In addition to the work cited above, the reviewer became aware of a new book entitled *Bioluminescence: Current Perspectives,* edited by K. H. Nealson and published in 1980 by Burgers, Minneapolis. Also, Marcel Dekker will soon be publishing a book on CL and BL analysis edited by T. Carter and L. J. Kricka.

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