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CHEMILUMINESCENCE ANALYSIS BASED ON
TWO PHASE REACTANT SYSTEMS

By

Thompson M. Freeman
B.S. (Chemistry) Augusta College, 1975

A DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
Graduate School
Department of Chemistry

May 1981

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ABSTRACT
CHEMILUMINESCENCE ANALYSIS BASED ON
TWO PHASE REACTANT SYSTEMS

by

Thompson Freeman

The University of New Hampshire, 1981

A new approach to analysis with chemiluminescent detection is proposed and demonstrated. Two Phase Chemiluminescence utilizes an immobilized reagent bulk phase and a sample phase which contains the analyte. The analyte is permitted to diffuse into the reagent phase under controlled conditions. A chemiluminescent reaction occurs in the reagent phase, which is quantitated and related to the analyte concentration in the sample phase. A general theory is presented to describe the relationship between analyte concentration and chemiluminescent intensity.

The reaction between luminol and hydrogen peroxide was used in a probe configuration. The reaction was catalyzed by peroxidase immobilized on one end of a fiber optic. A non-linear response for hydrogen peroxide was found at a pH of 9 and 10^{-3} M luminol concentration. The detection limit for peroxide was found to be 10^{-6} M.

The reaction between adenosine triphosphate (ATP) and firefly luciferine was monitored in a flow cell configuration. The firefly luciferase was immobilized in the presence of luciferin. A detection limit for ATP under nonoptimized conditions was estimated at 2×10^{-8} M ATP. With the current design, a calibration curve for ATP concentration could not be constructed due to the decay of the signal intensity with exposure of the reagent phase to ATP.

The chemiluminescence of a peramino ethylene; 1,1',3,3', tetraethyl $\Delta^{2,2'}$ bi(imidazolidine) (EIA); was used to develop an oxygen sensor. A 10% EIA solution in hexane was immobilized behind a Teflon membrane. Oxygen diffusing through the membrane reacted with the EIA producing a signal proportional to the oxygen partial pressure in both the gaseous and aqueous phases. The

detection limit for oxygen in the gas phase was estimated to be about 1 ppm(V/V).

CHAPTER ONE

INTRODUCTION TO CHEMILUMINESCENCE

Chemiluminescence (CL) is the emission of light from an electronically excited molecule generated by a chemical reaction. Emission may be from an electronically excited fluorescent product or a fluorophor excited by energy transfer from the electronically excited product. Thus, any reaction which occurs at room temperature with the emission of light is chemiluminescent. Bioluminescence (BL) is a subclass of CL in which the light is derived from a biological reaction.

CL processes are of interest for several reasons. Chemiexcitation for CL involves fundamental molecular processes. The study of CL provides access to elements of reaction mechanisms and molecular properties. Emergency light sources may be formulated from efficient CL reactions. Classification of different BL reactions indicates the relationships between different bioluminescent organisms

(1). CL and BL may be utilized to detect and measure trace analytes. The analytical literature has been reviewed by several authors over the last few years (1-8). Two phase chemiluminescence is a new approach to this application.

CL intensity reflects the rate of excited state production, since deactivation of the excited state is usually faster than the production of the excited state. Two analytically important consequences follow from this. The usual CL analysis is performed under analyte limiting conditions to ensure psuedo-first order kinetics with respect to the analyte and the CL intensity is then directly proportional to the analyte concentration. In order to maximize precision, the reagents must be mixed reproducibly.

The instrumentation for CL can be quite simple. A reaction chamber, either a test tube or a flow cell, is placed in a light tight chamber. Emitted light is observed by a photomultiplier (P.M.) tube connected to the necessary read-out electronics. Since the CL emission is radiated in all directions, the P.M. tube is commonly placed as close as possible to the reaction zone in order to obtain as high a collection efficiency as possible. A high voltage supply for the P.M. tube completes the usual instrument. In addition to instruments designed specifically for CL, liquid scintillation counters have often been adapted for CL assays

(7).

Analytical CL is described by (4,7)

$$I_{CL} = \phi_{CL} \phi_{det} V \quad (1)$$

where I_{CL} is the CL intensity in Einsteins/sec, ϕ_{CL} is the CL efficiency (the fraction of reacted molecules which result in emission), ϕ_{det} is the detector efficiency (the fraction of photons detected per photon emitted), and V is the rate of production of the excited state in moles/sec. ϕ_{CL} depends upon the specific reaction and the reaction conditions. ϕ_{det} depends on the optical arrangement. Either the peak CL intensity, which occurs soon after the mixing of the reagents and before appreciable reactant consumption has occurred, or the integral of the CL intensity over time is used for quantitation. Measurement of peak height is faster, but is susceptible to mixing errors. The integral of intensity over time is less sensitive to mixing problems, but is very sensitive to the effects of side reactions (7).

Three reactions have been utilized in this research. Analysis techniques have been investigated for hydrogen peroxide using the luminol reaction, ATP using the firefly luciferin-luciferase reaction, and oxygen by the oxidation

of a peramino ethylene. These reactions will be discussed in terms of their chemistry and their analytical characteristics.

CL of Luminol

Luminol (3-amino phthalhydrazide) (Figure 1) is a classic CL reagent for solution analysis.

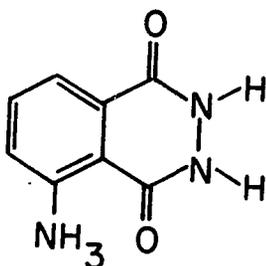


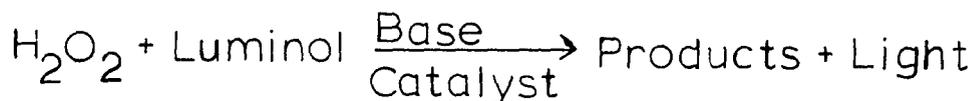
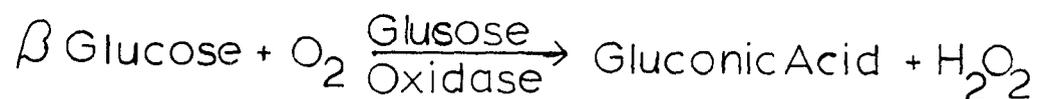
FIGURE 1

Luminol CL may be generated by several different oxidizing reagents including oxygen, hydrogen peroxide, iodine, and chlorine (9-14). The reaction may occur in either protic or aprotic media. Several metal ions and/or their complexes have catalytic properties when H_2O_2 is utilized as the oxidizing agent. Also, several enzymes can catalyse the reaction with H_2O_2 . Because so many species induce CL, luminol is relatively unselective for analytical purposes. The emitter is the aminophthalate ion (20).

Luminol CL occurs under basic conditions, usually between a pH of 10 and 12 in an aqueous phase. There is often a significant background signal due to oxidation by dissolved oxygen, which will degrade the detection limit that can be achieved with luminol. Less basic conditions than pH 10-11 will result in a lower background signal, but will also decrease CL intensity. Such a strategy seldom results in an improvement in the detection limit for peroxide.

Luminol has been utilized in a large number of assays. Luminol, oxidized by H_2O_2 , has been used to measure trace metals by means of their catalytic properties (14), as an endpoint detection system for titrimetry (15), and for the measurement of molecular oxygen (16). Luminol has been coupled to peroxide producing processes for the quantitation of biologically important compounds, such as glucose, amino-acids, and uric acid (21-24). Isoluminol has been investigated as a possible immunoassay label (25). Such an application could result in a lowered usage of nuclear isotopes for immunoassay. The removal of the radioactive material from these reagents is desirable due to restrictions on the disposal of radioactive waste. A detection limit for peroxide of 10^{-8} M has been achieved with luminol (7).

Coupling the luminol reaction to peroxide producing reactions is potentially useful, since some biologically important analytes react with molecular oxygen to produce hydrogen peroxide. For example, glucose, in the presence of the enzyme glucose oxidase produces peroxide (21-23). Difficulties in coupling the luminol reaction to peroxide producing reactions have been encountered. The problems are exemplified by the efforts to couple glucose oxidase to the luminol reaction for the quantitation of glucose.



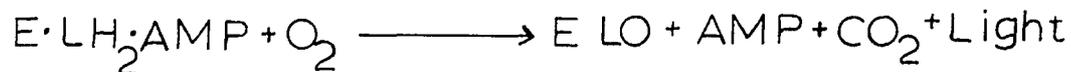
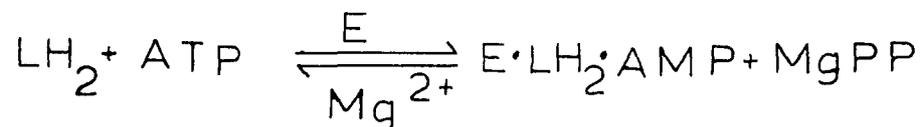
The major difficulty in this scheme, and it is common to many other schemes involving oxidase enzymes, is the pH incompatibility. Glucose oxidase has a pH optimum of about 6, and is completely deactivated by a pH slightly greater than 8. Luminol CL efficiency is very low in the pH range of 6-8, having an efficiency optimum of about pH 11.

Two routes for coupling these reactions are conceivable. A compromise pH can be utilized, losing some of the inherent sensitivity of CL in the process. The second approach is to perform two separate reactions. The oxidase reaction is carried out under optimum conditions and

pH raised (21-23). This approach has been used in essentially all applications to date.

Firefly Bioluminescence

The firefly reaction involves three substrates, luciferin (Figure 2), adenosine triphosphate (ATP), and molecular oxygen in the presence of luciferase enzyme and Mg^{2+} ions. The overall reaction is (26)



where LH_2 is luciferin, E is luciferase, AMP is adenosine monophosphate, MgPP is magnesium pyrophosphate, and LO is oxyluciferin (the emitter of the reaction) (Figure 2). In neutral and basic solutions, the emitted light is the usual yellow-green (λ_{max} 563nm.). The pH optimum is about 7.5. With sufficient care, this reaction can be used to measure as low as 10^{-14} mole of ATP (26). Several conditions must be met to achieve this low detection limit. The presence of heavy metals or an acid pH must be avoided, since the emission will be red shifted about 50 nm under these conditions. Since P.M. tubes are generally less sensitive in the red, such a large red shift would cause an

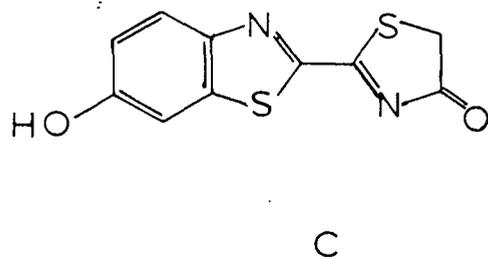
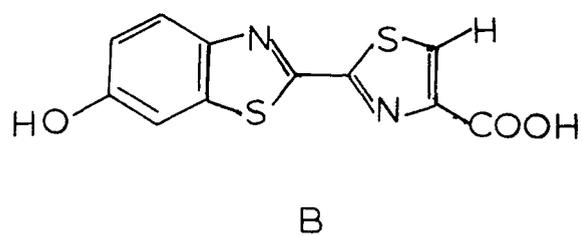
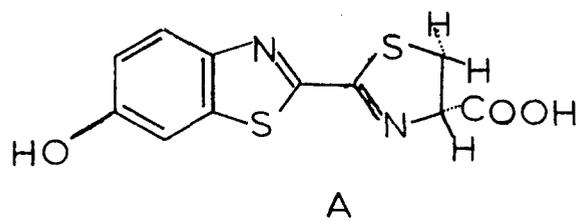
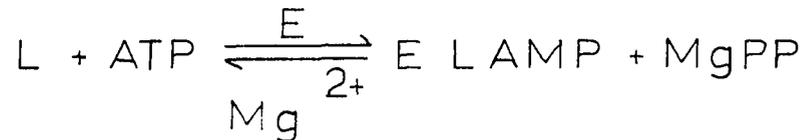


FIGURE 2

Structures of Several Luciferin Species

- | | |
|---|------------------|
| A | Luciferin |
| B | Dehydroluciferin |
| C | Oxyluciferin |

appreciable decrease in assay sensitivity. The purity of the reagents must be high to avoid side reactions. The presence of other enzymes can lead to rephosphorylation of ATP precursors which happen to be present in the solution. The presence of dehydroluciferin (Figure 2) will inhibit the reaction by forming a more stable, nonreactive enzyme complex by the reaction shown:



where L is the dehydroluciferin and the other symbols are as before. Addition of excess pyrophosphate can reverse this inhibition, but pyrophosphate will reverse the desired reaction with luciferin. Luciferase is a sulfhydryl containing enzyme and is inhibited by reagents interacting with these groups. The anions SCN^- , I^- , NO_3^- , and Br^- are also inhibitors.

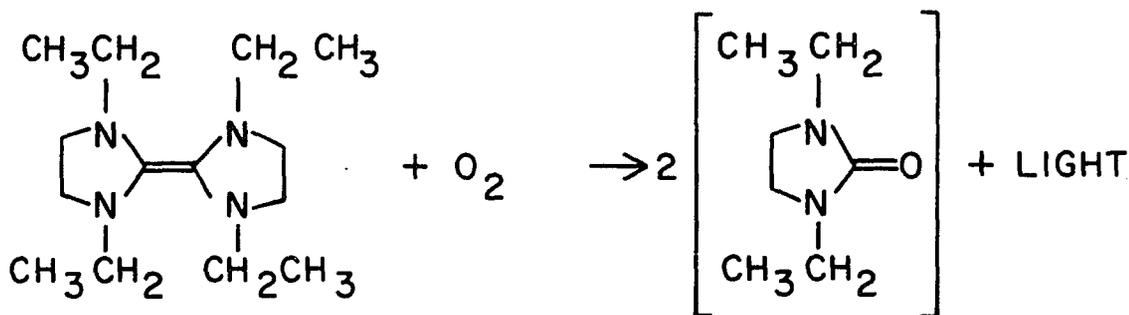
While the addition of luciferin is unnecessary if a crude firefly extract is used, the addition of luciferin will increase the emission intensity by a factor of about 4. Of course, luciferin must be added after purification of the luciferase enzyme.

Firefly luciferase is highly specific for ATP, but other nucleotides stimulate light emission (6,27). The luciferin-luciferase reaction can be used to estimate biomass, since the levels of ATP are similar among various organisms. The firefly reaction has also been applied to determine the activity of enzymes that utilize or produce ATP. ATP may also be bound to a ligand for use in immunological assays (6,27).

The luciferin-luciferase reaction has been used in a two phase analysis system by immobilizing the luciferase to the surface of alkylamine glass beads glued to a capillary tube (28). The peak light intensity is reported to be linear with respect to ATP solution concentration between 10^{-8} M. and 10^{-5} M. The enzyme is stable for extended periods of time when stored at 4°C in a phosphate buffer. One limitation of this approach is the difficulty in positioning the glass rod reproducibly within the sample chamber. A possibly more serious limitation is the time dependent response of the system as a depletion layer grows at the surface of the rod.

Peraminoethylene Chemiluminescence

The oxyluminescence of tetrakis(dimethylamino) ethylene (TMAE) was first discovered by Pruetz and co-workers in 1950 (29). Since then the oxidation of many other non-aromatic tetraaminoethylenes has been demonstrated to produce chemiluminescence. The overall reaction is shown for 1,1',3,3' tetraethyl- $\Delta^{2,2'}$ -bi(imidazolidine) (EIA).



The imidazolidone is the only isolable product except in the case of TMAE. The substituted urea is the predominant product of TMAE oxidation (30).

The oxidation of tetraamino ethylenes is unusual in that a bright CL emission is obtainable at very high reagent concentrations, or even using the neat liquid. This quality, along with the ready availability of oxygen, made these compounds of interest for chemical lighting applications. Since a large molar amount of reagent could be contained in a small volume, the integrated light output

of such a device should be high (1,31-38). To date, however, no device based upon this chemistry has seen commercial application, due in part to quenching by the reaction product and catalysis with quenching of the emission by protolytic substances. The difficulty of long term airtight storage probably contributes to the lack of commercial success .

The linear relationship between oxygen partial pressure and the CL intensity (35-38) make this reaction attractive for possible quantitative analysis of oxygen. Since CL is obtained only with oxygen, such a device should be quite selective (30). Reaction with other oxidizing reagents (halogens, permanganate) would severely shorten the useful lifetime of the reagent. This problem could be solved by physically isolating the ethylene reagent from the sample.

TWO-PHASE CHEMILUMINESCENCE FOR ANALYSIS

Two phase CL is a new approach to CL analysis. A reagent which is either chemiluminescent itself or an enzyme catalyzing a CL reaction is physically immobilized, while an analyte solution is presented to the interface between the two phases. The physical nature of the interfacial material is chosen to permit the analyte to diffuse through it under a concentration gradient. Once in the reagent phase, the analyte undergoes a CL reaction. The CL intensity is then quantitated. Figure 3 illustrates this schematically.

The characteristics of a Two Phase CL system will depend on both the chemistry of CL and the rate of mass transfer across the interface. It will also depend on whether the reagent phase is a surface phase or a bulk phase, since the analyte may diffuse into the interior of the bulk phase. The projects described in this dissertation all involve bulk reagent phases.

Two Phase CL has several potential advantages. These advantages include the recovery of expensive reagents localized in the reagent phase, the ease of obtaining reproducible mixing, the possibility of continuous sensing, and the possibility of tailoring the phase interface to

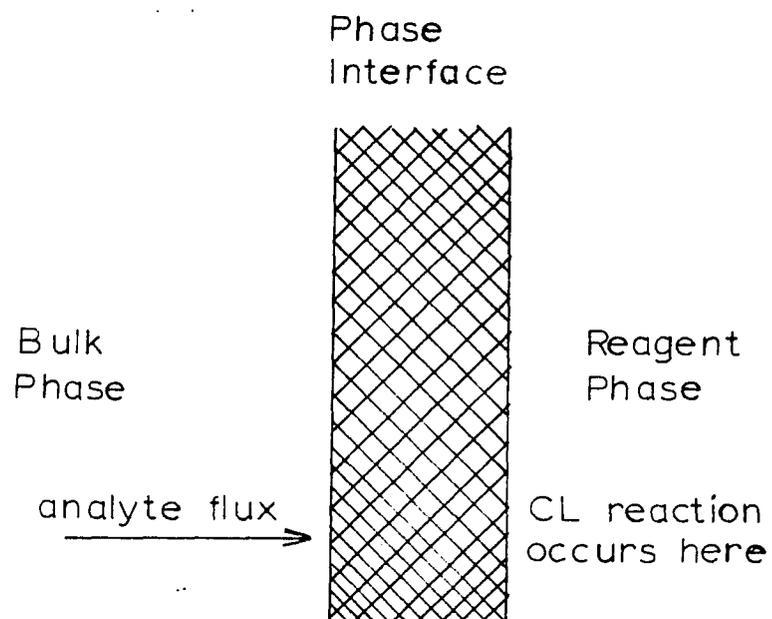


FIGURE 3

Schematic of TWO PHASE CHEMILUMINESCENCE

This figure shows the relationship between the two phases and emphasizes the direction of analyte flux.

exclude potential interferences.

Since at least one reaction component for the CL emission is immobilized, this component can be removed from the reaction and reused. When the component is expensive, as is the case for many enzymes, this approach would significantly reduce the cost per analysis.

As was stressed earlier, the manner of mixing affects the quality of the data derived from a CL assay. Mixing in Two Phase Chemiluminescence is by diffusion across a phase boundary under conditions of a dynamic steady state. This results in a signal intensity which is independent of elapsed time. Under such conditions, the intensity of the CL signal in a Two Phase system will depend upon the analyte concentration in the bulk phase and the phase interface thickness.

Another advantage is the ability to exclude potentially interfering substances from the reactant phase. For example, in the oxidation of peraminoethylenes, the presence of other oxidizing substances will increase the rate of reagent consumption, and, therefore, decrease the device's lifetime. The existence of the gas permeable membrane between the reactant phase and the analyte phase assures that nonvolatile substances such as MnO_4^- are excluded from the reaction. Similarly, a dialysis membrane between

the reactant phase and the analyte phase will protect an immobilized enzyme from being inactivated by proteolytic enzymes in the sample.

The objective of my research was to characterize the advantages, and disadvantages of the Two Phase CL approach to analysis. The theory to describe the steady-state response of a generalized Two Phase CL system is developed in chapter two. Chapter three covers a two phase system for peroxide analysis based on peroxidase catalysis of luminol CL. Chapter four describes a two phase system for ATP determination based upon the luciferin-luciferase reaction. A two phase oxygen sensor based upon peraminoethylene CL is the subject of chapter five.

CHAPTER TWO

THEORY OF TWO PHASE CHEMILUMINESCENCE

In this technique, the analyte is initially in solution with no analyte present in the reagent phase. Any non-immobilized reagents will be present in equal concentrations in both phases. The analyte will diffuse into the reagent phase and react. A conceptual concentration profile is shown by the solid line in Figure 4. The analyte is consumed as it diffuses through the immobilized reagent phase. If the reaction rate is rapid relative to diffusion, the analyte concentration will fall to zero at some depth into the reagent phase. At steady state, the rate of the analyte entering the reagent phase will just equal the rate of analyte consumption over the entire reaction volume. Therefore, the measured CL intensity will be the sum of intensity from each of the reaction volumes along the concentration gradient.

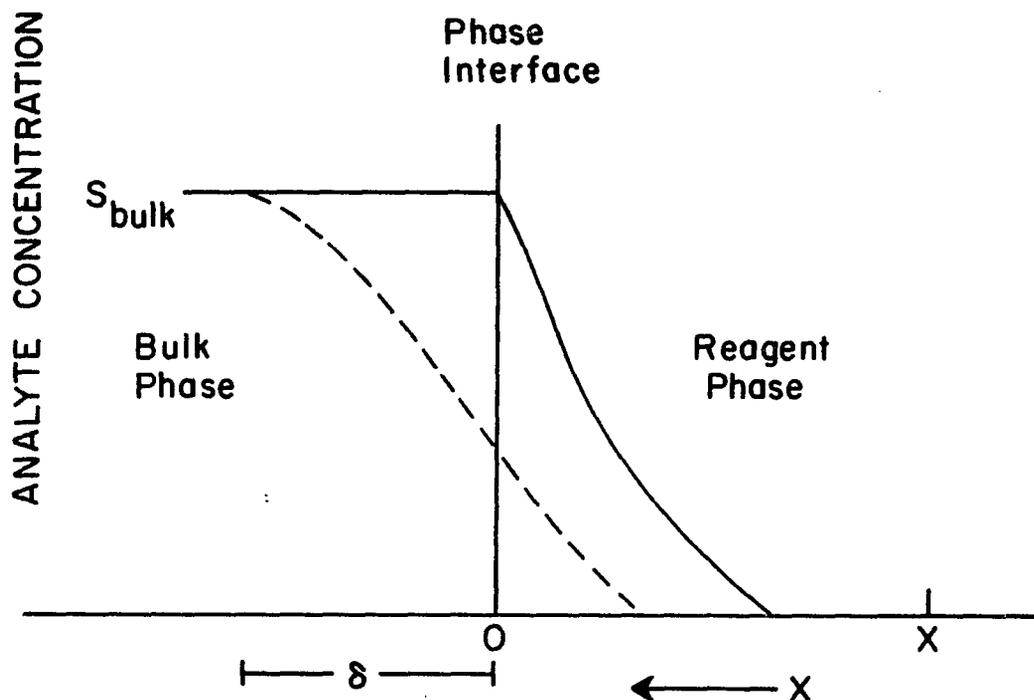


FIGURE 4

Concentration Profile of TWO
PHASE CHEMILUMINESCENCE

The solid line indicates the concentration profile assuming that the bulk phase mass transfer is fast enough to be neglected. The dotted line indicates the concentration profile when the reaction is sufficiently rapid to deplete the surface layer of the analyte. δ is the Nernst diffusion layer thickness. X is the thickness for the reagent phase.

While this discussion has been brief, the terms required to describe a steady state, two phase CL system have appeared; the rate of the analyte diffusion, the kinetic parameters of the reaction, and the physical layout of the system. A formal theory to describe steady state intensity will be developed. Since molecular diffusion is a slow process as compared with convection, two phase CL may be expected to exhibit lower intensities than one phase CL for equivalent analyte concentrations. This drawback may be overcome, in part, by utilizing a large interfacial area.

Three major assumptions have been made in developing a general theory for Two Phase CL. First mass transfer of the analyte to the interface is assumed to be sufficiently efficient to insure an interfacial concentration equal to that of the bulk solution. In practice, the analyte solution is stirred or pumped to insure that convective mass transfer in solution is much greater than the diffusional process within the bulk of the immobilized phase. Therefore, no depletion of the analyte should occur on the bulk phase side of the phase interface. This assumption will be dealt with subsequently. Second, the analyte is assumed not to partition between the two phases. For the work described here, this assumption appears reasonable. Third, the CL reaction is assumed to have psuedo-first order kinetics. For this to occur in enzymatic reactions, the

substrate has to be present in a concentration less than the Michaelis constant K_m . Since the analyte concentration will drop to zero at some point within the immobilized region, this assumption is reasonable.

An analogous problem has been solved by Blaedel et al (39) for the case of the steady state response of an enzyme electrode. Using the assumptions stated above, they have shown that the analyte concentration as a function of depth within the reagent phase is

$$S(x) = S_{\text{bulk}} \frac{\cosh \sqrt{\frac{K}{D_s}} x}{\cosh \sqrt{\frac{K}{D_s}} X} \quad (2)$$

The terms are defined and dimensioned as follows: $S(x)$, the analyte concentration at position x (mole/liter); S_{bulk} , analyte concentration in the bulk solution (mole/liter); X , the immobilized phase thickness (cm); D_s , the diffusion coefficient for the analyte in the immobilized phase (cm^2/sec); K , the pseudo-first order rate constant (1/sec).

Because CL is inherently a rate process, we are interested in the number of events per unit time, V , occurring in the entire reagent phase. Since we are

assuming a pseudo-first order process for the CL reaction, for each infinitesimal volume in the immobilized phase

$$R = K S \quad (3)$$

where R is the rate of the reaction (moles/liter-sec), and S is the effective concentration of the analyte. Equation 2 shows that S is an exponential function of depth in the immobilized phase. The integral of S(x) from 0 to X times the interfacial area A replaces S in equation 3, which changes R to V, producing the expression

$$V = A K \int_0^X S(x) dx \quad (4)$$

Equation 4, in an incomplete form, indicates the total number of events occurring in the volume of the immobilized phase. Substituting equation 2 for S(x) in equation 4

$$V = A K \int_0^X S_{\text{bulk}} \frac{\cosh \sqrt{\frac{K}{D_s}} x}{\cosh \sqrt{\frac{K}{D_s}} X} dx \quad (5)$$

Rearranging equation 5 results in

$$V = A K S_{\text{bulk}} \frac{1}{\cosh \sqrt{\frac{K}{D_s}} x} \int_0^x \cosh \sqrt{\frac{K}{D_s}} x \, dx \quad (6)$$

Evaluating the integral yields

$$V = A K S_{\text{bulk}} \frac{\sinh \sqrt{\frac{K}{D_s}} x}{\cosh \sqrt{\frac{K}{D_s}} x} \left[\frac{K}{D_s} \right] \quad (7)$$

Which can be rearranged to

$$V = D_s K A S_{\text{bulk}} \frac{\sinh \sqrt{\frac{K}{D_s}} x}{\cosh \sqrt{\frac{K}{D_s}} x} \quad (9)$$

When the values of $\sqrt{K/D_s} \gg 2$, the sinh and cosh terms are essentially equal and drop out of the equation. This corresponds to the situation where the immobilized phase thickness and the reaction rate are sufficiently large so that the analyte is completely reacted before it has a chance to diffuse through the phase.

Thus far, it has been assumed that no concentration gradient is formed in the analyte phase at the surface of the reagent phase. In practice, if the reaction in the immobilized phase is fast, analyte will be consumed at a fast enough rate to form a significant concentration gradient across the interface. The resulting analyte depletion at the interface is shown by the dotted line in figure 4. In this case, the assumption $S(0) = S_{\text{bulk}}$ is invalid. Therefore, a new variable, S_{surface} must be defined as the steady-state concentration of the analyte at the interface. If the solution is stirred, the Nernst diffusion layer concept (40), commonly used in electrochemical theory, may be applied. The equation for a one dimensional model is

$$V' = \frac{A D_s (S_{\text{bulk}} - S_{\text{surface}})}{\delta} \quad (10)$$

where V' is the number of moles of analyte reaching the surface per unit time, A is the interfacial area, D_s is the diffusion constant of the analyte in solution, and δ is the Nernst diffusion layer thickness, or the thickness of the membrane forming the interface. The diffusion layer thickness is a function of how well the solution is mixed (Figure 4).

Equation 10 may be modified by setting S_{bulk} of equation 9 equal to S_{surface} of equation 10 and cancelling the hyperbolic functions. At steady-state, the number of moles of analyte reaching the immobilized phase equals the number of moles reacting within the immobilized phase, i.e. $V=V'$. By simplifying and rearranging equation 9, substituting S_{surface} for S_{bulk} ,

$$S_{\text{surface}} = \frac{V}{A K D_s} \quad (11)$$

Substituting equation 11 into equation 10 and rearranging

$$V = \frac{K D_s A (D_s / \delta) S_{\text{bulk}}}{K D_s + (D_s / \delta)} \quad (12)$$

Equation 11 is a complete expression which relates the integrated reaction rate of the reaction with an immobilized reagent to the pseudo-first order rate constant, analyte concentration the analyte diffusion rate, and the quality of mixing.

Several efficiency factors must be considered to relate the CL signal intensity to the integrated reaction rate. By substituting into equation 1, one gets

$$I = \phi_{CL} \phi_{det} \frac{\sqrt{K D_S} A(D_S/\delta) S_{bulk}}{K D_S + (D_S/\delta)} \quad (13)$$

Avogadro's Number is required to convert the units of I_{CL} from moles of photons/sec to photons/sec. As expected, the CL intensity is proportional to the bulk concentration of the analyte.

If $\sqrt{K D_S} \ll D_S/\delta$, the signal intensity will be limited by the kinetics of the CL reaction. The intensity will depend upon the pseudo-first order rate constant. The overall intensity will be lower than in the other limiting case. The signal will not demonstrate a dependence upon the mixing efficiency. When $\sqrt{K D_S} \gg D_S/\delta$, the signal will become mass transfer-limited. This circumstance is analytically desirable since a greater intensity and better sensitivity may be achieved. The signal intensity will depend upon the mixing efficiency but will be independent of reaction rate.

This model may be extended to the case of an enzyme-catalyzed reaction utilizing Michaelis-Menten kinetics. Since the pseudo-first order rate constant K is equal to V_{max}/K_m , at substrate concentrations less than a few percent of the K_m substitution results in

$$I = \phi_{CL} \phi_{det} \frac{\sqrt{\frac{V_{max} D_s}{K_m}} A \left[\frac{D_s}{\delta} \right] S_{bulk}}{\sqrt{\frac{V_{max} D_s}{K_m}} + \left[\frac{D_s}{\delta} \right]} \quad (14)$$

A quantitative theoretical model of the time response for Two Phase CL has not been developed. However, it is possible to delineate some of the qualitative aspects concerning the factors affecting the response times of these devices.

Initially, a two phase CL device will have a concentration profile in the form of a step function. Under the influence of concentration gradients, the profile will start to become a smooth curve, which will achieve a steady-state form after some period of time. This time period will be a function of the depth of the concentration profile ($\delta + x$) and the diffusion coefficient of the molecule, assuming no complicating equilibria exist. The depth of analyte penetration into the immobilized phase should be a function of the reaction rate, the diffusion coefficient, and the analyte concentration in the bulk phase. If the reaction is sufficiently fast, $\delta \gg x$, then the response time should become independent of the reaction rate.

CHAPTER THREE

INTRODUCTION TO THE CHEMILUMINESCENT- FIBER OPTIC PROBE

An immobilized CL reagent is used in two phase chemiluminescence. This reagent may react directly with the analyte to produce emission, or it may catalyze the CL reaction between the analyte and a second reactant. A useful property for the immobilized reagent is a high molecular weight. High molecular weight species are easily trapped in the fibrous network of an immobilizing gel or are easily retained by a dialysis membrane. BL reactions, and some CL reactions, are catalyzed by high molecular weight species known as enzymes.

Enzymes have enormous analytical potential. Enzymes are generally very specific for their substrates. The catalyzed reaction occurs under chemically mild conditions. These qualities enable the analyst to employ simple methodologies in very complex matrices without loss of specificity or complicating side reactions. A difficulty of enzyme techniques has been the high cost of enzymes of sufficient purity to use in analysis. A potential solution

to the cost problem is to immobilize the enzyme since an immobilized enzyme may be reused (41-43).

Enzyme electrodes are analytical devices which utilize immobilized enzymes. These devices have an immobilized enzyme over the surface of an electrode. The enzyme reacts with the analyte and either consumes a second, electroactive substrate or produces an electroactive product (39).

The Chemiluminescent-Fiber Optic Probe (CL-FOP) is a device which is similar to the enzyme electrode. An immobilized enzyme phase covers the active face of the "sensor". The analyte diffuses into the enzyme phase, undergoes a reaction, and yields a product which emits light. The emitted light can then be quantitated.

The expected advantages of a CL-FOP are the sensitivity of CL reactions, and the conservation of enzymes. Since BL enzymes are expensive, initial characterization of the CL-FOP was with oxidation of luminol by hydrogen peroxide. This reaction is catalyzed by peroxidase, which is a robust and inexpensive enzyme.

EXPERIMENTAL

Reagents

All reagents and their sources are listed in Table 1.

Two procedures were used to prepare the immobilized reagent phases. For some experiments, a weighed amount of enzyme was dissolved in the same buffer used to make the sample solution. This solution served as the reagent phase. Separation of the reagent phase from the analyte solution was by means of a dialysis membrane between the two phases. Physical entrapment of the enzyme in a polyacrylamide gel was used in most experiments (42). The preparation of the gel was an adaptation of a method reported to form a stable, clear gel (42). The gel was formed from a 50 ml solution of either 0.1 M borate or 0.1 M phosphate buffer, containing 0.12 g N,N-methylene-bisacrylamide, and 2.38 g acrylamide. The buffer pH and luminol concentration were chosen to match the respective sample solution values. Typical values were 10^{-3} M luminol and pH 9. The buffer solution was heated to 60°C to dissolve the acrylamide. The solution was then cooled to 35°C and filtered to remove undissolved solids. This solution was stable at 4°C for at least two days. The gel was formed after addition of the enzymes to the stored solution. Approximately 1 mg/ml of both riboflavin

TABLE 1

List of Reagent Suppliers for CL-FOP

| REAGENT | SOURCE |
|---|--------------------------|
| Horseradish Peroxidase (91 Purpurogallin Units/mg) | Sigma type I |
| Glucose Oxidase (130 units/mg) | Sigma type VII, |
| Boric Acid | "Baker Analysed" reagent |
| Sodium Phosphate monobasic | "Baker Analysed" Reagent |
| Potassium Hydroxide | "Baker Analysed" Reagent |
| N,N-methylene- bis acrylamide | Aldrich |
| Acrylamide | Aldrich |
| 30% Hydrogen Peroxide | "Baker Analysed" Reagent |
| Glucose | Aldrich |
| 3-aminophthalhydrazide | Aldrich |
| Potassium Ferricyanide | "Baker Analysed" Reagent |

and potassium persulfate were added to effect the polymerization of the acrylamide and the bisacrylamide. Polymerization was accomplished in a water saturated nitrogen environment by irradiating the gel solution with a Hg pen lamp. The gel was about 1 mm thick and appeared to be uniform. Unused gel was stored in a buffer- luminol solution at 4°C.

A diagram of the apparatus for forming the immobilized enzyme gels is shown in Figure 5. A stream of nitrogen gas is then passed into a glass chamber, about 12.7 cm long and 5 cm in diameter. The open end of the chamber is closed with a one hole rubber stopper. The gel is formed on the top side of a piece of plexiglas, which has been milled to a depth of 1 mm and placed within the chamber. U.V. radiation to induce polymerization was supplied by a Hg pen lamp, with the shield removed, held over the glass chamber by a ring stand. The entire apparatus, except for the nitrogen cylinder, was kept in a hood. The face of the hood was covered with paper to reduce the stray U.V. radiation.

Buffer-luminol solutions were prepared by combining appropriate amounts of the acid in deionized water with a basic solution containing luminol. 0.5 g/liter Na_2EDTA was added, and the solution titrated to the desired pH with base. pH paper was used to monitor the pH of the solution.

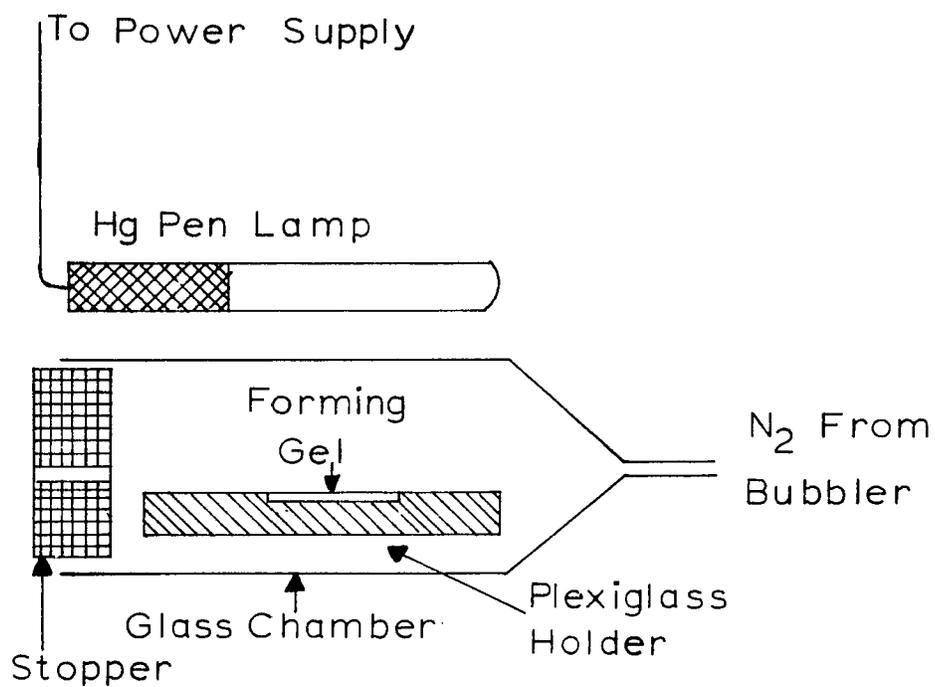


FIGURE 5

Apparatus for Immobilizing Enzymes
in a Polyacrylamide Gel

The EDTA was added to complex any trace metals in the solution thus inactivating their catalytic properties, and reducing the background signal due to metal catalyzed luminol CL.

Analyte stock solutions were prepared by dilution with deionized water. Standard solutions were then prepared by dilution of the initial stock solution. Analytical samples were the result of adding a portion of stock solution to 10 ml of buffer solution. All analyte concentrations refer to the concentration of the analyte at the final diluted concentration.

Apparatus

The original apparatus is diagrammed in Figure 6. The sample solution was contained in a 15 ml Pyrex beaker. The beaker was set in a hole cut into corrugated cardboard. Both beaker and cardboard were glued to the top of a magnetic stirring plate. PVC electrical tape around the edges of the cardboard eliminated light leaks and tape over the cardboard provided a measure of protection from spills. A 2x10 mm stirring bar was the only size found which achieved good stirring without either spinning free or vortexing part of the solution out of the beaker. Solution withdrawal was accomplished with a small diameter tube

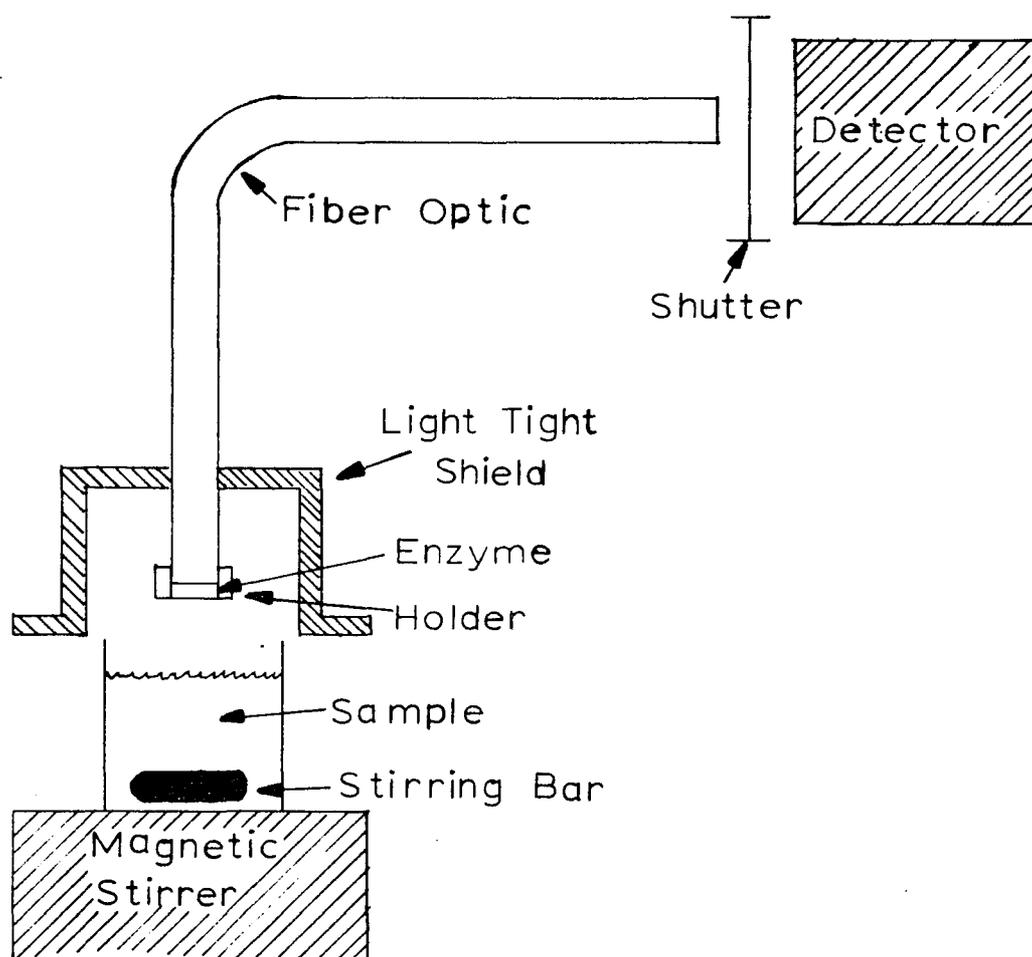


FIGURE 6

Diagram of CHEMILUMINESCENT
FIBER OPTIC PROBE apparatus

fastened to a syringe.

The fiber-optic (FO) (Corning, 2 ft length, 1/8 in. diameter, numerical aperture 0.63, acceptance angle 39° , transmittance 0.63 at 500 nm.) was slip-fitted into a light shield. The immobilized phase was held in place by a Nylon net from a stocking fitted between two concentric 1 cm long pieces of tygon tubing. Dialysis membrane, when used, was held by the same holder as the gel. The other end of the FO was inserted into an adapter fitted to a 35 mm camera shutter. The shutter was taped with electrical tape to the vapor barrier of a P.M. tube (EMI 9558 qa, S-20 response) housing. A Styrofoam outer housing allowed the P.M. tube to be cooled with crushed dry ice to reduce the dark current noise. At 1800 V, the room temperature dark current was about 10^{-8} A with a peak-to-peak noise component of $2-3 \times 10^{-9}$ A. After cooling with dry ice, the dark current averaged 9×10^{-10} A with a noise component of 3×10^{-10} A peak-to-peak. The P.M. tube output was connected to a 610c Keithly Electrometer. The electrometer output was connected to a Heath SR 255 A/B strip chart recorder.

In order to perform a crude temperature dependence study of the probe, an ice jacket was constructed for the light shield with plastic bags. After they were filled with ice, the bags were taped to the exterior of the light

shield. All solutions were kept in an icebath prior to use.

After the majority of the work was completed, the P.M. tube, P.M. tube housing, shutter, and electronics were changed. The end of the fiber-optic was connected with a shutter, which fitted on a Products for Research refrigerated P.M. tube housing (model TE-10143). The P.M. tube was an RCA PF1012 tube with a 1/4 inch photocathode and S-20 response. The high voltage supply was set at 1800 volts. The power supply and electrometer were consolidated into a SPEX digital photometer (model DPC 2) which has the capability of photon counting. The cooling capabilities of the P.M. tube housing were not employed since the room temperature dark current of the RCA tube was less than 10^{-11} A, the lowest current readable on the SPEX photometer.

Procedures

Measurements of CL intensity with respect to peroxide concentration were performed by standard addition of 25 μ l peroxide solutions to ten ml of buffer/luminol solution. The light shield was placed over the beaker, the shutter opened, and the resulting signal recorded on the chart recorder. Separate gels, matching the sample solution in pH and luminol concentration, were made for each determination

of the effect of pH, luminol concentration, and enzyme concentration. The temperature dependence experiment was performed at 4°C and 24°C. The experimental set-up was cooled by the ice jacket as described earlier. Except for one set of experiments, stirring was at the highest rate possible.

While using the original equipment, the above procedures were followed on all but three sets of experiments, two of which involved attempts to change the chemistry of the system. The first change attempted was the substitution of Lucigenin (Figure 7) for luminol .

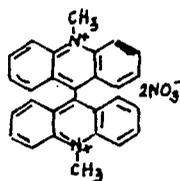


FIGURE 7

Peroxide was added by standard addition to a buffer/lucigenin solution of pH 9. The second set of experiments used luminol as the analyte, instead of peroxide. The peroxide was held at a concentration of 10^{-2} M, and the concentration of luminol varied. The solution was buffered at pH 9.

The few experiments performed on the new equipment included experiments on catalysis by ferricyanide, hemin, and peroxidase. The analytes used were glucose and hydrogen peroxide. The procedure for each experiment will be described in the appropriate Results section.

RESULTS AND DISCUSSION

pH and Luminol Dependence

Initially, it was anticipated that there would be a marked pH dependence of the probe response. This is due to the difference between the optimum for the peroxidase enzyme, which is pH 7 (43), and the optimum pH for luminol CL, which is between pH 10 and 11. An early experiment was performed to locate the optimum pH where luminescence is acceptably intense while maintaining sufficient enzyme activity to insure a quick response.

At the time this early experiment was carried out, it was also not known what effect the luminol concentration had upon the CL signal of the probe. Therefore, concurrently with the pH dependence study, a luminol concentration dependence study was performed. This resulted in a two dimensional mapping experiment. The test conditions were pH's of 8, 9, and 10, each for luminol concentrations of 10^{-4} M, 10^{-3} M, and 10^{-2} M. Since response time had to be traded for signal intensity. An optimum set of conditions was selected for further characterization. The criteria for selection of the optimum were signal intensity and time to reach steady-state.

The pH effect is illustrated by the data for 10^{-3} M luminol in Figure 8 and the relative intensity values in Table 2. At pH 8, the signal reaches a steady-state very quickly, reflecting the higher peroxidase activity at lower pH's. However, the signal intensity is relatively weak, due to the inefficiency of luminol CL at this low pH. The situation is reversed at pH 10. The long period of time required to reach a steady-state intensity is the result of the low enzyme activity. Since the enzyme is less active, the peroxide must diffuse to a greater depth in the gel before being totally consumed. The high pH, however, also causes the luminol CL process to be more efficient than at low pH's. Signal intensity at pH 10 is almost eight times the value at pH 8 for the same luminol concentration. At a pH of 9, the time required to reach steady-state is greater than for pH 8, but less than for pH 10, having a value between 30 and 45 seconds. For 10^{-3} M luminol the intensity at pH 9 is greater than at pH 10. At pH 9 peroxidase is a better catalyst, compensating for the higher CL efficiency at pH 10.

The probe response dependence upon luminol concentration was less dramatic than the dependence on pH. Nevertheless, some effect was observed, and is shown in Table 3. At pH 9, increasing the luminol concentration simply improved the response times. The probable reason for

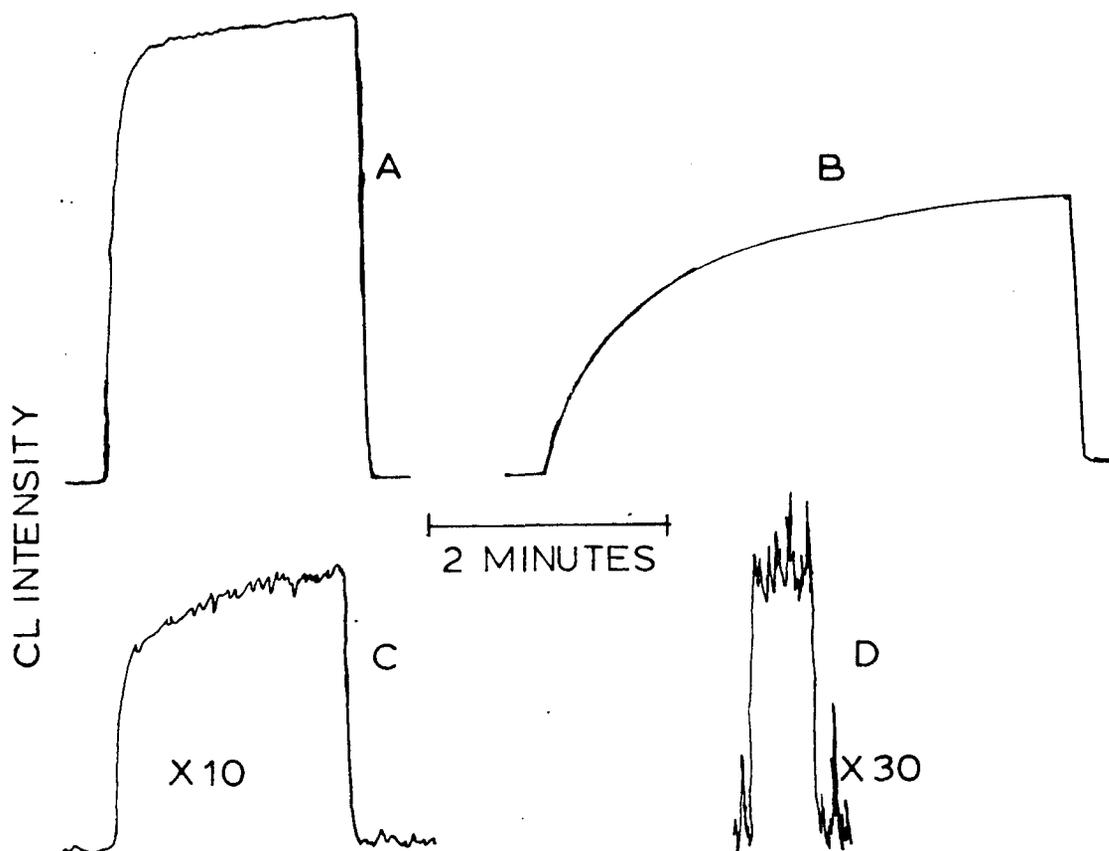


FIGURE 8

Response of CL-FOP Under Different Conditions

CL intensity vs. time under different conditions:

- (a) pH 9, 10^{-3} M luminol, 10^{-4} M H_2O_2
 - (b) pH 10, 10^{-3} M luminol, 10^{-4} M H_2O_2
 - (c) pH 8, 10^{-3} M luminol, 10^{-4} M H_2O_2
 - (d) pH 9, 10^{-3} M luminol, 10^{-5} M H_2O_2
- All data for a polyacryamide gel containing 1mg/ml horseradish peroxidase.

TABLE 2

Relative Intensity as a Function of pH and Luminol

| pH | Emission Intensity for: | | |
|----|------------------------------|------------------------------|------------------------------|
| | 10^{-2}M Luminol | 10^{-3}M Luminol | 10^{-4}M Luminol |
| 8 | 0.003 | 0.089 | — |
| 9 | 0.88 | 0.99 | 0.50 |
| 10 | 1.00 | 0.72 | 0.10 |

All intensity values are normalized relative to the intensity for 10^{-2}M luminol at pH 10.

Table 3

Relative Response Time as a Function of pH and Luminol

90% Response times for:

| pH | $10^{-2}M$ | $10^{-3}M$ | $10^{-4}M$ |
|----|------------|------------|------------|
| | Luminol | Luminol | Luminol |
| 8 | — | 0.19 | — |
| 9 | 0.18 | 0.30 | 0.51 |
| 10 | 0.36 | 1.00 | 0.72 |

All values are normalized relative to the response time of $10^{-3} M$ luminol at pH 10.

this is that luminol is also a substrate in this reaction. Therefore, an increase in the luminol concentration will increase the overall reaction rate, thereby decreasing the time required to reach a steady state response. For pH 10, the 10^{-3} M luminol concentration is the slowest responding set of conditions.

The maximum steady-state signal occurs at 10^{-3} M luminol at both pH 8 and 9. Maximum steady-state response at pH 10 is with 10^{-2} M luminol. Rationalizing these results is difficult, but may be due to the nature of the peroxidase-luminol reaction (44). There is a free radical intermediate which results from the reaction of an intermediate form of the enzyme and a luminol molecule. This free radical eventually reacts with a free peroxide molecule. It is reasonable to expect that at pH's 8 and 9, where the enzyme is relatively active, an appreciable concentration of the free radical could accumulate. At luminol concentrations above 10^{-3} M, the concentration of free radical intermediates could be large enough to allow side reactions to become kinetically important, leading to a quenching of the signal. At a pH of 10, the enzyme is relatively inactive, and the free radical intermediate never achieves a high enough concentration to suffer from these side reactions.

Since luminol concentration and pH affect the CL intensity, all gels were formed from the same buffer as the test solution. The gel was also soaked in the test solution as a further precaution. Under these conditions, any concentration gradients in the unused gel should be eliminated.

Concentration gradients can form, however, with sufficiently high concentrations of peroxide due to the depletion of luminol. The peroxide molecule is considerably smaller than the luminol molecule, and should diffuse more rapidly in the gel. Since the stoichiometric ratio is two peroxides:one luminol, under conditions of diffusion-limited mass transport, luminol should become depleted within the reaction zone. For conditions of 10^{-3} M luminol in a pH 9 buffer, the 10^{-4} M peroxide signal decays at a rate of about 1% per minute. For a 10^{-5} M peroxide signal, the rate of decay is much smaller.

Traces A and D in Figure 8 also show an interesting effect of peroxide concentration upon the response time. For our purposes, response time is the time between the insertion of the probe into the test solution and the attainment of a horizontal portion of the tracing. At 10^{-5} M peroxide (trace D), with a pH 9 and 10^{-3} M luminol solution, the response time is typically 3-5 seconds. This

compares to a response time of 50 seconds for 10^{-4} M peroxide under the same conditions.

Precision and the Effect of Stirring

The CL-FOP is expected to be highly sensitive to the rate of stirring of the solution. Recalling equation 14

$$I = \phi_{CL} \phi_{det} \frac{\sqrt{K D_S} A(D_S/\delta) S_{bulk}}{K D_S + (D_S/\delta)} \quad (14)$$

it can be seen that when $\sqrt{V_{max} D_S/Km} \gg D_S/\delta$, the CL intensity will be dependent upon δ . When δ is decreased in magnitude, the CL intensity rises. Since the diffusion layer thickness is a function of the stirring rate, the CL-FOP should be very sensitive to variations in stirring when mass transfer-limited. If the probe is experimentally sensitive to changes in the stirring, it is a good indication that the peroxidase concentration is sufficient to deplete the hydrogen peroxide concentration at the gel/solution interface.

An experiment was done to investigate the dependence of the CL-FOP to stirring and a possible technique for improving precision. Signal intensity was halved by decreasing the rate of stirring from the highest setting to the lowest setting that could be counted on to continue

mixing. Stopping stirring altogether resulted in a very small signal with 10^{-4} M peroxide. The intensity would return to its original (stirred) value upon resumption of stirring. These results led us to glue the beaker to the stirring plate, in order to obtain the most reproducible stirring possible with the equipment at hand. The within run precision of the intensity values under these conditions was about 10% relative standard deviation (RSD) for five replicate measurements of 10^{-4} M peroxide. As will be discussed later, the RSD in terms of concentration is less than 10% because the probe's response to H_2O_2 is nonlinear.

While investigating the effect of stirring on the CL intensity, a dialysis membrane was placed over the enzyme gel at the gel/solution interface in an attempt to improve precision. It was expected that the signal intensity would decrease and the precision would improve due to the larger diffusion layer imposed by the membrane.

The presence of dialysis membrane decreased the signal by a factor of 0.62, while improving the precision of measured intensity from about 10% to 5% RSD. A sequence of five replicate measurements were made in each case. The presence of a dialysis membrane also increased the response time of 10^{-4} M peroxide signals by about 15 to 20 seconds.

The dialysis membrane appears to function in this case by effectively making a thicker diffusion layer, since small molecules are free to diffuse through the pores of the membrane. While the dialysis membrane adds to the thickness of the diffusion layer, the membrane does not affect the variability of the diffusion layer. Therefore, the relative uncertainty of the diffusion layer is decreased by the addition of the dialysis membrane. This results in improved precision. The increase in response time is due to the increase in the layer's thickness. More time is required to establish a steady state concentration gradient over a long distance than a short distance.

Temperature Studies

Enzyme activities are generally very temperature dependent, the rate of the reaction often doubling for each 10°C increase up to the point where the protein starts to thermally denature. Equation 14 predicts that when the CL-FOP is kinetically limited, the CL intensity will vary as the $\sqrt{V_{\max}}$. Thus, for an approximately 20°C change in temperature, if the probe is kinetically limited, the CL intensity should change by a factor of 2. Diffusion processes have much lower temperature coefficients. Thus a diffusion limited probe should be essentially independent of temperature since the $\sqrt{V_{\max} D_s / Km}$ term drops out

of equation 14.

Because the light shield and stirring plate had no provisions for operation above or below room temperature, a well controlled temperature study could not be conveniently designed. Therefore, two easily approximated temperatures, 0°C and 20°C (room temperature) were chosen. The low temperature was obtained by keeping stock solutions in an ice bath, and constructing an ice jacket for the light shield. The temperature of the solutions in the reaction beaker was kept in this manner at about 4°C . The warmer temperatures were about 23°C , also measured in the reaction beaker. Given the precision of the CL-FOP measurement and the experimental difficulty in obtaining well controlled intermediate temperatures with the equipment at hand, the two temperature data points were taken to locate rather large effects of temperature.

The recorded CL intensity for the cooled solutions was the same as the CL intensity recorded for the room temperature solutions within the precision of the measurement. Since a reaction limited probe would be expected to vary by a factor of about 2, this experiment reinforces the idea that the probe is mass transfer limited. A probe which is independent of temperature will have some desirable properties. Temperature independent probes will

be the most sensitive configuration, since the reaction rate can not be increased any further. The temperature independent probe also makes simpler demands upon the design, since no provision for temperature control need be made.

Effect of Enzyme Concentration

The CL intensity should be independent of enzyme concentration for a mass transfer limited system. This can be seen from equation 14a where $V_{\max} D_s / K_m \gg D_s / \delta$,

$$I_{CL} = \phi_{CL} \phi_{det} A (D_s / \delta) 6.02 \times 10^{23} S_{bulk} \quad (14a)$$

As long as the enzyme concentration is high enough so that the signal is mass transfer limited, the CL intensity will be independent of the enzyme concentration. However, when the enzyme concentration is reduced so that mass transfer control will no longer exist, the CL intensity should decrease.

Response time may also be expected to be independent of enzyme concentration, since in a mass transfer limited probe, response time would be expected to depend only on factors influencing the growth of the diffusion layer.

Experimentally, steady-state CL intensity and the response times are essentially constant for 0.5 mg enzyme/ml and 3mg enzyme/ml gel at pH 9 and 10^{-3} M luminol. However, for 0.1 mg enzyme/ml of gel, the steady-state intensity was decreased by 20% and the response time increased three-fold.

Calibration For Peroxide

Based upon experience with luminol and other catalysts, the statement that the luminol-peroxidase reaction followed Michaelis-Menten kinetics, and the results of the general model developed earlier, the CL intensity was expected to be linearly proportional to hydrogen peroxide. Therefore, experimental verification of system linearity was considered less interesting than other effects, and was delayed until other factors of the probe response had been investigated. However, response to peroxide was found to be highly nonlinear. The log-log plots (Figure 9) of the experimental calibration curves do not have a slope of one, contrary to the original expectations.

Figure 9 is a log H_2O_2 concentration vs. log response plot for several enzyme concentrations at pH 9 and 10^{-3} M luminol. An enzyme solution was included to check the possibility of a chemical interference from the

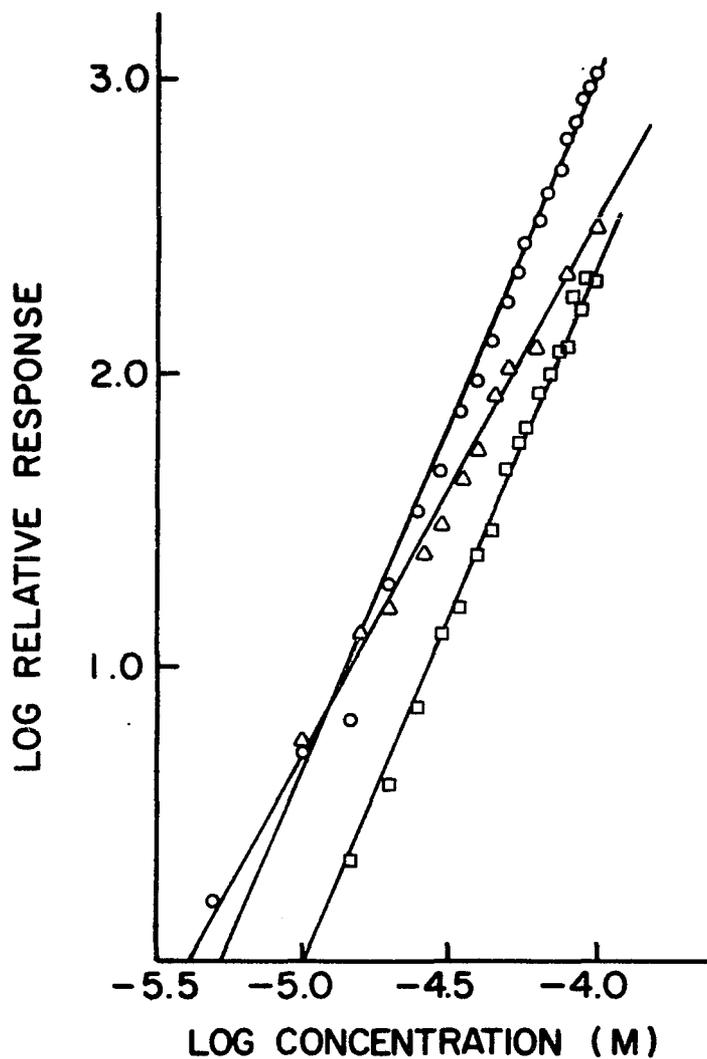


FIGURE 9

Plot of Log CL Intensity vs. Log Peroxide Concentration

Log CL intensity vs. Log peroxide concentration for three probes.

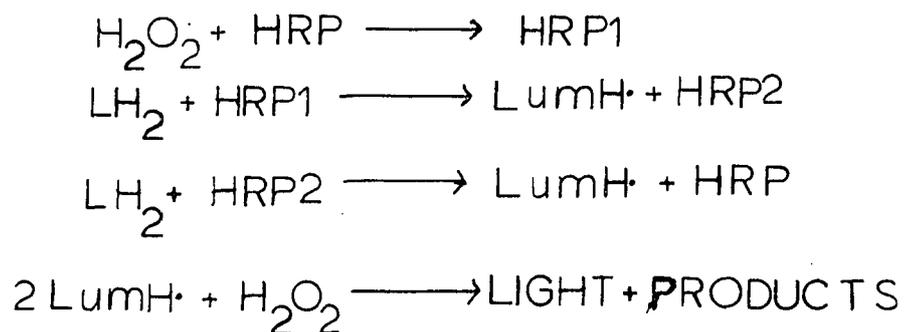
□-□-□ 1mg/ml horseradish peroxidase in polyacrylamide gel.

O-O-O 1mg/ml horseradish peroxidase in buffer entrapped behind dialysis membrane.

Δ-Δ-Δ 0.1mg/ml horseradish peroxidase in a polyacrylamide gel.

polyacrylamide gel. The apparent (least squares) slope for the 1 mg enzyme/ml gel curve is 2.30 while for the enzyme solution it is 2.35. The fact that these slopes are almost the same indicates that the gel matrix is not involved. Similar slopes were also observed for 0.5, 2, and 3mg enzyme/ml gel preparations. Therefore, the explanation for this nonlinearity must lie in the basic mechanism of the luminol-peroxidase reaction.

A mechanism for the CL oxidation of luminol by peroxide catalyzed by peroxidase has been proposed (44)



where HRP is horseradish peroxidase, 1 and 2 indicate intermediate forms; LumH \cdot is an intermediate free radical, and LumH $_2$ is molecular luminol. The light producing step is second order in the intermediate radical.

The luminol-peroxidase reaction can be described by Michaelis-Menten kinetics under conditions where the HRP concentration is much less than the peroxide concentration. This condition is not met in the CL-FOP, and the reaction

kinetics differ from the Michaelis-Menten model. From the mechanistic considerations, one half of the peroxide entering the gel reacts with the enzyme, and one half reacts with the intermediate radical. At steady-state one half of the peroxide present in any location of the gel is responsible for producing twice its own concentration of intermediate radicals as a first order reaction with respect to peroxide. The second portion of peroxide is then reacted in a reaction which is second order in intermediate radical. The mass transfer limitation of the probe complicates the interaction between the first and second reactions.

A second unpleasant surprise is that the log-log plots of Figure 9 are slightly curved when tested by a test of runs. The reason for the curvature is speculative.

The minimum detectable concentration (signal/dark current noise=1) is 10^{-6} M H_2O_2 when the P.M. tube is cooled with dry ice. Since there is no background emission in this configuration, the detection limit depends upon the magnitude of the signal relative to the detector noise.

The Lucigenin Probe

Due to the nonlinearity of the CL-FOP using peroxide as the analyte in the presence of luminol, a different approach was tried. Lucigenin (10^{-3} M) was substituted for the

luminol in the buffer solution.

The lucigenin-peroxide reaction is known to require a more basic pH than the luminol-peroxide reaction for optimum CL efficiency (45). The enzyme concentration was 3 mg enzyme/ml gel and a peroxide concentration of 10^{-4} M was used. Under these conditions, the signal intensity was of the same magnitude as the noise of the uncooled P.M. tube, about 4×10^{-9} A. Due to the inability to obtain an adequate signal, this approach was discontinued.

The Luminol Probe

Since luminol is also a substrate in the peroxide - peroxidase - luminol reaction, a possible configuration of the CL-FOP would be analysis for luminol. If such a probe were made, and if it had sufficiently low detection limits, a CL-FOP could become a chemiluminescent detector for an immunoassay technique.

In order to test the feasibility of this approach, one set of conditions was tried. The peroxidase concentration on the probe was 3 mg enzyme/ml gel. The pH was buffered to 9, as a reasonable compromise between good CL efficiency and good enzyme activity. Peroxide was 10^{-2} M in the buffer solution. Luminol was added in 25 ul increments of luminol stock solution.

Two problems with this line of research became evident. First, when the enzyme gel was in contact with the buffered peroxide solution, a background signal of about 5×10^{-8} A was observed. This background was not stable with time, and was quite noisy. The second problem was the reaction of luminol and peroxide in solution with the generation of CL. For a 10^{-4} M luminol sample, this resulted in a signal of about 10^{-8} A when the solution was observed with the fiber optic alone. Due to the unstable background, this approach was discontinued.

The Glucose Probe

A final series of experiments was performed after the assembly of the new equipment. The purpose of the experiments was the fabrication of a CL-FOP sensitive to glucose. A pH of about 8 was chosen in order to retain some glucose oxidase activity. Since the efficiency of luminol emission is low at this pH, the first effort was the testing of alternative catalysts. This was done in the hope of finding the most effective possible catalyst under these conditions. $K_3Fe(CN)_6$, Hemin, and peroxidase were compared for emission intensity. Maximum intensity was observed with peroxidase.

A gel was created which was 0.1g/ml of glucose oxidase and 0.01mg/ml of peroxidase. The buffer was a 0.05M phosphate solution at a pH of 8. This system had a very slow response time of about 15 minutes for the 90% response. A log-log plot of the intensity as a function of glucose concentration showed a slope of 2.0 over the range of $4-16 \times 10^{-4}$ M glucose. Due to the high concentration of glucose required to achieve a signal, this project was terminated.

CHAPTER FOUR

INTRODUCTION TO THE CHEMILUMINESCENT IMMOBILIZED REAGENT FLOW CELL

The Chemiluminescent-Immobilized Reagent Flow Cell is similar to the CL-FOP and utilizes the firefly reaction in a flow cell configuration. The flow cell was designed to overcome a limitation of the fiber optic portion of the CL-FOP. Above about 1 cm diameter, the fiber bundle becomes stiff and hard to manipulate. However, it may be desirable to have a surface area which requires about a 2.5 cm diameter optical path. Such a large interfacial area should exhibit greater sensitivity than a smaller area of similar composition.

A flow cell of the design used is expected to have significant advantages in addition to the larger area over the fiber optic probe. A flow cell based upon a channel plate for the sample phase and a Plexiglass immobilized reagent cell is more versatile than the probe, since the reagent cell may be milled for a range of interfacial areas. The flow cell also utilizes the simplest immobilization technique possible, entrapment behind a semipermeable

membrane.

The firefly reaction was used as the BL reaction for this cell. Since ATP is part of a large number of reactions used for biochemical assays (6,7), a sensitive detector for ATP could find wide application. The firefly reaction is a sensitive indicator of ATP concentration, but is limited in application due to its high reagent costs. A simple immobilization technique, if the technique is conservative enough of reagents, would bring the relative cost down to a more practical level. The BL intensity of the firefly reaction is a linear function of ATP concentration, which is desirable due to the simplicity of calibrating the device. Because the firefly reaction is kinetically slow, it is expected to yield a device which is kinetically controlled, as opposed to the mass transfer control of the peroxide CL-FOP. This kinetic control will damp the pulsations of a peristaltic pump.

Inspection of the mechanism for firefly BL reveals several potential difficulties. The formation of the excited state is dependent on the concentration of an enzyme-substrate complex (26). This complex is in turn dependent upon equilibria with the native luciferase enzyme concentration, the luciferin concentration, and the pyrophosphate concentration in addition to the concentration

of ATP. Since luciferin is quite expensive, it is hoped that the luciferase enzyme binds luciferin tightly enough that additional luciferin is not needed. If luciferin must be added to the system, the oxyluciferin-luciferase complex may present problems by blocking the turn-over of the enzyme. The situation is further complicated by diffusion of the low molecular weight species under the influence of concentration gradients. Clearly, the interrelationships between chemical species of the firefly reaction in a Two Phase system are complex.

EXPERIMENTAL

Instrumental

The system for immobilizing reagents and observing CL emission is shown in exploded form in Figure 10.

The CL reactions were performed in the Plexiglass holder which fitted to the face of an aluminum channel plate. A piece of dialysis membrane was placed between the two parts to separate the immobilized reagent and the analyte stream. The immobilized reagent was held in shallow grooves milled into the Plexiglass. The analyte stream flowed in a spiral channel found on the face of the aluminum plate. The surface area of the channel is estimated to be 4.5 cm^2 . The flow channel was filled and emptied from the reverse side of the plate by holes drilled into the plate at either end of the channel. An aluminum shield and compression ring permitted the holder and plate to be bolted together. Sufficient pressure was applied to form a liquid tight seal between the two pieces. The holder also provided a light shield for the device when the assembly was bolted to the face of the P.M. tube housing (Products for Research Model TE-104-TS). The P.M. tube was an EMI 6255B with an S-20 response. A SPEX DPC model 2 and Heath strip chart recorder completed the electronics of the device.

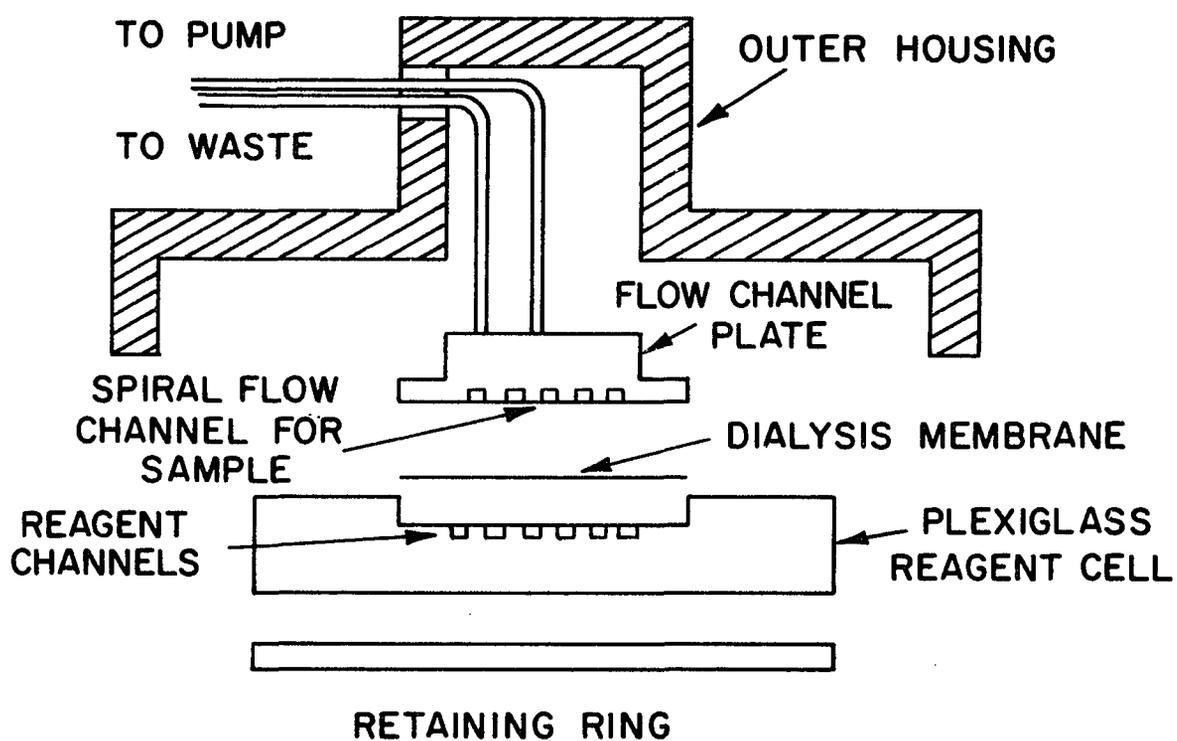


FIGURE 10

Exploded view of Chemiluminescent - Immobilized
Reagent Flow Cell.

The analyte solution was pumped past the immobilized phase by a Cole-Palmer peristaltic pump (model 7545-10) equipped with two model 7013 pump heads, which provided a total flow of about 13 ml/min at the highest setting. Altex Teflon tubing connected the pump to the flow plate, and the flow plate to the waste.

pH measurements were made with an ORION model 501 meter.

Reagents

The reagents and their suppliers are listed in Table 4.

The immobilized phase was formed by combining weighed quantities of enzyme preparations with a small volume (usually 0.5 ml) of the analyte/buffer solution without any ATP. The resulting mixture was mixed with a Pasteur pipet, and pipetted onto the Plexiglass holder. Since the holder had a capacity of about 0.2 ml, the quantity transferred was in excess of the need. The purpose of utilizing an excess was to avoid the entrapment of air bubbles in the immobilized phase during the assembly of the cell.

The desired flow system is shown in Figure 11. A nonbuffered solution of ATP is pumped from the sample container while an equal flow of buffer is pumped from a

TABLE 4

List of Reagent Suppliers for the Flow Cell

| Reagent | Source |
|--|--------------------------|
| Boric Acid | "Baker Analysed" Reagent |
| Horseradish Peroxidase | Sigma type I |
| 3-aminophthalhydrazide | Aldrich |
| 30% Hydrogen Peroxide | "Baker Analysed" Reagent |
| Firefly Tails Extract | Sigma |
| Adenosine 5'Triphosphate | Sigma |
| LUMIT HS | LUMAC |
| HEPES [4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid] | Aldrich |
| KOH | "Baker Analysed" Reagent |
| MgCl ₂ | "Baker Analysed" Reagent |
| Pyrophosphatase | Sigma |
| Luciferin | Sigma |
| Albumin | Sigma |
| Apyrase | Sigma |

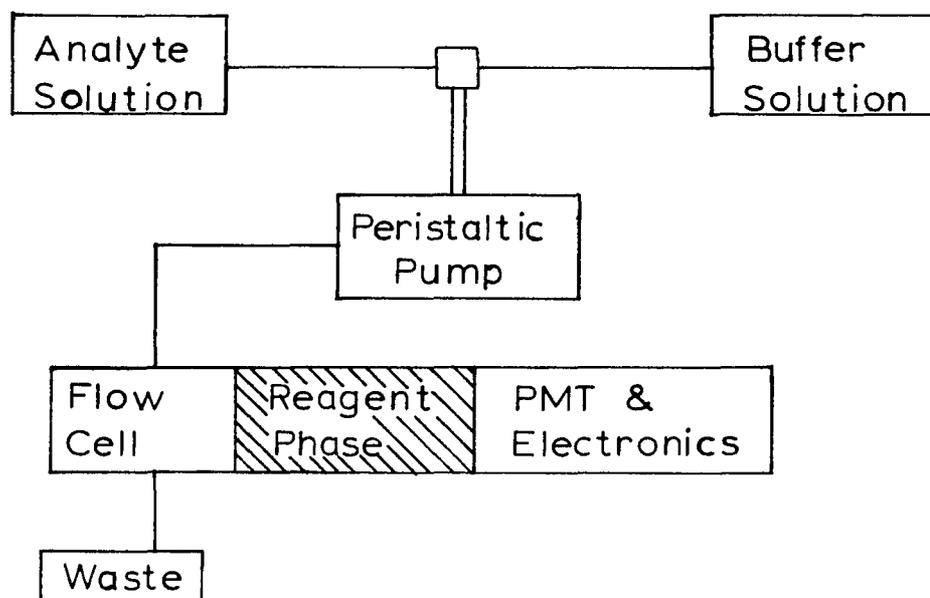


FIGURE 11

Flow System of the Chemiluminescent-
Immobilized Reagent Flow Cell

reagent reservoir. The two streams are mixed and passed through the flow cell proper. The spent solution is then sent to waste. All experiments reproduced the effect of the dilution of the buffer with the unbuffered sample in the analyte phase.

In several early experiments, the ATP/buffer solution was recycled in order to conserve limited reagents. In order to reproduce the concentrations to those of the desired flow system, the buffer components were made up at 1/2 strength. In order to maintain the character of the analyte phase after equilibration with the immobilized reagent, the desired quantity of ATP was added as a dry powder. This procedure was adopted to avoid changing the analyte phase by dilution. Only one ATP concentration per experimental run was possible with this approach.

Experiments without recycling were conducted in the manner indicated by Figure 11. ATP samples were made up from a fresh stock solution with deionized water. No buffer system or other reagent was added to these solutions. The buffer, prepared fresh for each experimental run, contained all reagents which were not immobilized. These reagents were added as dry powders.

The analyte phase conditions at the dialysis membrane were usually pH 7.5, which was maintained by 0.025 M HEPES buffer. 10^{-2} M Mg^{2+} (added as $MgCl_2$) was also present.

Procedure

Sample recycle experiments had the following structure. After the immobilized reagent was loaded into the Plexiglass carrier, the reaction assembly was bolted together, and screwed to the front of the P.M. tube housing. Once this was finished, the P.M. tube high voltage was turned on, and 50 ml of buffer were pumped through the system. Buffer was pumped from both the buffer and the sample lines of the pump, with the waste line inserted into the buffer container creating a closed system. After the dark current of the P.M. tube had decayed to a low value (usually about 1.5 hrs), a weighed quantity of ATP would be added to the buffer reservoir, using a small quantity of buffer to rinse the ATP over. The signal was recorded for the desired length of time.

The alternative technique was as follows. Buffer was diluted with deionized water (1:2), and was used in preparing the immobilized phase. After sealing the assembly up, and turning the P.M. tube high voltage on, a small

quantity of the diluted buffer was pumped past the immobilized reagent while the dark current decayed. When the dark current decayed to a low value, the reagent line was placed into the selected buffer solution, while the sample line was used to sample several ATP solutions. Between ATP samples, deionized water was used to wash out the sample line, and maintain the ionic strength of the buffer.

RESULTS

Luminol-Peroxide Response Time

In an early experiment, a small diameter enzyme reservoir (0.6 cm diameter) with the luminol-peroxide CL system was used. This experiment was conducted to estimate the response time of the flow reagent with a well characterized immobilized reagent and to establish that the flow cell worked. The immobilized phase was 0.5 mg of peroxidase enzyme per ml of buffer placed behind a dialysis membrane. The buffer solution was a 0.1 M borate buffer, adjusted to pH 9, with a luminol concentration of 10^{-3} M. EDTA was added to reduce background produced by metal ions. The sample was about 10^{-5} M peroxide in distilled water.

The recorder trace of Figure 12 shows that the response time of the flow system is similar to the CL-FOP. Throughput with the flow cell is slightly higher than with the CL-FOP because changing the sample is much simpler. The 90% rise time is on the order of 12 seconds. The chart speed was too low to estimate the 90% fall time. The S/N is about 300 for the 10^{-5} M H_2O_2 samples, which suggest a detection limit (S/N=1) of about 5×10^{-7} M (sample) for these non-optimized conditions. This result is better by a factor of 2 than the detection limit of the CL-FOP and

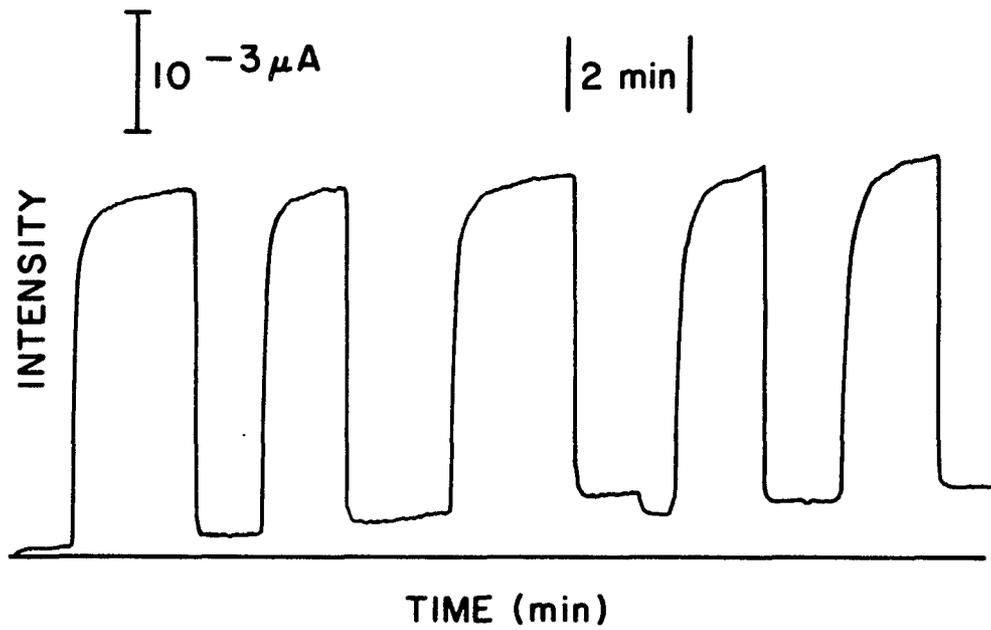


FIGURE 12

Typical Response for Peroxide

Response time of the Immobilized Reagent Flow Cell. Conditions are pH 9, 10^{-3} M luminol, 10^{-5} M H_2O_2 .

reflects the larger area of the reactant phase.

These measurements confirmed that the flow cell was performing as expected.

Firefly Reaction - Initial Experiments

A series of experiments was initiated to gain operating experience and to discover what conditions were needed for the emission of light. All of the experiments were conducted in a batch mode where the sample and buffer solutions were combined. This solution was then recycled. The pH of the 0.05 M HEPES buffer was 7.0 and the ATP concentration was 10^{-4} M. The buffer solution containing ATP had a volume of 30 ml. Conditions in the reagent phase varied, including the dry reagent of the firefly extract (FFE), 13.5 mg of FFE per ml of buffer, and 27.5 mg of FFE per ml of buffer. The concentrated FFE solution gave the highest intensity of the three samples, with the dilute solution and the dry powder having about 1/2 this intensity. The dry powder gave the longest lived emission, followed by the concentrated solution. The dry powder was also the more difficult of the two methods of reagent addition. These results indicated that reagents would be best handled as liquids, and that a high reagent concentration was desirable.

Since the signal decay could have been due to the aging of the FFE (due to denaturation of the enzyme or to the decomposition of the luciferin), a pair of experiments were performed to check the room temperature stability of the FFE. Two FFE samples of 39.9 mg/ml were prepared. One was used immediately, the other was left at room temperature for 6 hours. The initial intensities for both extracts were comparable to within 5%. This was taken as an indication that the FFE reagent was stable at room temperature over the time span of the expected experiments.

A final experiment, utilizing LUMIT H.S. reagent, was undertaken to get an idea of the time response of the firefly system, and to try this highly purified reagent. The buffer for this run was 0.025 M HEPES which was 10^{-2} M in $MgCl_2$. The pH was adjusted to 7.5, which is the suggested condition for the LUMIT H.S. reagent. A pump speed which resulted in a flow of 9.4 ml per minute was chosen to conserve the buffer. The 90% rise times ran about 4 minutes while the 90% fall times varied between 4 and 20 minutes. An insufficient number of samples were run to observe whether the variation was concentration dependent. The signal intensity for a 10^{-5} M ATP sample fell from an initial value of 0.35 uA to 0.005 uA in three hours. Two experiments utilizing the firefly extracts gave similar results, although with slower response times. The FFE

reagent, however, gave a much flatter background, and is considerably less expensive than the LUMIT H.S. reagent. For these reasons, further work with the LUMIT H.S. reagent was suspended.

2⁵⁻¹ Factorial Experiment

A 2⁵⁻¹ factorial experiment was conducted to examine the effects of five independent variables (Table 5) upon the initial BL intensity and upon the integrated intensity over the space of several hours. Figure 13 shows the shape of an intensity vs. time curve. The peak intensity, reached within a few minutes of adding ATP to the analyte phase, is considered the initial intensity. The integrated intensity is the sum of instantaneous values at short intervals along the decay curve. The P.M. tube dark current was subtracted from each value before the value was summed. The presence of "high" levels of luciferin, Mg²⁺, pyrophosphatase, and temperature were expected to result in a more intense initial signal with a 10⁻⁴ M ATP sample. High values of the first three should favor the formation of the enzyme intermediate complex, while a high temperature would encourage its decomposition. Albumin was added to the analyte stream to investigate the possibility that luciferin dialysis into the analyte stream could be controlled by changing the solution characteristics.

TABLE 5
Results of 2^{5-1} Factorial Experiment
Effects of Independent Variables

| Variable | Maximum Intensity | Integrated Intensity |
|------------------|-------------------|----------------------|
| Luciferin | 1.65 | 300 |
| Pyrophosphatase | -0.32 | -38.4 |
| Mg ²⁺ | 0.30 | 12.7 |
| Albumen | -0.21 | -64.2 |
| Temperature | 0.02 | -57.6 |

A positive slope indicates that a high value of the independent variable results in an increase in the value of the dependent variable.

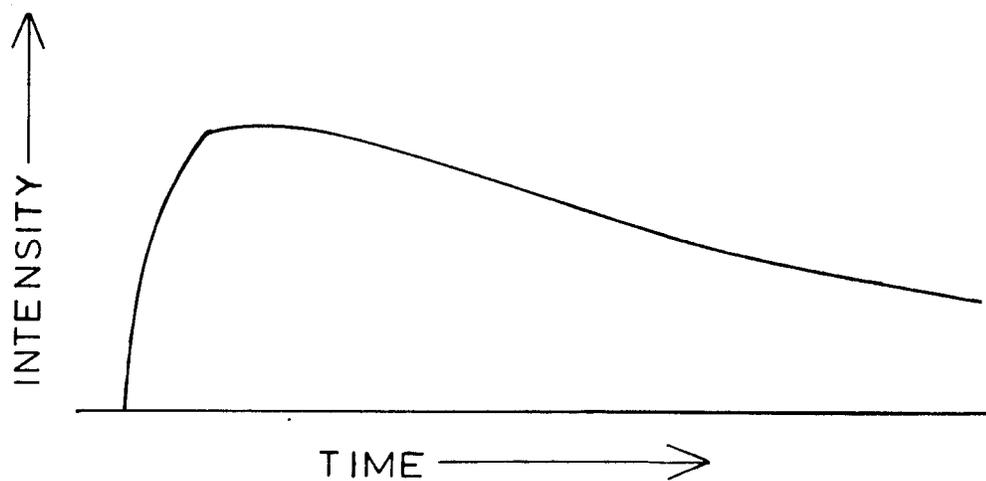


FIGURE 13

Trace of Recycled ATP Signal Decay

The results of this experiment are summarized in Table 5. High luciferin and high Mg^{2+} concentrations appear positively related to the initial intensity. Albumin in the sample stream and a high level of pyrophosphatase both appear to decrease the initial intensity. High luciferin concentrations increase the 6 hour integrated intensity by a large factor. Mg^{2+} concentration appears to have little effect upon the total amount of light available from the system. The presence of high values of temperature, pyrophosphatase, and albumin reduce the integrated light intensity. The interaction values are also fairly large in most cases.

Intensity decay was due primarily to the consumption of luciferin. An alternative was checked by an assay of the ATP. The FFE was used with a Chem-Glo photometer, using diluted samples of the ATP solution. The relationship between the peak intensities and the ATP standard indicate that ATP depletion in the sample stream was not significant.

Improved Response Times: Use Of Apyrase.

The rise and fall times using FFEAS the immobilized reagent are exceedingly slow, on the order of 10 minutes for the 10^{-4} M ATP solutions used in the previous examples. The reason for this slow response is that a buffer is added

to the FFE reagent to further slow down the already slow kinetics of the firefly reaction. Therefore, ATP molecules must diffuse even further into the immobilized phase before reacting. It was hypothesized that the addition of an ATP hydrolysing enzyme (apyrase) in the enzyme phase would shorten the excessively long response times of the device. This improvement would be at the expense of intensity, since the luciferase would be exposed to a lower level of ATP in the enzyme phase.

A series of experiments were conducted to test this hypothesis, and to gauge the size of the effect. The conditions were a pH 7.5 buffer, 0.05 M HEPES and 0.02 M MgCl_2 with 1 mg of luciferin added per liter of buffer. The ATP samples were 10^{-5} M and 5×10^{-6} M. The immobilized phase was 10 mg firefly extract/0.5 ml of buffer. The apyrase levels were varied from 0 to 1 mg/0.5 ml of enzyme phase.

The results, averaged for each run, are shown in Figure 14. As can be seen, intensity decreases by roughly the same proportion as the response times.

A problem with these trials is that the intensity for two similar ATP samples decreases with cumulative exposure of the immobilized reagent to ATP, a problem which was observed in earlier experiments. This exposure-dependent

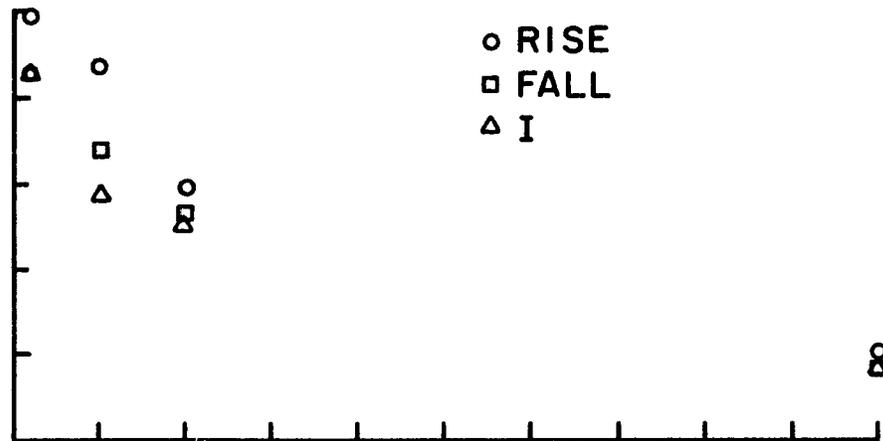


FIGURE 14

Effect of Apyrase on Response to ATP

Graph showing the relationships between Apyrase concentration in the immobilized reagent, the observed intensity, and the 90% rise time. Intensity and rise time are normalized with respect to their largest value. Conditions are 1mg/liter luciferin, 0.05M HEPES buffer, 0.02M MgCl₂, pH 7.5 buffer; and a 10⁻⁵M ATP aqueous sample.

intensity decay also made it impossible to obtain a calibration plot for ATP. It is therefore impossible to prove that the device is being operated under non-ATP limiting conditions, a hypothesis which is suggested by the relative intensities of adjacent peaks. As a result, future experiments were conducted at lower ATP concentrations.

Relationship Between Luciferin, Luciferase, Apyrase 2³

Factorial Experiment

The intensity of the ATP signal will depend upon the presence of a large amount of the intermediate complex, while the response time should depend upon the instantaneous rate of ATP consumption. Intensity and response time are both a function of the concentration of luciferin, luciferase and apyrase concentrations. A 2³ factorial experiment was run to study these effects.

The buffer used was 0.05 M HEPES, 0.02 M MgCl₂, and pH 7.5. ATP solutions of 10⁻⁶ and 2x10⁻⁶ M were prepared in deionized water. When the response time was sufficiently fast, ATP solutions of 0.5x10⁻⁶ and 1.5x10⁻⁶ M were added to the experiment, as a check on the consistency of the data at different ATP concentrations. The pump was set to its highest speed. Luciferin levels were 1.5 mg/l buffer (high), or 0.5 mg/l buffer (low).

Apyrase levels were 0.75 mg (high) or 0.25 mg (low) per 0.5 ml of enzyme solution. The firefly extract reagent, after being extended with the LUMIT H.S. due to low supplies, was 30 mg (high) or 10 mg (low) per 0.5 ml of enzyme solution. The enzyme phase was made up with buffer diluted to 1:2 the original concentration.

Table 6 provides a summary of the results. (A "+" indicates a desirable relationship.) Apyrase has the expected effect of improving the response time while decreasing the signal intensity. High apyrase also decreases the relative intensity decay, most likely due to the lower amount of ATP reacting with the luciferase than would otherwise occur. A high level of both the firefly "extract" and the luciferin resulted in a more consistent signal over several trials and a larger signal intensity. These last two variables are also associated with a longer set of response times.

Pyrophosphatase Results

A final pair of experiments was performed to discover if the build-up of pyrophosphate in the reagent phase is responsible for the continued decrease of intensity with time of this device. The intensity, rise time, and fall time of a sequence of samples were recorded for two

TABLE 6

Summary of 2^3 Factorial Experiment
Effects of the Independent Variables

| Variable | I | I/I | 90%rise | 90%fall |
|-----------------|---|-----|---------|---------|
| Luciferin | + | + | | |
| Firefly Extract | + | + | - | - |
| Apyrase | - | - | + | ++ |

+ desirable effect

- undesirable effect

different reagent phases. Both phases were based upon 15 mg firefly extract (extended with the LUMIT H.S.) and 1.5 mg apyrase enzyme mixture. To one sample, about 2 mg of the pyrophosphatase preparation was added. Luciferin was added to 1 mg per liter of buffer used. The ATP samples were made by dilution from 10^{-4} M stock with deionized water.

Figure 15 shows the results, in relative units, for 10^{-6} M ATP.

The addition of pyrophosphatase enzyme yields a system with a larger initial intensity, and similar response time compared to a similar system without the enzyme. However, the pyrophosphatase system suffers from a marked loss of intensity over the experimental run, and becomes somewhat slow responding by the end of the experiment. Due to its high emission intensity, the pyrophosphatase enzyme system gave a Signal/Noise of about 5 with a 10^{-7} M ATP sample. This indicates a detection limit of about 2×10^{-8} M ATP.

The results of this pair of experiments provide a strong indication that the oxyluciferin complex is the major cause of the loss of intensity upon exposure to ATP. If pyrophosphate were building up in the immobilized phase, the addition of the pyrophosphatase enzyme should result in increased intensity over the entire experimental run. Rather the converse occurs, with a large intensity at first,

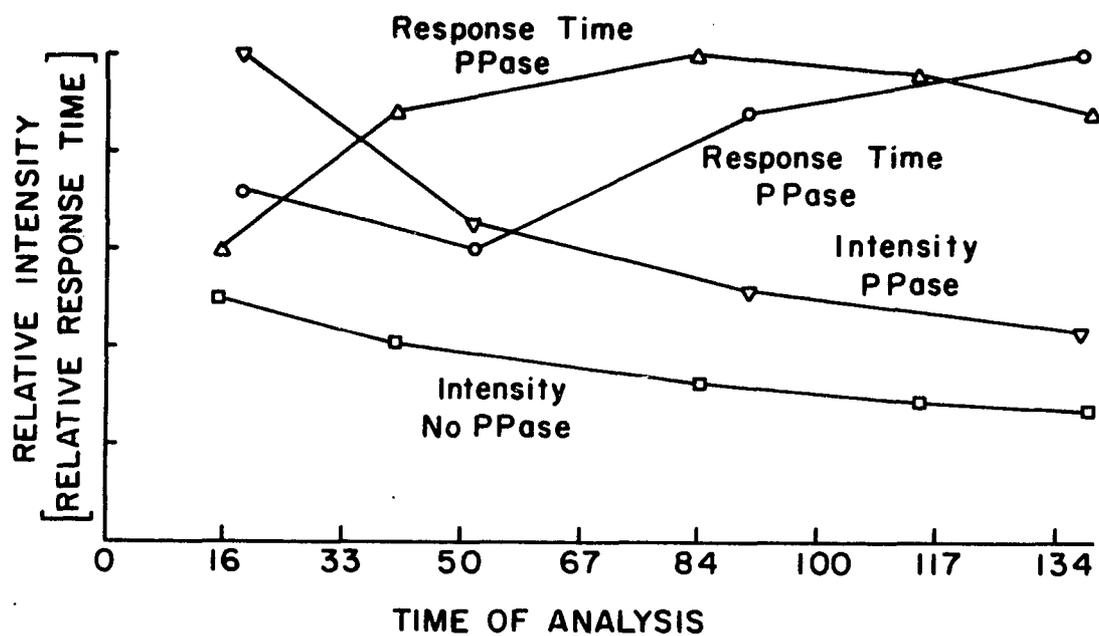


FIGURE 15

Comparison of Signals Showing Effect of Pyrophosphatase

Comparison of two sets of signals (2×10^{-6} M ATP), with and without the addition of pyrophosphatase.

but a very large loss of intensity upon further exposure. Apparently, the presence of pyrophosphate in low concentrations helps disrupt the oxyluciferin-enzyme complex, permitting the enzyme to bind a fresh molecule of luciferin.

DISCUSSION

The ATP sensor presents a complex situation for the experimenter seeking to utilize it as an analytical technique. This state of affairs is due to the number of equilibria and side reactions occurring in a non-homogeneous phase. There are at least five species undergoing diffusional processes, four of which are involved with equilibrium reactions with the luciferase enzyme. This results in several enzyme species. The integrated rate of the decomposition of the last enzyme-substrate complex is the value which is proportional to the intensity, but obviously this will be a function of the diffusing species, and any other enzymes co-immobilized with the luciferase.

The following conditions, in broad outline, are needed to achieve a useful device. Luciferin is needed in the solution, very likely at an order of magnitude greater concentration than used here. Apyrase may be used to improve the device's response time, provided that the samples are sufficiently concentrated. The Mg^{2+} concentration should be at least 0.01 M at the reaction site, and possibly higher. Additionally, some method is needed to remove the oxyluciferin from the enzyme phase, and thus stop its inhibitory effects.

Both luciferin and luciferase are needed in large quantities in order to favor the formation of the intermediate enzyme complex with ATP. High luciferin concentrations will also help counterbalance the effect of transient pyrophosphate concentrations upon this equilibrium. The time stability of the intensity over many samples will also be enhanced by high concentrations of both substances.

A Mg^{2+} concentration of about 0.01 M is needed to favor the formation of the enzyme intermediate. Since this concentration is considerably larger than the concentrations of the other reagents, and Mg^{2+} is not consumed in the reaction, the effect of varying the concentration is not obvious.

The apparent competitive inhibition of the reaction by oxyluciferin is currently a very serious obstacle to the practical application of this device. Some means of washing the oxyluciferin out of the enzyme phase is needed. One possible technique would be periodic dialysis against a buffer containing pyrophosphate and adenosine monophosphate (AMP). A second approach, which would result in lower intensities, would be to add pyrophosphate to the usual buffer.

A second major obstacle to the device's practicality is the cost of luciferin. A method must be devised to obtain high local concentrations in the enzyme phase which are less wasteful of luciferin than the current technique. One possibility could be to allow the luciferin from a reagent stream to diffuse through the enzyme phase, and out through the analyte mobile phase. It is not inconceivable that this type of approach could include reagents for the removal of the oxyluciferin product also.

CHAPTER FIVE

CHEMILUMINESCENT SENSOR FOR OXYGEN BASED ON PERAMINOETHYLENES

Several methods are available to the analyst for the determination of oxygen in both gaseous and aqueous samples. The Clark electrode, based upon the electrochemical reduction of oxygen at a noble metal electrode after diffusion through a barrier membrane, is one of the more common techniques. There are several problems associated with the Clark electrode, which prompts the search for substitute techniques. The Clark electrode is sensitive to the presence of other oxidizing gases in the sample, e.g. Cl_2 which will also be reduced at the cathode, thus giving a falsely high value. Another type of interference will be gases which react with the electrode proper, e.g. H_2S , which results in a poisoned electrode (46). It is desirable to overcome these defects in an O_2 sensor. Neither of the above problems of the Clark

electrode would be expected with a sensor based on peraminoethylene CL, since no oxidant is known other than O_2 which will generate luminescence. Additionally, the oxidation of peraminoethylenes is fast. A device based upon their CL should operate under mass transfer limited conditions.

The most studied peraminoethylene is tetrakis (dimethylamino)ethylene (TMAE). TMAE is commonly synthesized utilizing a bomb technique. Therefore, synthesis of this reagent is not readily accomplished by us. The other members of this class of ethylenes may be synthesized in good yield by a variety of methods. One, 1,1',3,3' tetraethyl $\Delta^{2,2'}$ -bi(imidazolidine), was chosen to initiate the research. During the course of this research it was discovered that TMAE is available through Armegeeddon Chemical Company, Durham, N.C.

EXPERIMENTAL

Instrumentation

An early version of the sensor was constructed along the lines of the CL-FOP. A reagent reservoir (Figure 16) was constructed and fitted to a Nylon block. The Fiber Optic (FO) was fitted behind the reagent reservoir, and held in place with PVC tape and clay. The Nylon blocks were dyed black and then wrapped in PVC electrical tape to ensure a dark environment for the CL reaction. At first, the FO was the 1/8 inch diameter optic used in the first project. A later version utilized a 1/4 inch diameter FO and a scaled up reagent reservoir. Experience with this device, including the freezing of the neat EIA in the capillaries when attempting to load the reservoir, and the inability to achieve a usable signal intensity more than once, resulted in a complete redesign of the device.

Except as noted, the instrument and gas delivery system in Figure 17 were used. Air (or O₂) is taken from the compressed gas cylinder and passed through a calibrated Matheson Model 602 rotameter, and then passed through a needle valve. This valve arrangement was chosen to minimize the effects of pressure fluctuations downstream from the meter. N₂ from the other cylinder is mixed with the air

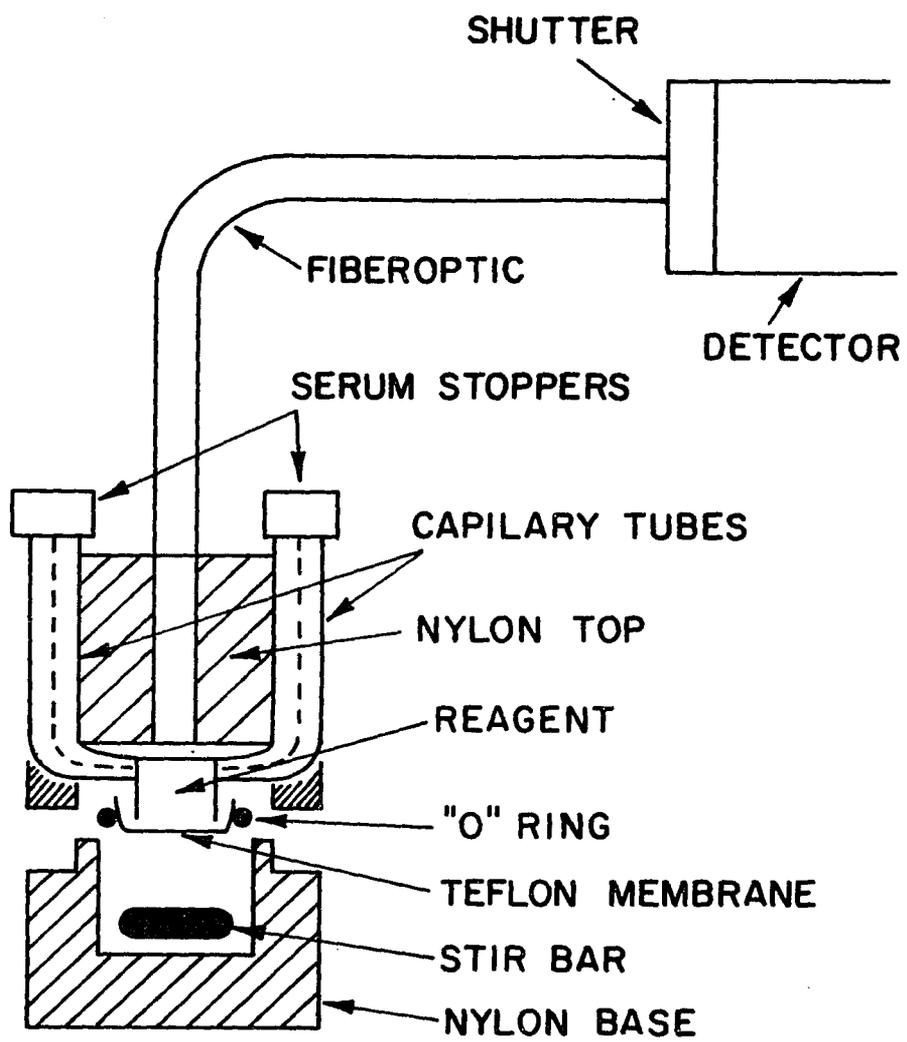


FIGURE 16
Initial EIA Apparatus

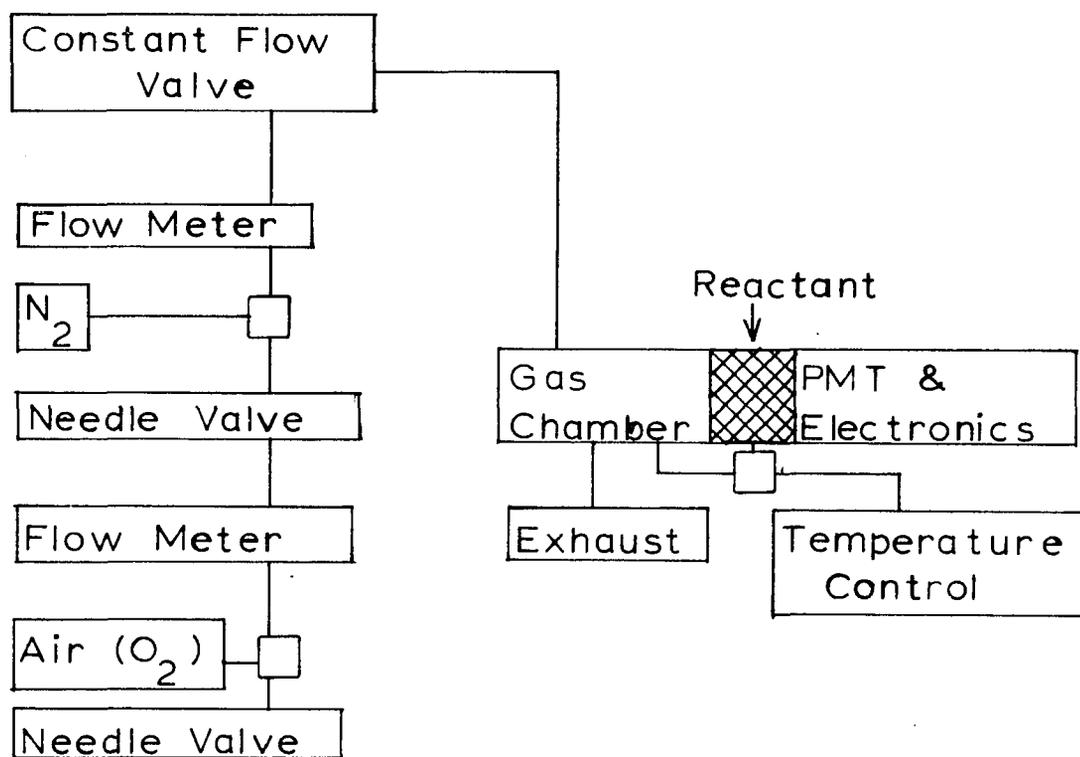


FIGURE 17

Flow Diagram of Sensor and Gas Flow System

or O₂ stream, and the combined mixture is passed through a second rotameter to a constant volume valve (Brooks Instrument Co.). The second flow meter served no quantitative purpose, but provided a crude check on the stability of the combined flow stream. The gas stream passed into the sample chamber of the reaction housing (Figure 18) where the O₂ in the gas stream could diffuse across the Teflon membrane. The observed CL signal was created by reaction with EIA in solution. The CL signal was observed by a P.M. tube located behind the glass cell in a refrigerated housing (Products For Research Model TE-104-TS). Gases were vented to the atmosphere. The total flow rate of gases was slightly less than 1.5 liters/min. At the gas flow rates used in this study, the use of plastic tubing in the gas delivery system is not expected to introduce significant errors due to O₂ diffusion through the plastic.

The intensity of the CL signal was observed by an EMI 6255B photomultiplier tube with the photocurrent displayed on a SPEX Digital Photometer (Model DPC2). Reaction temperature was controlled by pumping thermostatted water through copper tubing wrapped around the exterior of the aluminum housing. Black cloth covered the reaction chamber/P.M. tube assembly. EIA solutions in the reagent cell were stirred by a Corning PC351 stir/heating plate

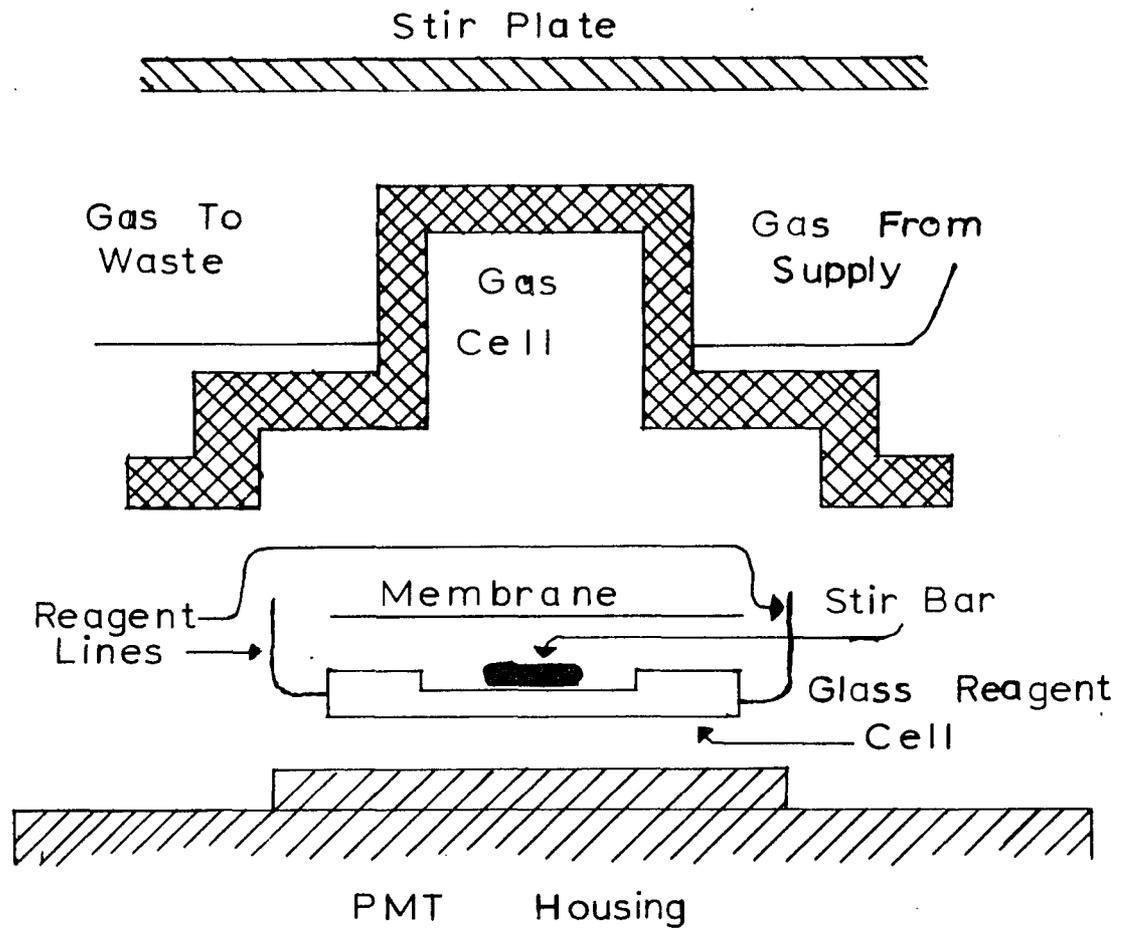


FIGURE 18
Exploded View of Gas and Reagent Cells

coupled to a magnetic stir bar within the reagent cell. The purpose of stirring was to reduce the O_2 partial pressure on the reagent side of the membrane. However, CL intensity remained constant when stirring was turned off, and thus it appears that stirring of the reagent is unnecessary.

The membrane was 1 mil thick FEP Teflon with an area about 10 cm^2 .

Reagents

All gases were supplied by Linde, and were used as received.

The EIA reagent was prepared by a modification of a literature technique (47). N,N' ethylenediamine (Aldrich) was refluxed under N_2 with an equal volume of N,N' dimethylformamide dimethylacetal (Aldrich) in a 100 ml flask. Methanol was distilled off at 65°C . The reaction was heated further until the distillate reached a temperature in excess of 150°C . At this time, heating was stopped, and the reaction allowed to cool to room temperature under N_2 . After the reaction was cool, the N_2 tank was disconnected and a vacuum pump attached. EIA was vacuum distilled under a pressure of between 0.5 and 1.0 torr at a temperature of 80°C . The light yellow product was collected in a Schlenk flask, and stored as a hexane

solution (Baker Analysed HPLC Reagent) to prevent freezing. When exposed to air, this solution exhibited a bright yellow emission. Dilution with hexane shifted the emission to a blue-green color.

All dilutions were carried out in a glove bag swept with N_2 .

Procedure

The procedure for measuring O_2 with the sensor was as follows. The gas and reagent sides of the membrane were flushed with N_2 from the gas delivery system. The reagent cell was disconnected from the N_2 and filled by syringe with the desired EIA solution. To avoid trapping gas bubbles in the cell, the entire assembly was tilted during the loading process. Not more than 6 ml of EIA solution were used in a given injection. Initial CL emission was allowed to decay to a low, steady value against N_2 . This emission probably arises from O_2 diffusing through the Teflon portions of the system, and traces of O_2 in the nitrogen gas. If temperature control was used, it was set during the 0.5 hr that the initial decay typically required. The specific sequence of gas flow rates which comprised the experiment was then carried out. For the case of the intensity decay experiments, O_2 flow was set, and the

signal recorded for 12 hr on a strip chart recorder. In calibration experiments, air flow would be set to zero flow, and the P.M. tube current recorded. The needle valve for air would be quickly opened (3-5 seconds) until air flow was constrained by the constant volume valve. The P.M. tube current was again recorded when the signal value had stabilized. Air flow was then set to the desired value. When the P.M. tube current had stabilized, the current was again recorded. This process was repeated, starting with the zero air flow rate. This cycle would be continued until the experiment was completed.

Calibrations against an aqueous phase sample were performed slightly differently from gas phase calibrations. For these experiments, the reaction/P.M. tube assembly was inverted so that the water sample rested upon the Teflon membrane. An air calibration was performed upon the EIA sample before water was injected into the gas sample tubing with a syringe. Due to the high gas flow rate, the water had to be replenished at least as often as every other calibration point in the experiment. The rate of response was much slower for a liquid phase due to the time required to exchange the old and new gases in the liquid.

After an experiment was completed, spent reagent solution was flushed from the glass cell with 25-30 ml of hexane.

RESULTS

Initial Experiments

An experiment was designed to determine how four variables influenced the observed CL intensity and the rate at which the CL intensity changed with time. The four variables were chosen due to their expected importance. The independent variables which were selected for investigation were the O₂ pressure (actually the flow rate of the gas), the concentration of the EIA in the reagent reservoir, the concentration of the imidazolidone product from the reaction, and the concentration of an alcohol (t-butanol). A factorial design was chosen, with high and low setting for each variable. The EIA concentrations were chosen to bracket a 5% (wt/wt) solution in hexane, the high value being about 10% and the low value being about 1%. O₂ flows were chosen to approximate an atmosphere of pure O₂ and one of 20% O₂ (atmospheric pressure) N₂ being used as the diluent gas. The high setting for the alcohol and the imidazolidone were 10⁻³ M in hexane. The low settings in both cases were the absence of the compound. A center point in triplicate was included in the design but not used. All experiments were randomized using a random number generator.

In each experiment the reagent chamber was filled with the solution under investigation. The desired O₂ flow was set, and intensity was recorded over 12 hours. The strip chart traces were then replotted on the University's DEC SYSTEM 10 computer using a library plotter program. Data reduction consisted of overlaying the high and low traces for each of the variables. A variable was considered significant when all eight cases showed the same results (ie. the high setting of the variable was always more intense than the low setting). The results are shown in Figure 19.

By these criteria, the experiment showed significant dependence for both dependent variables on the levels of EIA concentration and the O₂ flow. A high O₂ flow results in a larger signal, but also results in a greater rate of decay of CL intensity over 12 hours. A high EIA concentration also increases the CL intensity, while tending to retard the decay in intensity. Under the conditions used, and by the criteria selected for significance, neither the alcohol nor the imidazolidone concentration were accepted as significant.

The shape of the decay curve reflects the mechanism by which the decay occurs. Therefore, the shape of the curve was investigated. It is expected that the decay in

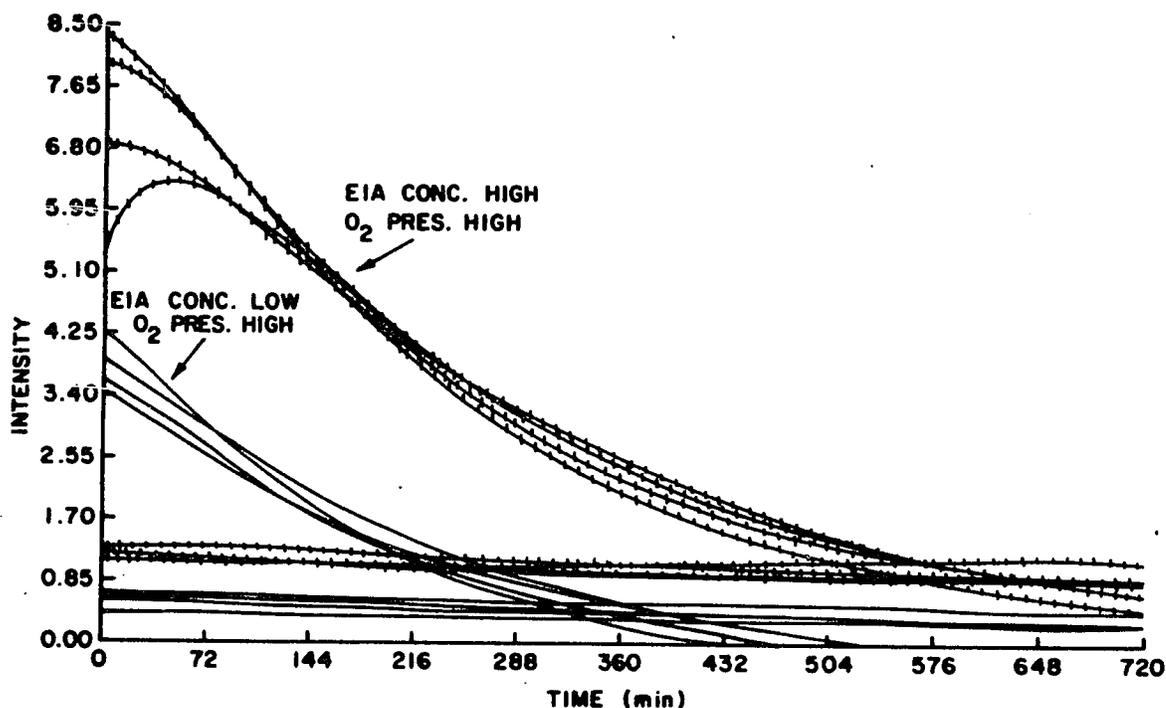


FIGURE 19

12 Hour Signal Decay Curves for Oxygen Sensor

This figure is an overlay of sixteen 12 hour tracings for the decay of the CL sensor. The top cross hatched set of curves are all from samples with a high EIA concentration (about 10%) performed at a high O₂ partial pressure. The top set of plain curves represent the decays for low EIA solutions at the same high O₂ partial pressure. The bottom two sets of traces represent similar experiments at a low O₂ partial pressure, where the cross hatching still represents the high EIA concentration.

intensity is due to the consumption of EIA or quenching by the buildup of the imidazolidone product, or a combination of the two processes (30,35-38).

Quenching by the imidazolidone product is expected to follow a Stern-Volmer type process. After considering the reaction, it is obvious that the concentration of the product is proportional to the length of time that the device has been exposed to oxygen at a constant pressure. A Stern-Volmer quenching mechanism results in an intensity inversely proportional to a term containing the quencher concentration. From these considerations, a plot of the reciprocal of the intensity vs. time will be linear if quenching is the dominant cause of the decay. When the decay curves are replotted as the reciprocal of intensity (Figure 20), they are curved. Thus, while quenching by increased product concentration may occur, it is certainly not the major reason for the intensity decay. This is consistent with the observation that 10^{-3} M imidazolidone did not have an observed effect on the intensity vs. time curves.

Another possibility is that the intensity decay is due to EIA consumption. The concentration of EIA is inversely proportional to O_2 exposure (and the product concentration), while the CL intensity is proportional to

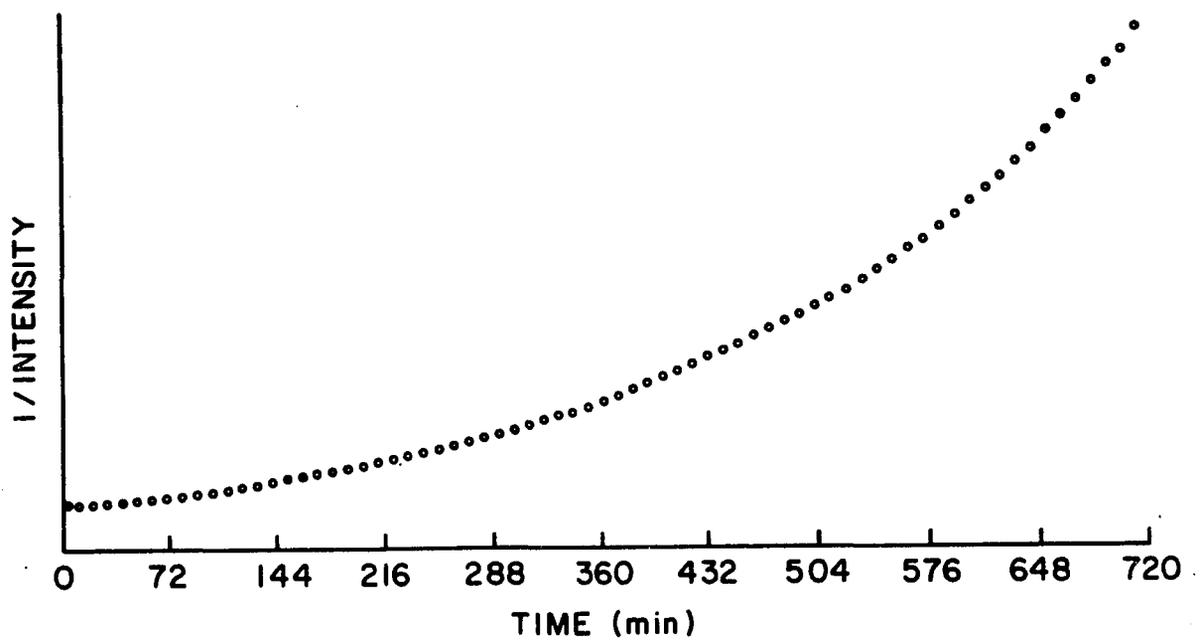


FIGURE 20
Stern-Volmer Plot of Sensor Signal Decay

the square of the EIA concentration. Therefore, the shape of the decay curve should be that of a second order polynomial. When the data is fit by a least squares routine, the resulting fit to the model is much better than in the case of the Stern-Volmer quenching, although the fit is not perfect (Figure 21). This is indicative that the rate of intensity decay is primarily due to EIA consumption.

CL intensity decayed much more rapidly than expected, based upon calculations using known rates of oxygen permeation through Teflon membranes. Apparently, the hexane solution of EIA interacts with the FEP Teflon to enhance oxygen permeability. As will be considered in the discussion, it would be preferable to work at a much lower O_2 permeation rate in most practical situations.

Response To Oxygen / Temperature In The Gas Phase

CL intensity as a function of O_2 partial pressure and temperature was investigated next. For this experiment, the flow meter in the air line was calibrated by water displacement. The valve in the flow meter was replaced by a needle valve placed into the tubing after the flow meter, minimizing the effects of variable back pressure upon the calibration of the flow meter. Also of interest were the

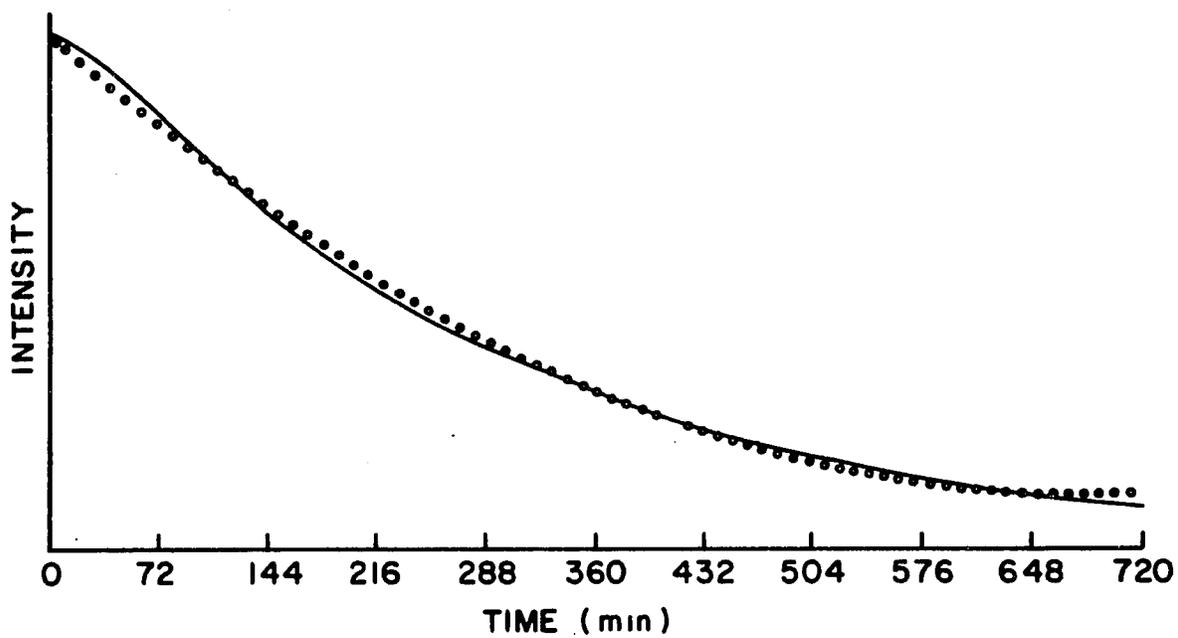


FIGURE 21

Quadratic Regression on 12 Hour Intensity
Curve

- + Least Squares Estimated Intensities
- Experimental Values

effects of temperature and O₂ flow rate upon the rise and fall times of the signal.

The effect of temperature on the calibration curve can be seen in Figure 22. The variation in slopes for the replicate calibrations at the same temperature reflect variations in reagent quality. The effect of temperature on the sensor is most likely due to the temperature dependence of the diffusion of a gas through a membrane. This will follow the equation (46):

$$D = D_0 \text{ EXP } (-E_d/RT)$$

where D is the diffusion coefficient at the experimental temperature, D₀ is the diffusion coefficient at a reference temperature, E_d is the activation energy for the diffusion process in the membrane, R is the gas constant, and T is the absolute temperature. This type of process is capable of initiating temperature effects of 1-6% per degree. The observable temperature effect will also include any changes in CL efficiency with temperature.

All calibrations resulted in linear calibration curves over the limited range studied, although the slopes between cases were highly variable. Correlation values were consistently better than r=0.995 for 14 points. The value

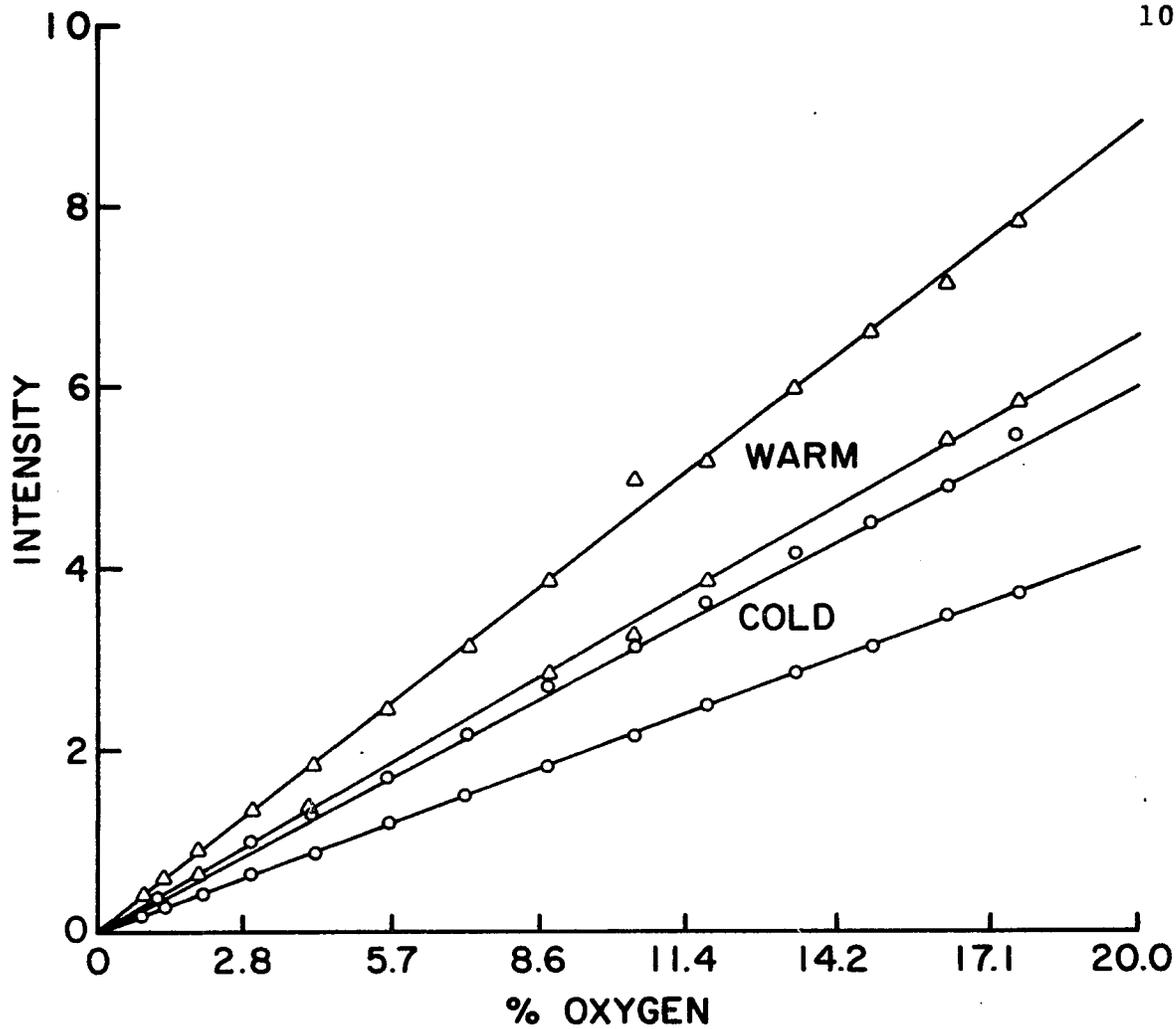


FIGURE 22

Effect of Temperature Upon the
Calibration Curve for Gas Phase Oxygen

+ 40°C Calibrations
x 15°C Calibrations

of the signal obtained with a pure air flow did not change appreciably during the course of the experiment, about 2 hours.

A detection limit of 1 ppm(V/V) O_2 in the gas phase was estimated making two important assumptions. First, it was assumed that response to oxygen continues to be linearly proportional to the O_2 partial pressure at low values. Second, it was assumed that the ability to measure small oxygen levels was limited by the P.M. tube darkcurrent noise. In practice, a significant level of CL is observed with a N_2 gas in the sample chamber. This signal is attributable to residual oxygen as a contaminant in the nitrogen plus oxygen that diffuse through the plastic tubing of the gas handling system.

The effect of temperature upon the rise and fall times (time to reach 90% of the final value) was small. At $15^\circ C$ both the rise and fall times ran about 20-24 seconds. At the higher temperature, these responses were decreased to only about 10 seconds. The improvement in the response time reflect the kinetics of both the diffusion process and the kinetics of the reaction. Typical rise and fall times as well as typical precision are illustrated by the recorder tracing for six consecutive measurements of 20% O_2 (Figure 23).

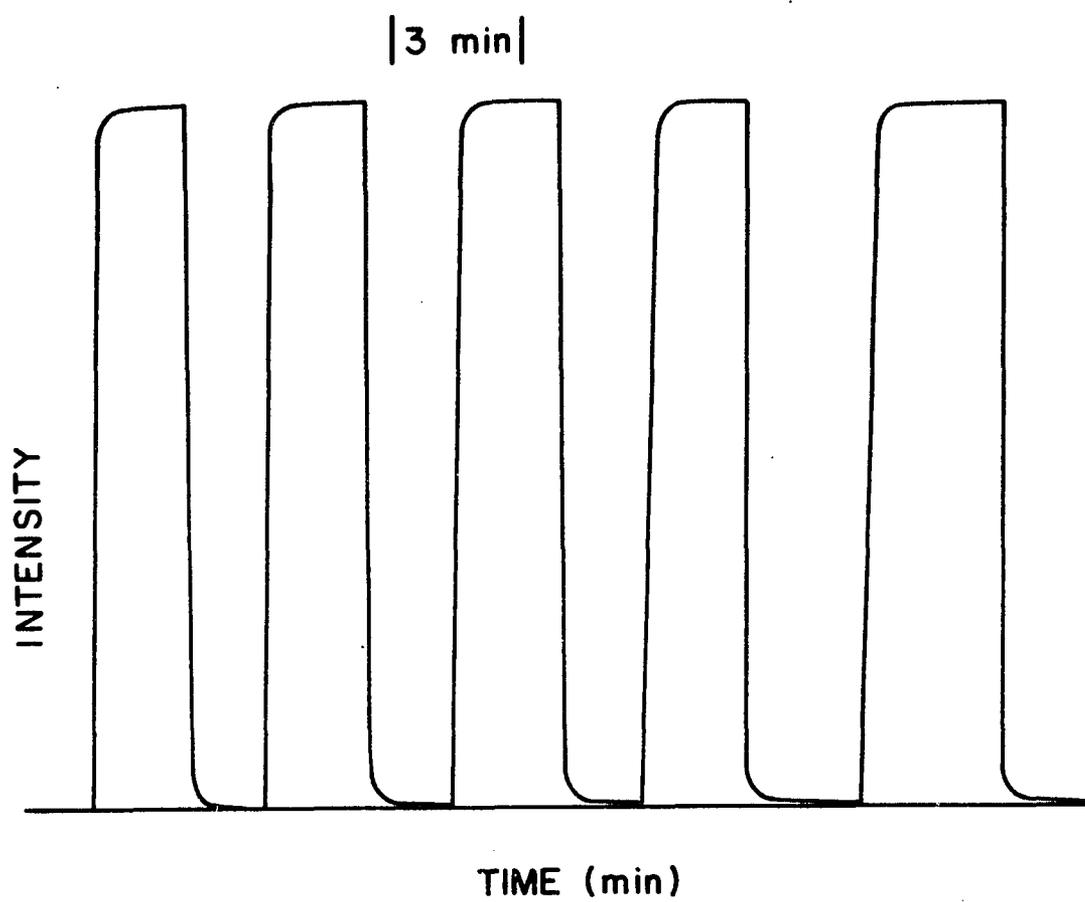


FIGURE 23

Recorder Trace for 90% Response Times
for a Gas Phase Sample

Liquid Phase Calibration

The calibration experiments were repeated with water in the gas chamber as opposed to gas. Figure 24 shows a calibration curve in water compared to a calibration curve for O_2 in air using the same reagent. The calibrations in water show a decreased linearity, which in part is due to the extended time required for the experiment, about 5 hours. The signal from an air stream decreased significantly during the course of the experiment, which alters the slope of the calibration curve.

The slope of the analytical curve in water is much less than in air. This is a mass transfer effect. Due to the high O_2 permeation rate through the membrane, the concentration of O_2 at the membrane surface is depleted. This effect is greater in aqueous samples than in gas samples because mass transfer in liquids is much slower than in gases. Saturating gas phase samples with water vapor had no effect on CL response.

Using the conditions of the experiment illustrated in Figure 24, the detection limit for O_2 in water in terms of partial pressure will be about four times greater than the corresponding detection limit in air. It should, however, be recognized that relative detection limits in air and water will be a function of membrane permeability and

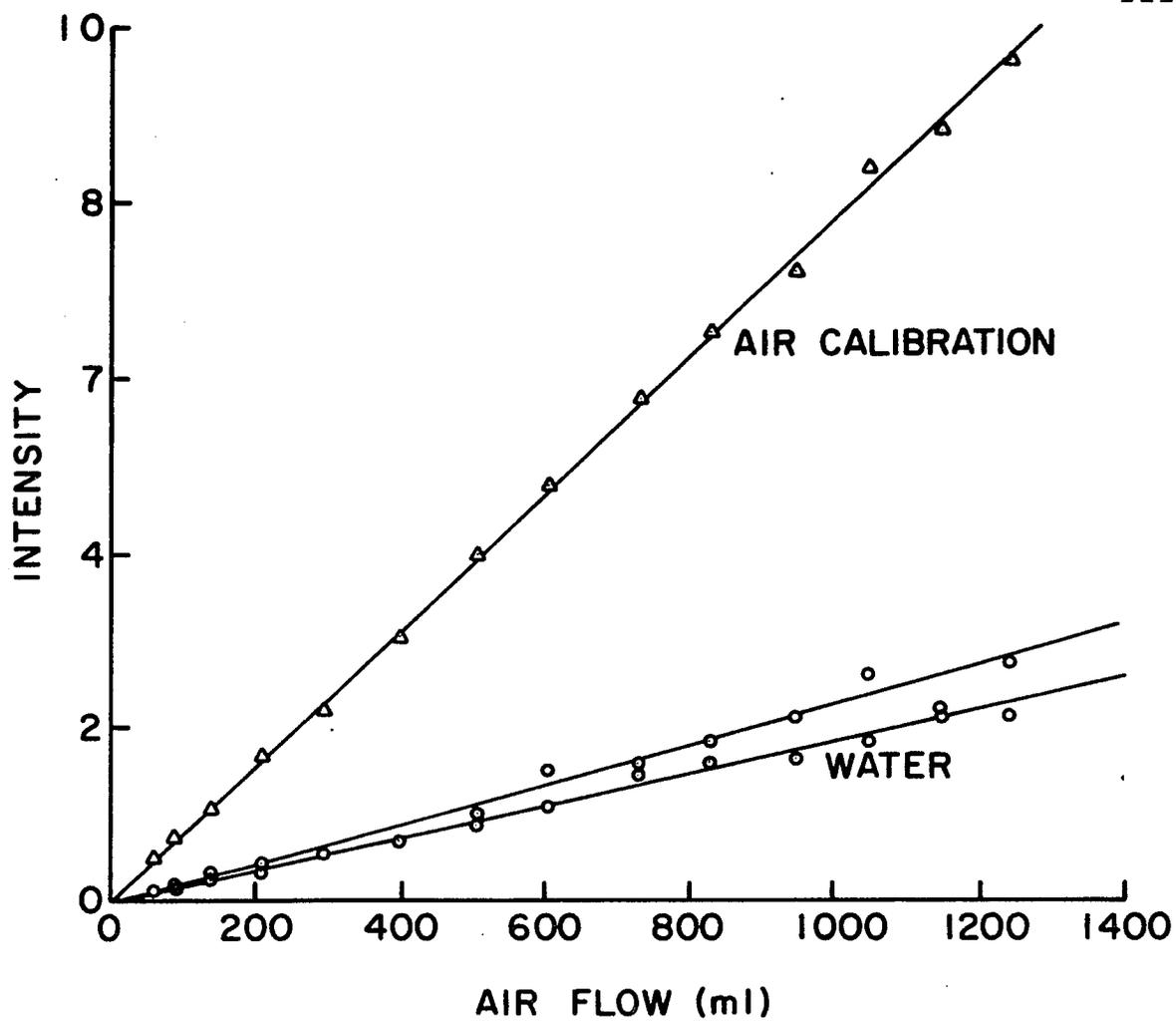


FIGURE 24

Comparison of Gaseous and Aqueous Sample Phases
at 20°C

stirring rates. The estimated detection limit is cited to indicate the magnitude of the CL signal relative to variations in detector noise under a particular set of conditions. In practice, the detection limits will depend upon a number of factors, including membrane permeability, area, and the freedom from background oxygen contamination.

DISCUSSION

Oxygen is most frequently measured using an oxygen electrode. This electrode is similar to the CL device. In both devices, the oxygen diffuses through a membrane and then reacts to yield a measurable signal. Both devices measure oxygen partial pressure rather than concentration. In both devices, response varies considerably with temperature, requiring a correction factor for accurate measurements. Both devices have similar response times. Like the electrode this device appears to be mass transfer limited.

There are also some significant differences between an oxygen electrode and the CL sensor. The CL oxygen sensor as described here is more sensitive than the typical oxygen probe, but is more sensitive to changing response due to reagent consumption. If the CL oxygen probe is operated with a less permeable membrane and/or a smaller membrane area for the same reagent volume, its response will be less sensitive, but longer lived. For example, if the rate of oxygen consumption is decreased by a factor of 100, the CL oxygen probe should operate for two to three months without requiring new reagent. It would still have a detection limit comparable to many oxygen electrodes. Further

improvements may be realized by substituting pure TMAE for the 10% EIA solution used in the version described here. TMAE is a non-viscous liquid at room temperature while EIA is a solid.

The oxygen electrode is subject to interference from oxidizing gases, such as chlorine, which can diffuse through the membrane and be reduced at the cathode. It is also subject to interference from reducing gases such as H_2S which poison electrode response causing inaccurate measurements. These gases are not expected to interfere with the CL oxygen probe since they do not react with tetraamino ethylenes to yield CL.

The CL oxygen sensor requires that ambient light be excluded. This is its most serious limitation relative to the oxygen electrode. This problem could be eliminated if an opaque membrane material were found. The CL oxygen sensor also cannot be turned off like the electrode. It should be stored in an oxygen-free environment when not in use to prolong its lifetime.

CHAPTER SIX

SUMMARY

We have investigated the possibility of using two phase CL for analysis. Three different reactions have been adapted for these measurements. While the results are promising, further research is required before these systems can be applied to practical problems.

The most promising system is the oxygen sensor based on the oxidation of EIA. It compares favorably in sensitivity and response time to the oxygen electrode and is expected to be interference free. Further improvements in sensitivity and lifetime are expected if TMAE is used instead of EIA. Further work is required to find membrane materials that will be less permeable to oxygen so that the CL reagent is consumed slower. Also, the calculated detection limit needs to be confirmed experimentally as does the presumption that the CL oxygen sensor is not subject to interference.

ATP measurement based on immobilized firefly luciferase requires substantial improvement to be practical. The two main problems are the slow response times due to the slow

kinetics of the firefly reaction and the change in response due to luciferin consumption and oxyluciferin buildup. Conceivably, these problems can be dealt with by adding an appropriate amount of ATP-degrading enzyme and by devising a means of replenishing the luciferin supply. If these problems can be solved, this will be an attractive system for ATP analysis because it permits reuse of the firefly luciferase yet does not require a difficult immobilization procedure.

The peroxide probe based on luminol has been demonstrated to have an acceptable detection limit with a good response time. However, probes based on coupled enzyme catalysed reactions will be limited to enzyme reactions that have sufficient activity in the pH 8 to 9 range.

Currently, the flow cell design is more useful for two phase CL measurements than the fiber optic probe. The availability of a large surface area and good optical efficiency are the two most important factors in choosing the flow cell over the probe. Sample handling is easier with the flow cell also.

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