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FLOW SYSTEMS FOR CLINICAL ANALYSIS USING IMMOBILIZED ENZYMES AND CHEMILUMINESCENCE DETECTION (CHROMIUM, GLUCOSE)

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University of New Hampshire PH.D. 1983

FLOW SYSTEMS FOR CLINICAL ANALYSIS USING IMMOBILIZED ENZYMES AND CHEMILUMINESCENCE DETECTION

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B.S., LeMoyne College, 1977

DISSERTATION

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in
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This dissertation is dedicated posthumously to my parents, whose love, support, encouragement and understanding were invaluable in helping me pursue my education.

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FLOW SYSTEMS FOR CLINICAL ANALYSIS
USING IMMOBILIZED ENZYMES AND
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University of New Hampshire, December, 1983

Analytical systems used in a clinical setting should offer high sample throughput and ease of operation, while at the same time providing selective, sensitive, accurate and precise determinations of clinically important analytes. Described in this study are two flow systems based on chemiluminescence (CL) detection that provide the features important in a practical clinical method: a flow injection analysis (FIA) system and a two phase flow system. Both systems take advantage of the specificity of enzyme reactions.

FIA systems for the determination of Cr(III) and glucose, based on luminol CL were developed. The Cr(III) system used EDTA as a masking agent to eliminate interferences from other metal ions, making it selective for Cr(III). The FIA system for glucose determination in biological fluids was based on the use of glucose oxidase, which was covalently immobilized to controlled porosity glass beads. The immobilized enzymes were packed into a column incorporated into the FIA system. Optimum operating conditions, detection limits and linear ranges were determined. The system was capable of sampling rates as high as 240 samples/hour. There was no significant difference at the

95% confidence level between CL-FIA results for blood glucose determination and results obtained by a commercially available reference method. The immobilized glucose oxidase was quite stable and could be reused for many assays.

The two phase flow system work employed a custom-designed flow cell that featured enzymes immobilized by entrapment by a membrane. Analyte in the flow stream could diffuse under a concentration gradient across the membrane into the reagent cell. Theory describing the performance of the cell was was developed. The system was characterized using the peroxid se-catalyzed luminol reaction, firefly bioluminescence (BL) and bacterial BL. The kinetics of the luminol and firefly reactions were varied in order to observe the effect of reaction rate on response time. Rise and fall times to steady state emission were measured, as were the relative intensities for the various reaction conditions. Rise times as fast as 40 seconds were observed for the firefly reaction when 10 mg/mL of apyrase was added to the system. The bacterial BL system exhibited much slower response than the modified firefly systems, but the bacterial system was more stable over time. Response time in the bacterial system was a function of NADH concentration. The performance of these systems for ATP and NADH determination was investigated, and approximate detection limits and linear ranges were determined.

CHAPTER 1

CHEMILUMINESCENCE

Introduction

The phenomena of chemiluminescence (CL) and bioluminescence (BL) have fascinated mankind since the first firefly was seen. Recently, there has been an increase in the attention given to these interesting reactions because of their potential application to clinical analysis. This interest is confirmed by the large number of recent reviews dealing with analytical applications of CL and BL (1, 2, 3, 4, 5, 6, 7).

CL is light emission accompanying a chemical reaction. BL is a special case of CL, being CL observed in or derived from living organisms. The two phenomena are essentially the same from the point of view of the analyst. In this dissertation, CL will often be used in reference to both. Methods based on CL offer several important advantages to analytical and clinical chemists.

Principles

CL emission occurs when a chemical reaction yields an electronically excited product which can emit light itself or transfer its energy to another molecule that can emit. This process can be summarized as

$$A + B \longrightarrow C^* \qquad (1.1)$$

$$C^* \longrightarrow C + \text{light} \qquad (1.2)$$

A and B are the CL reactants, C^* is the electronically excited product, and C is the ground state product. The emission process is analogous to

fluorescence. The distinction between CL and fluorescence is in the means of obtaining the excited state. Energy required to produce the excited state in CL is derived from a chemical reaction, rather than from irradiation. This process is referred to as chemiexcitation.

A reaction must meet three requirements in order to produce CL (2, 7): 1) sufficient energy to populate an excited state must be available from the chemical reaction, 2) the reaction pathway must favor formation of excited state product, and 3) the electronically excited state must be capable of emitting a photon itself or transferring its energy to some other molecule that can emit.

The efficiency of CL, ϕ_{CL}, can be defined as follows:

$$\phi_{CL} = \frac{\text{\# photons emitted}}{\text{\# molecules reacted}} \qquad (1.3)$$

The expression for ϕ_{CL} includes the efficiency of population of the excited state, ϕ_{ex}, and the efficiency of emission, ϕ_{FL}, of the excited excited state, ϕ_{ex},

$$\phi_{CL} = \phi_{ex} \frac{\text{\# excited states formed}}{\text{\# molecules reacted}} \times \phi_{FL} \frac{\text{\# photons emitted}}{\text{\# excited states formed}} \qquad (1.4)$$

BL reactions are more efficient than CL reactions. The firefly reaction, for example, has a ϕ_{CL} of about 0.88, or 88% (1). Bacterial BL reactions typically have efficiencies on the order of 0.05, most non-biological CL reactions have efficiencies of about 0.01 or less (7, 1).

Chemical Analysis Based on CL and BL

The intensity of CL, I_{CL}, depends on both the efficiency of the reaction and the rate at which electronically excited product is formed,

as shown below:

$$I_{CL}\ \frac{\text{photons}}{\text{sec}} = \phi_{CL} \times \frac{dC}{dt}\ \frac{\text{molecules reacted}}{\text{sec}} \qquad (1.5)$$

In practice, the measured intensity also depends on detector efficiency and the geometrical efficiency of the instrumental arrangement (photons reaching detector/total photons emitted).

When using CL for chemical analysis, the reaction conditions are adjusted to make CL intensity a function of analyte concentration. This is accomplished by choosing concentrations such that the analyte is the limiting reagent in the CL reaction. All other necessary reactants are present in excess. For the case where the CL reaction is first order in analyte, equation 1.5 reduces to the following expression for I_{CL} at any time t after mixing of analyte with the other reagents:

$$I_{CL(t)} = K \phi_{CL} [A]_t \qquad (1.6)$$

where K is a rate constant and $[A]_t$ is analyte concentration as a function of time.

There are two basic approaches to CL measurement. Most assays involve single phase CL, in which all reactants, including the analyte, are mixed in a single solution. However, there is growing interest in two phase CL, which involves contact between two distinct phases. These two approaches may be referred to as homogeneous and heterogeneous assay systems, respectively.

Homogeneous CL Systems:

There are two basic approaches to homogeneous CL assay. The first, and most commonly used approach, is to mix analyte rapidly with

reagents and measure the intensity of the resulting light as a function of time. The second approach involves mixing analyte and reagents continuously in a cell in a flow system. This produces a steady state light output which is recorded. The steady state is reached when the rate of analyte mixing with reagents is equal to the rate of analyte consumption plus the rate at which analyte leaves the reaction cell. These two approaches have been termed the "batch method" and the "continuous method" (2). The batch method has been the method of choice for most solution phase analyses, while the continuous method has seen more application in automated instruments and in gas phase CL (2). Details of the continuous method will not be discussed further here, although certain features of this approach, such as the attainment of a steady state, will appear in the discussion of two phase systems.

When an analyte solution is mixed with a solution of CL reagents, an intensity-time curve of the general shape shown in Figure 1-1 is obtained. The exact shape of this curve is determined by the reaction kinetics, the mixing process, and any variations in ϕ_{CL} that may occur during the course of the reaction. Changes in ϕ_{CL} could be caused by quenching of emission by products or reactants. In such cases, changes in ϕ_{CL} will be observed as the concentration of quencher changes during the reaction (2).

There are two ways to analyze the intensity-time curve obtained in a batch-type CL assay. The intensity can be measured at a specific time after mixing, or the curve can be integrated for a specified amount of time. Often, the maximum intensity will be measured and related to analyte concentration. Use of peak intensity offers the advantages of speed (since the entire intensity-time curve need not be recorded),

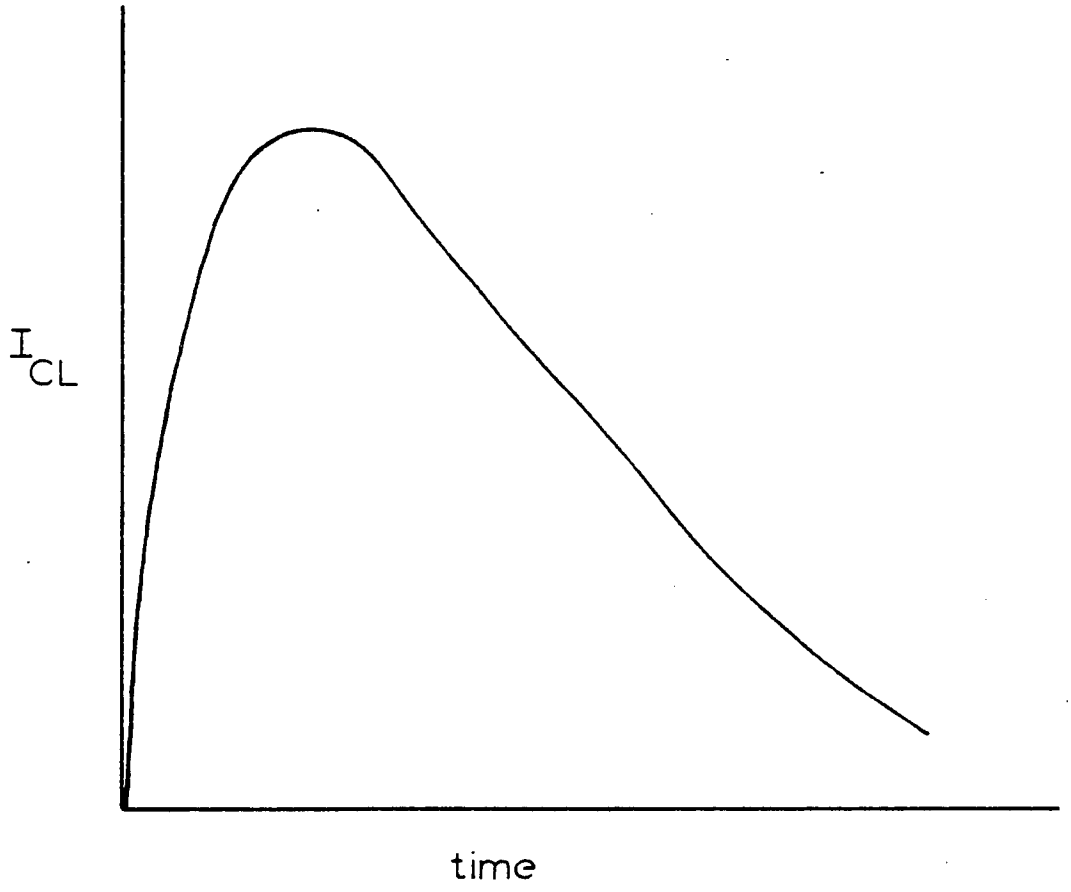

Figure 1-1. Generalized intensity-time curve for a CL reaction.

convenience and high sensitivity. In addition, effects from possible side reactions that produce or consume analyte are systematically reduced (2). There is a disadvantage to using the intensity maximum, however. Since this type of CL assay is really a form of kinetic analysis, any factor, such as temperature variation, that affects reaction rate will affect the maximum intensity (2).

When using the maximum intensity method, it is essential that the mixing of analyte and reagents be highly reproducible, otherwise, the precision of the method will be intolerably poor. Variations in mixing can have significant effects on the intensity-time curve. It is preferable to choose a system in which mixing is rapid compared to the reaction kinetics, because the effect of mixing on the intensity-time curve is not so great as it is when mixing rate and reaction rate are comparable (2).

The alternative to relating maximum intensity to concentration is to integrate all or part of the intensity-time curve. The integrated intensity can be expressed as

$$I_{CL(int)} = \int_{t_1}^{t_2} \phi_{CL(t)} \frac{dC^*}{dt} dt \qquad (1.7)$$

Integrated intensity can then be related to concentration. Equation 1.7 has been solved for first order kinetics assuming ϕ_{CL} is constant (2). The integrated I_{CL} will be independent of reaction rate if the entire intensity-time curve is integrated. This is a type of "equilibrium" or "end point" analysis (2). Results will be somewhere between pure end point and pure kinetic analysis if only part of the intensity-time curve is integrated, and the effects of reaction rate on this integrated

intensity can be determined only if the rate parameters and integration interval are known (2).

One of the simplest devices for a batch type CL measurement is shown in Figure 1-2. The sample is injected by a syringe through a septum into a cell containing reagent solution. The cell is mounted in front of a photomultiplier tube (PMT). In this case, one depends on the force of the injection to provide adequate mixing. While this is often a safe assumption when using reactions with slow kinetics, it is very difficult to obtain good precision when using reactions with fast kinetics. Automated injectors based on this type of arrangement offer somewhat more satisfactory results, but still suffer from a lack of convenience and precision for fast reactions.

The recently introduced technique of flow injection analysis (FIA) offers many advantages as a system for making batch type CL measurements. FIA is somewhat similar to continuous flow analysis developed by Skeggs (8), but involves no air segmentation. Samples are injected by a sample valve directly into a reagent stream, and mixing is accomplished by natural hydrodynamic forces in the flowing stream. Important advantages of FIA include high sample throughput, small sample sizes, simple instrumentation, versatility and ease of operation. Of particular interest to the analyst using CL systems is the fact that in FIA mixing is highly reproducible and easily controlled. In addition, sample can be introduced to reagents remote from the detector, thereby reducing light leak problems.

Some applications of FIA with CL detection have been reported (9, 10). FIA with CL detection for the determination of Cr(III) and glucose are the subjects of Chapter 2.

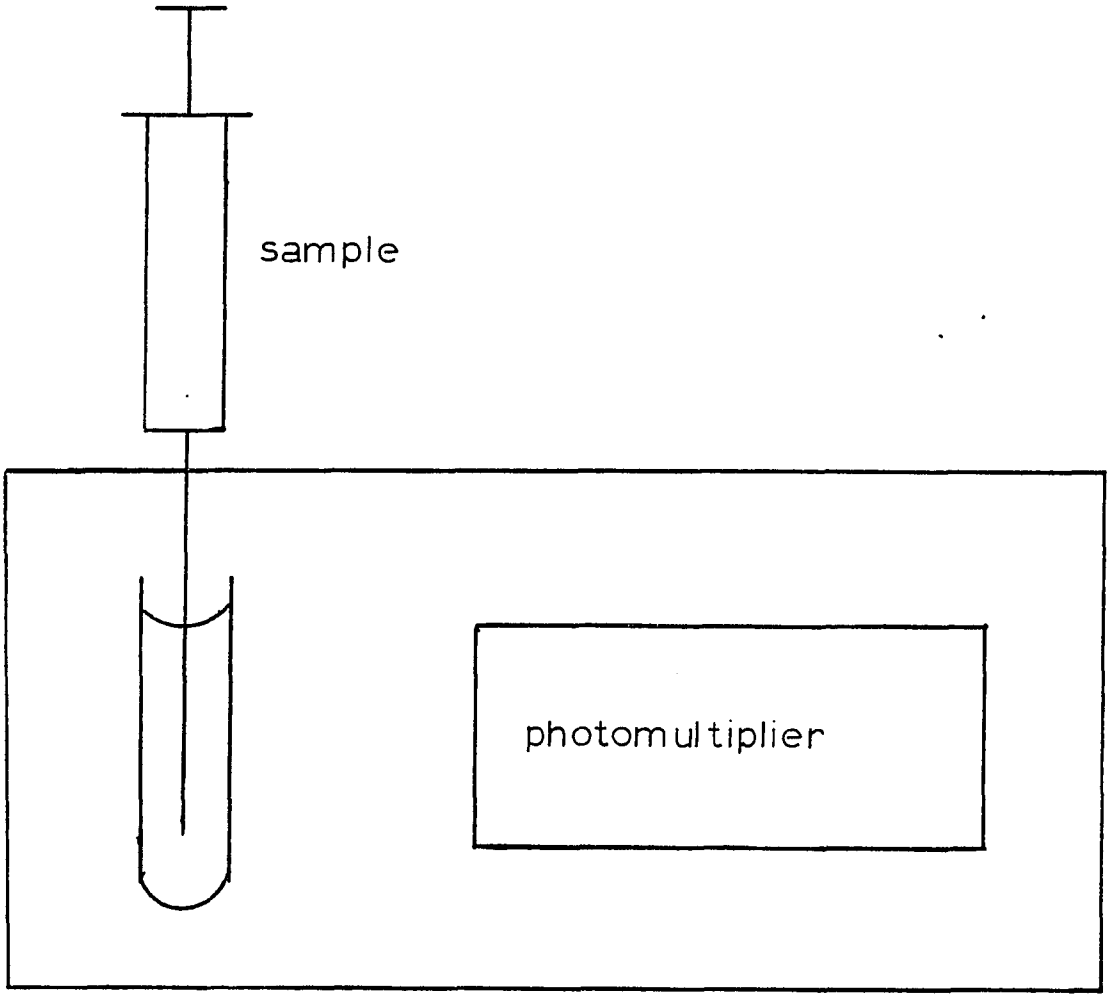

Figure 1-2. Simple system for measurement of CL. Photomultiplier and cuvette containing reagents are mounted in a light-tight housing. The needle of the sample syringe is inserted through a septum.

Heterogeneous Systems:

Many CL analyses based on contact between two distinct phases have been reported recently. An example of this approach is the use of immobilized enzymes as solid phase catalysts for solution reactions (11, 12, 13, 14, 15, 16, 17). The CL intensity in such cases depends on both the kinetics of the CL reaction and the efficiency of the mass transfer process that brings analyte and reagents to the interface between the two phases (2). Immobilized reagents may be chemically bound to a solid matrix (such as glass beads) or immobilized by entrapment in a solution phase separated from the analyte phase by a semi-permeable membrane. Analyte moves to the reagent phase under a concentration gradient. Non-immobilized reagents are present in both phases in equal concentrations.

For the case in which convection is the primary means of mass transfer in the analyte phase, reaction kinetics are first order in analyte, and analyte can diffuse from solution into the interior of the reagent phase. The relationship of CL intensity to reaction rate and mass transfer efficiency has been described theoretically (2, 18). When the rate of analyte reaching the reagent phase equals the rate of analyte consumption, steady state emission will be established. Equation 1.8 relates this intensity to analyte concentration.

$$I_{CL} = \frac{\phi_{CL} \quad KDc \quad A \quad (Dc/d) \; C_{bulk}}{KDc \quad + \quad Dc/d} \qquad (1.8)$$

I_{CL} is the steady state intensity, K is the rate constant for the light emitting reaction, A is the area of the phase interface, D_c is the diffusion coefficient of the analyte in the solution phase, D_c is the diffusion coefficient of analyte in the reagent phase, C_{bulk} is the bulk

concentration of analyte, and d is the thickness of the Nernst diffusion layer, i.e., the thin layer of solution at the phase boundary that is assumed to be unstirred. This equation assumes that there is no depletion of analyte in the bulk analyte solution. Equation 1.8 can be modified to account for such depletion, if necessary, for situations in which reaction kinetics are fast, stirring is efficient, and interfacial area is large (2).

When reaction rate is fast relative to mass transfer, $\sqrt{K\bar{D}_c} >> D_c/d$, and the $\sqrt{K\bar{D}_c}$ terms cancel. This means that mass transfer controls CL intensity, and small changes in reaction rate will not affect measured intensity. On the other hand, when mass transfer is fast relative to reaction rate, $D_c/d >> \sqrt{K\bar{D}_c}$ and the D_c/d terms cancel. Here, the steady state signal varies with $\sqrt{K}$ and therefore is less subject to fluctuations in reaction rate than a single phase kinetic assay in which intensity depends directly on the rate constant.

Another important advantage of using two phase systems is that it is usually possible to reuse the immobilized reagents for many analyses, thereby reducing reagent costs considerably. This is especially important for firefly and bacterial BL enzymes, which are quite expensive.

Advantages of CL Methods of Analysis

Instrumental Requirements

CL-based methods require simple and inexpensive instrumentation. The essential components include a system to mix analyte and reagents and a light detector (usually a photomultiplier tube). Optical resolution is not required for most assays.

Sensitivity

Low detection limits are possible with CL methods because it is easy to measure low levels of light with modern PMT's. For example, the BL determination of NADH is estimated to be some 25000 times more sensitive than the spectrophotometric determination of NADH (5). Detectability is often limited not by light measuring ability, but by reagent purity. Crude reagent preparations often exhibit background emission which dictates the detection limit. Detection limits are improving as more highly purified reagents become commercially available.

Linear Range

Because CL and BL are emission processes (as opposed to absorption), response to analyte concentration is usually linear over several orders of magnitude. This results in a linear dynamic range usually extending from the minimum detectable concentration to the point where it is no longer possible to maintain an excess of reagents over analytes (5).

Specificity

BL reactions are inherently specific. CL reactions generally are not, but can be coupled to enzyme catalyzed processes to take advantage of the specificity of the enzyme reaction.

Speed of Analysis

The speed of CL measurements depends largely on the CL reaction used and on the method of mixing. A rapid flash of light is observed in some reactions, and concentration may be related directly to peak height. Other reactions, such as firefly BL, rise more gradually to a maximum level and light emission may last for several minutes (5, 11,

19, 20, 21, 22), requiring complete or partial integration of the intensity-time curve.

Cost

As mentioned above, the cost of the necessary instrumentation is minimal. Reagent costs vary, depending on the reaction. Luminol, a common CL reagent, is fairly inexpensive, while firefly luciferase and luciferin cost in the neighborhood of $400 per gram or more, for highly purified reagents. The expense of these reagents is often tolerable because the high sensitivity of the methods allows the analyst to work with minimal amounts of the expensive reagents.

Stability

Most non-biological CL reagents are quite stable, but BL reagents do suffer from a lack of stability. Bacterial and firefly luciferase enzymes are especially prone to thermal denaturation, even at room temperature, although this is becoming less of a problem as highly purified and stabilized reagents appear on the market.

Toxicity

Most CL and BL reagents present no hazards for users or the environment (5). They are, therefore, promising as alternatives to radioisotope labels for immunoassay.

Disadvantages of CL Methods of Analysis

The high cost of BL reagents is a disadvantage, as mentioned above. Other major disadvantages of CL methods are the paucity of mechanistic data and the shortage of useful reactions.

Mechanisms

In spite of the fact that many papers have appeared exploring the basic chemistries of CL reactions (1, 19, 20, 21, 23-45), mechanisms are in many cases still poorly understood. This can make it difficult to predict and control the analytical behavior of a CL method (5).

Available Reactions

While many reactions exhibit CL, few are efficient enough to be useful for analysis. This limits the number of potential analytes which can be determined by CL methods. It is possible, however, to increase the number of substances determined by CL assays by using coupling reactions in which the analyte of interest reacts to produce or consume one of the CL reactants.

CL and BL Reactions

The work described in this dissertation is concerned with analytical applications of the following three CL systems: luminol CL, firefly BL and bacterial BL.

Luminol

Luminol CL was discovered by Albrecht in 1928 (46) and has been studied extensively (1). The CL reaction of luminol (5-amino-2,3-dihydro-1,4- phthalazinedione) is summarized in Figure 1-3.

The quantum efficiency, ϕ_{CL}, of the luminol reaction is about 0.01 (1). Hydrogen peroxide is the most common oxidant, although other oxidizing agents do react with luminol to produce CL (7).

The exact mechanism of the luminol reaction is unknown and kinetic data is unavailable for most oxidizing systems (2). It is known, however, that the aminophthalate anion, which is formed in the singlet

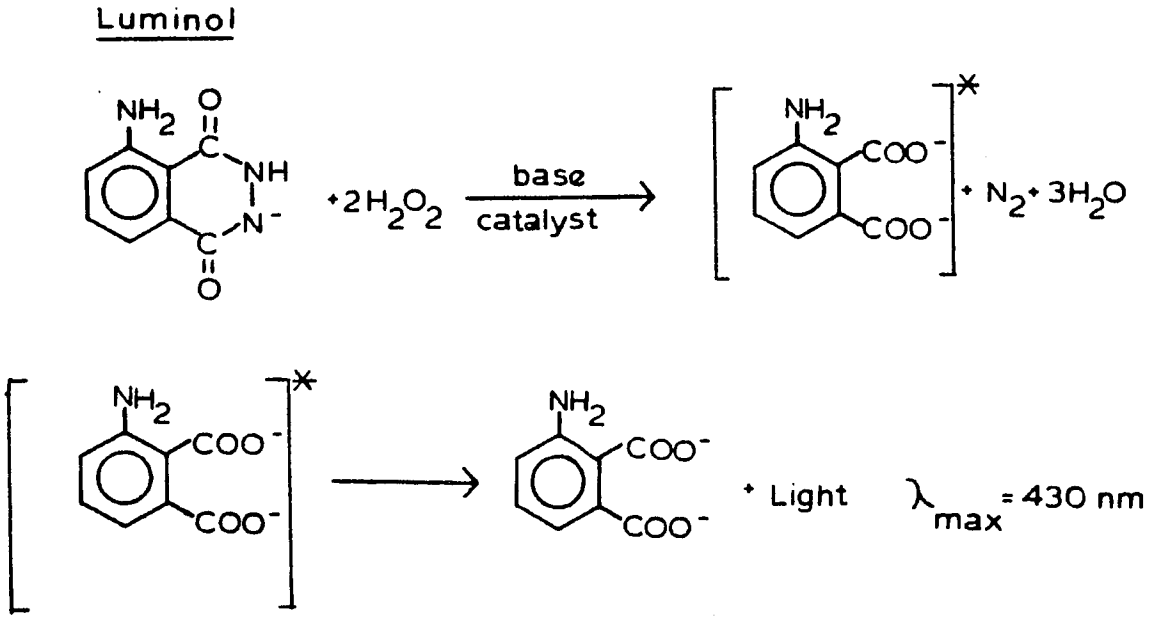

Figure 1-3. The luminol reaction.

excited state, is the emitter (3, 29).

Choice of catalyst/cooxidant. A third component is necessary if luminol and hydrogen peroxide are to react to an appreciable extent (27). This component may function as a catalyst, a cooxidant, or both. For example, peroxidase functions as a catalyst, since the enzyme is unchanged in the overall reaction. Peroxydisulfate is an example of a cooxidant, since it does not survive the reaction unchanged (47). Ferricyanide behaves as a catalyst/cooxidant, it is reduced to ferrocyanide as it oxidizes luminol, but is later reoxidized back to ferricyanide (47, 48).

The overall reaction requires a four electron oxidation of luminol. This would require two moles of peroxide per mole of luminol, as indicated in Figure 1-3, unless a cooxidant is responsible for part of the oxidation.

The catalyst/cooxidant influences reaction rate, efficiency, the order of the reaction, and the stoichiometry (47). Choice of catalyst/cooxidant is, therefore, an important consideration.

Several transition metal ions have been shown to catalyze luminol CL (27), with Co(II) and Cu(II) being the most efficient. While both Co(II) and Cu(II) have been investigated for use as catalysts in peroxide determinations (49), both have drawbacks. Response to peroxide concentration is non-linear with a copper catalyzed system. The main problem with cobalt is poor solubility in the high pH range where luminol CL is most efficient.

If hydrogen peroxide and luminol are present in excess, so that the transition metal ion catalyst is the limiting reagent, one can design a system for determination of trace metals (50, 51). For example, Cr(III)

can be determined by this approach. The Cr(III) assay will be discussed in Chapter 2.

Probably the most widely used catalyst/cooxidant for CL determination of peroxide is ferricyanide (2, 49, 52, 53, 54). The kinetics of the ferricyanide catalyzed reaction fall in a range that makes it quite useful for analytical purposes (47). The rate is sufficiently fast to provide a rise to maximum intensity within a few seconds or less, but not so fast that the emission becomes too highly sensitive to variations in the mixing of analyte with reagents. In addition, the reaction rate does not seem to be overly dependent upon temperature (47, 49), and it is psuedo-first order in hydrogen peroxide. One potential problem associated with the ferricyanide catalyzed reaction is that ferricyanide, molecular oxygen and luminol react to emit light in the absence of peroxide. This results in a high, but usually quite stable background signal, which in most cases can be easily subtracted.

pH. Luminol CL is most efficient in the pH range 10.4 to 10.8 (47, 49, 53, 54). Emission is fairly constant with pH over this range, but falls off rapidly above pH 11 and below pH 10. The best precision for luminol-based CL assays occurs between pH 10.4 and 10.8.

Luminol concentration. Luminol concentration is not a critical parameter, particularly when using the ferricyanide catalyzed reaction (47). CL increases with increasing luminol concentration, but background emission increases as well as signal from peroxide, so that the analyst does not really gain, after a point, by increasing luminol concentration. At high luminol concentrations the observed CL becomes more sensitive to variations in mixing, which leads to a loss of

precision as well as an increase in background emission. Luminol concentrations in the range 0.1 mM to 1 mM are generally used for peroxide determinations (47).

Coupled reactions. It is possible to extend the application of luminol CL assays to other analytes through coupling reactions. The substrates for a number of enzyme reactions that produce peroxide can be determined by coupling to the luminol reaction. This approach has been used for glucose and uric acid determinations (47, 49, 52, 53, 54, 55). A luminol CL system for glucose based on the use of glucose oxidase as the coupling enzyme is discussed in Chapter 2.

Firefly Bioluminescence

The firefly reaction has been the most intensively studied BL system (1,3). It is the most efficient luminescent reaction known, with a quantum efficiency of about 0.88 (1). There are many recent reviews dealing with this reaction (1, 2, 4, 20, 21, 22, 30), which is summarized below:

$$LH_2 + ATP + E \longrightarrow E{:}LH_2{:}AMP + PP \qquad (1.9)$$

$$E{:}LH_2{:}AMP + O_2 \longrightarrow E + [L{=}O]^* + CO_2 + AMP \qquad (1.10)$$

$$[L{=}O]^* \longrightarrow L{=}O + light \qquad \lambda_{max} = 562\ nm \qquad (1.11)$$

E is the enzyme firefly luciferase, LH_2 is firefly luciferin, PP is pyrophosphate and L=O is oxyluciferin. The asterisk denotes the excited state oxyluciferin emitter.

Analytically, the reaction is of greatest interest for ATP determination. Detection limits as low as 0.01 pmoles of ATP have been reported (2). Detection limits are limited by background emission (in

the absence of added ATP) from luciferase preparations. Response is linearly proportional to ATP concentration over several orders of magnitude (2).

While crude firefly luciferase preparations contain some luciferin, additional light output is observed if more luciferin is added to the system. This in turn leads to lower detection limits (2, 56). Although luciferases from different species of fireflies may have differences in their structures, the structure of luciferin is identical for all species (3). Highly purified luciferase preparations require added luciferin for light production. Synthetic luciferin is commercially available, albeit at an astronomical price.

Stability. The stability of luciferase preparations depends on the method of preparation. A purified preparation stable for several days at room temperature has been reported (57), and several preparations that are quite stable are now available commercially. For example, a lyophilized firefly luciferase preparation available from Lumac is reported to be stable for two years at 2-8^{o} C. The reconstituted reagent is stable at room temperature for 12 hours. These highly purified and stabilized reagents are the choice for clinical applications because they yield lower detection limits and are much more stable than crude preparations. They are, however, quite expensive.

Kinetics. Reports describing the kinetics of the firefly reaction have been published (19). Upon injection of ATP there is an initial lag time of about 25 msec. followed by a rapid rise to maximum intensity requiring about 300 msec (22). After the peak intensity is reached there is a fairly rapid initial decay, followed by a slower decay over a long

period of time. Figure 1-4 shows a generalized intensity-time curve for the firefly reaction. Product inhibition by pyrophosphate and oxyluciferin cause a decrease in intensity, ATP consumption has been shown to be minimal (2). Product inhibition can be minimized by using low ATP concentrations (2, 57). With crude luciferase preparations the intensity decays more rapidly due to the presence of ATP-consuming enzymes (2, 22).

Interferences. The firefly reaction is, for practical purposes, specific for ATP. There are, however, two major interferences (2). One problem arises from unwanted ATP generated by reactions catalyzed by contaminating enzymes present in crude luciferase preparations. This is not a problem with highly purified commercial preparations. A second problem is due to anion binding effects: anions such as citrate can bind Mg(II) and thus inhibit firefly BL (2). The standard additions method can be used to counter this effect.

Applications. The firefly reaction is widely used to determine ATP. Many applications involve biomass determinations. In addition, several substrates can be determined by the firefly reaction by coupling to other reactions that produce or consume ATP. A recent review summarizes many of these applications (2).

Bacterial Bioluminescence

Several species of marine bacteria bioluminesce. These BL bacteria may be found free-living in sea water or may exist as symbionts in the light organs of certain types of fish. Several recent papers and reviews deal with the chemistry of bacterial BL (6-8, 10-13, 15, 16, 17, 19, 20-24) and with the analytical applications of the reaction (2-5, 9,

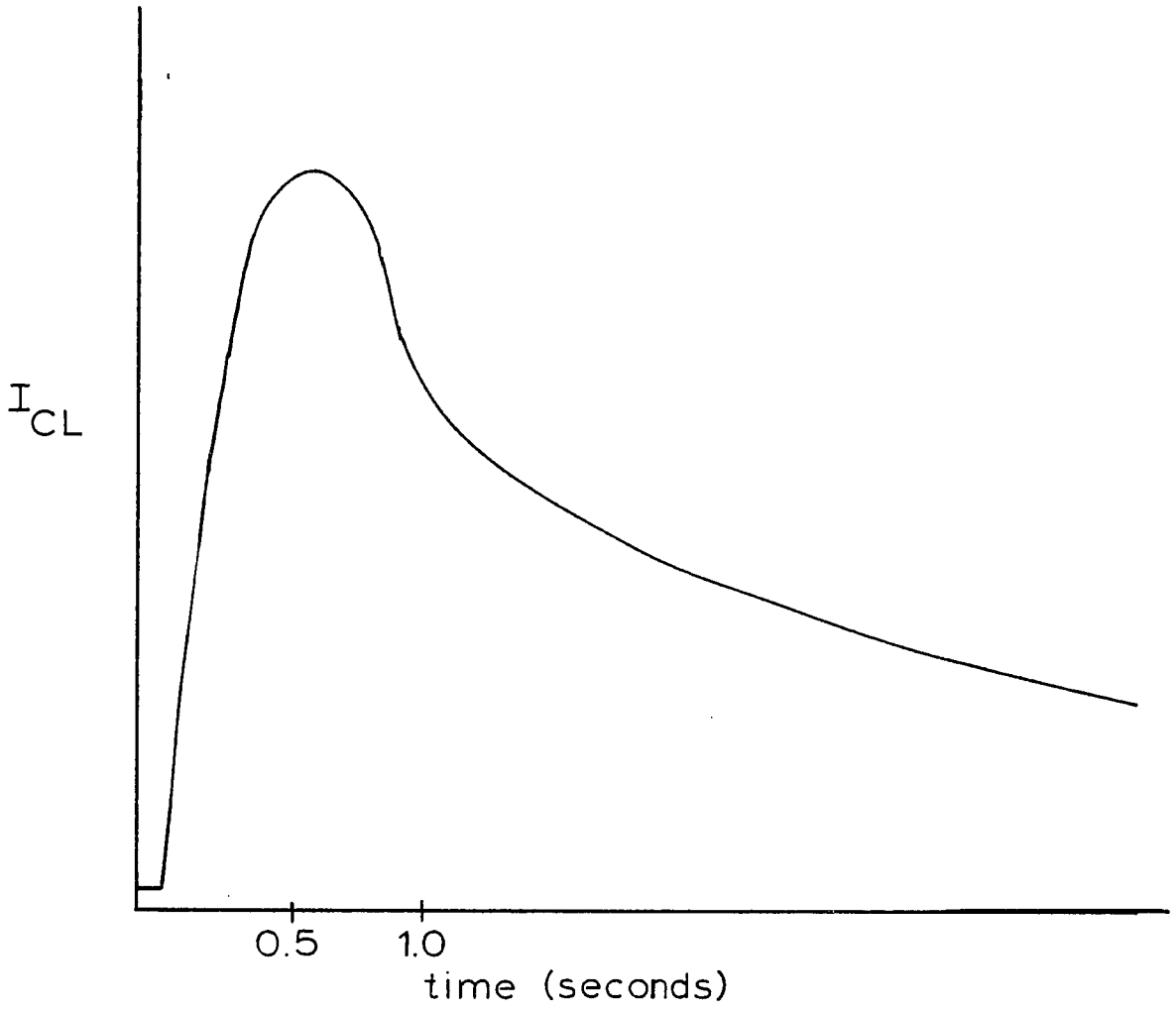

Figure 1-4. Typical intensity-time curve for firefly BL. Intensity is in arbitrary units.

13, 14). The basic reaction is shown below:

$$FMNH_2 + O_2 + RCHO \longrightarrow FMN + RCOOH + H_2O + light \quad (1.12)$$

$FMNH_2$ is reduced flavin mononucleotide and RCHO is a long chain aliphatic aldehyde. The reaction is catalyzed by the enzyme bacterial luciferase.

Light emission is rapid upon addition of $FMNH_2$ to a solution of luciferase and aldehyde. This is followed by a slow decrease in intensity. A generalized intensity-time curve is shown in Figure 1-5. The initial rise is due to rapid formation of the $FMNH_2$ - luciferase complex (2). The slow speed of the reaction of the enzyme-flavin complex to produce light accounts for the relatively slow decrease in intensity. Direct oxidation of $FMNH_2$ by oxygen is an important side reaction that consumes any $FMNH_2$ not bound quickly to the enzyme.

The stability of crude luciferase preparations is a potential problem because the enzyme is susceptible to heat denaturation even at room temperature (3). Purified luciferase preparations that are being made commercially available are reported to be stable for 24 hours at room temperature. Luciferase appears to be stabilized by phosphate ion and bovine serum albumin (BSA) (2, 3). Improved detection limits are another advantage to using purified reagents. The enzymes are highly specific for $FMNH_2$, although they do show weak activity toward other flavins and flavin analogs (3).

The clinical potential of bacterial BL is due to the fact that the luciferase reaction, (1.12), can be coupled to an oxidoreductase reaction which oxidizes NADH or NADPH while forming $FMNH_2$. This NAD(P)H:FMN oxidoreductase is found together with luciferase in luminous bacteria. The enzymes are separated during purification, but can be

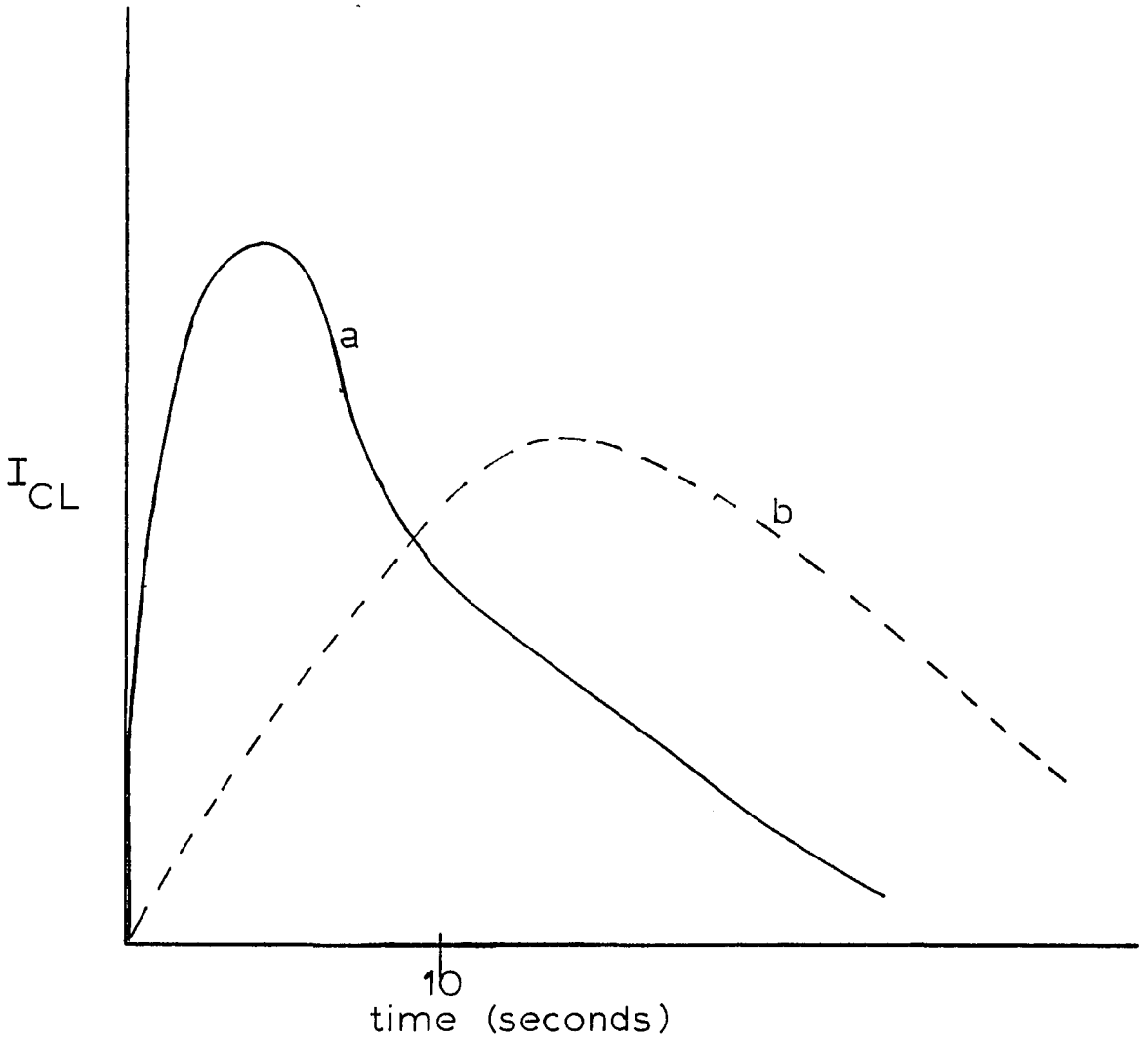

Figure 1-5. Typical intensity-time curve for bacterial BL. (a) is response when $FMNH_2$ is injected, (b) is response of oxidoreductase-coupled system using NADH injection (2).

recombined to form a coupled reaction system that responds to NADH or NADPH, depending on the particular oxidoreductase used. The oxidoreductase reaction is shown in equation 1.13.

$$NAD(P)H + FMN \longrightarrow FMNH_2 + NAD(P) \qquad (1.13)$$

The newly formed $FMNH_2$ is rapidly bound to luciferase to take part in the light emission reaction (1.12). In this coupled reaction scheme, the second reaction, 1.12, is relatively fast. It is possible to control overall system kinetics by varying the oxidoreductase activity (2). Figure 1-5 shows a typical response curve for the coupled system.

The NAD(P)H:FMN oxidoreductases are heat-labile, although perhaps not so much as luciferase (3), and they are stabilized by dithiothreitol (2). The oxidoreductases are highly specific for NADH or NADPH.

Bacterial BL has been used to determine NADH in various clinical assays (2-4, 16, 17, 59, 60, 61). Particularly interesting is the use of immobilized luciferase and oxidoreductase, permitting reuse of these expensive reagents (13, 14, 16, 17). The use of purified enzymes has resulted in detection limits below 10^{-15} moles of NADH (2, 60). Linear response to NADH concentration over several orders of magnitude is observed (5). There do not appear to be any serious interferences in the use of bacterial BL for NADH determination (2). Purified and stabilized luciferase-oxidoreductase preparations with long shelf lives are commercially available.

The fact that NADH can be determined by the bacterial BL system opens up a wealth of clinical applications for the reaction. In principle, any reaction which produces or consumes NADH or NADPH could be coupled to the bacterial BL system. In view of the number of enzyme

reactions that involve NADH oxidation and reduction, it is easy to imagine the far-reaching applications of bacterial BL. Examples of such possible coupling reactions include the use of lactate dehydrogenase (LDH) for lactate determination and alcohol dehydrogenase (ADH) for ethanol determination. Alternatively, LDH and ADH activities may be assayed. The feasibility of this approach has been demonstrated (2, 13, 14, 62). This application of bacterial BL does have problems, however, most notably the fact that most dehydrogenases have equilibria that favor NADH consumption rather than NADH production. This requires provisions for driving the reactions toward NADH production. Alternatively, the analyst could try to measure NADH consumption against a high background. The problems associated with this approach have been discussed (2).

Goals of this Project

The goals of the work discussed in this dissertation were to develop and characterize analytical systems based on CL and BL. For the most part, the research was aimed toward analytes of clinical importance. In a clinical setting, analytical methods must not only be accurate and precise, but also should be easy to perform, have high sampling rates, be sensitive and selective, and, ideally, require relatively simple and inexpensive equipment. Automated flow systems are very important in clinical chemistry because they offer many of these advantages. In this work, flow systems were used in conjunction with CL reactions in an effort to design clinically useful analytical systems for a variety of important analytes, while making use of the advantages of CL reactions combined with flow system instrumentation.

CHAPTER 2

FLOW INJECTION ANALYSIS WITH CL DETECTION

Introduction

Flow Injection Analysis (FIA) is an approach to automated analysis that is extremely well suited for use in assay systems based on CL detection. The combination of CL and FIA offers many advantages, combining the sensitivity of CL reactions with the easily controlled, reproducible mixing of FIA. The clinical laboratory especially can benefit from the high sample throughput and simple operation of FIA. The subjects of this chapter will be CL-FIA systems for the determination of Cr(III) and glucose.

Flow Injection Analysis

Basic Principles

Flow injection analysis is a relatively new form of flow analysis introduced independently by Ruzicka and Hansen (63) and Stewart (64, 65). It differs from the air-segmented continuous flow analyzers introduced by Skeggs (8) in that there is no air-segmentation in FIA. Rather than trying to minimize sample dispersion, as is done in traditional continuous flow analyzers, FIA actually depends on controlled dispersion to accomplish mixing of sample and reagents. Another important difference between FIA and air segmented systems is that in FIA the analyst observes transient peaks rather than steady state plateaus. The elimination of air segmentation in FIA means that

it is not necessary to introduce or remove air bubbles from the flow line, thereby greatly simplifying the instrumentation.

The simplest type of FIA experiment involves introducing a slug of sample, usually by means of a sample injection valve, into a flowing stream of carrier solution. The carrier may contain reagents with which the sample will react, or merely serve to transport the sample to a detector. The components of a simple FIA system are shown in Figure 2-1. As this figure shows, there are only three components required to construct an FIA system: a relatively pulse-free pump, a sample injection device, and a flow-through detector with some sort of data readout capability. Plumbing components, such as tubing and any necessary fittings, complete the apparatus. The components are essentially the same as one would find in many liquid chromatography systems.

The key to understanding FIA's advantages lies in a consideration of what happens to the sample slug once it is injected into the flowing stream under the prevailing conditions of laminar flow. A sample slug injected into the flow stream (Figure 2-2a) soon assumes a parabolic concentration profile (Figure 2-2b) because the sample solution near the walls of the tubing is retarded by friction with the walls while the solution at the center of the tube actually moves at twice the mean flow rate. The development of this parabolic profile dilutes the sample and spreads it out. Air segmented systems use air bubbles to reduce this effect and thus maintain high sensitivity and prevent sample carryover. FIA, however, takes advantage of this phenomenon, and controls sample dispersion by employing conditions that promote radial mass transfer.

Diffusion is an important mechanism contributing to radial mass

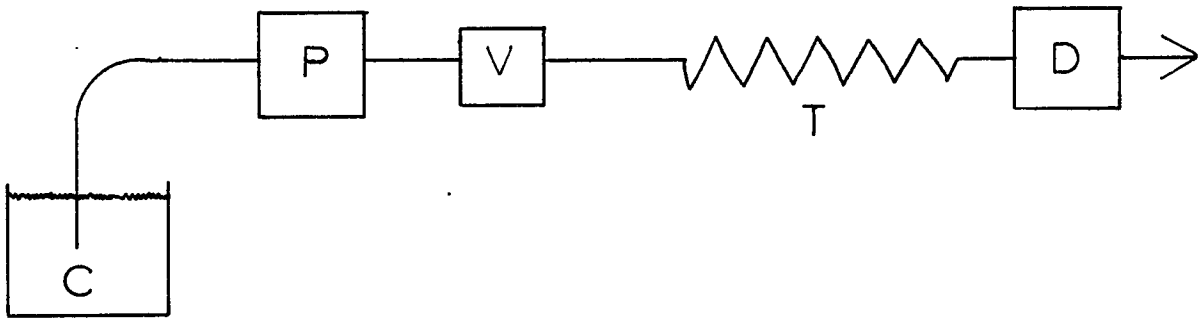

Figure 2-1. Components of a simple FIA system. C: carrier solution; P: pump; V: sample injection valve; T: tubing coil to enhance mixing; D: flow-through detector.

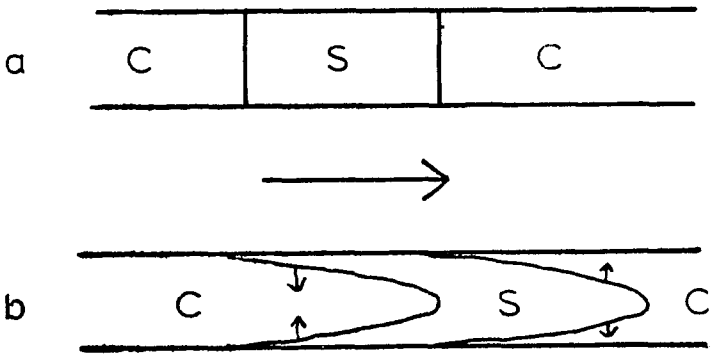

Figure 2-2. Development of parabolic concentration profile in a FIA system. Flow in the system is in the direction of the large arrow. (a) sample slug S when injected into carrier stream C; (b) concentration profile some time after injection; small arrows indicate radial diffusion.

transfer. Sample in the leading cone of the parabolic concentration profile will tend to diffuse under a concentration gradient to the less concentrated carrier region near the walls, while sample in the tail, retarded by the walls, will tend to diffuse toward the more dilute center (see arrows in Figure 2-2b). Thus, the sample from the leading cone diffusing toward the walls will be slowed down by friction, and the trailing sample will gradually move more rapidly with the flowing stream as it diffuses toward the center. This reduces sample zone spreading while at the same time causing sample and carrier to mix.

Radial mass transfer is enhanced by coiling the tubing between injector and detector. The magnitude of this effect depends on flow rate, tubing diameter, degree of coiling, and the diffusion coefficient (66, 67, 68).

As the sample zone moves down the tube from the injector, dilution and sample spreading increase (Figure 2-3), giving rise to the generalized FIA signal shown in Figure 2-4. Tailing is common because of the nature of the zone spreading. Control of dispersion is one of the critical requirements in FIA. Dispersion is defined mathematically as

$$D = C / C_{max} \qquad (2.1)$$

where C is the initial concentration of sample and C_{max} is the maximum concentration of sample that flows past the detector. FIA systems in which sample is diluted in a carrier of reagent would typically be designed to have a "medium" dispersion of about 3 to 10 (68). A dispersion of 3 is enough to mix sample and reagents adequately, without causing excessive loss of sensitivity because of too much dilution.

Factors to be considered when designing an FIA system include

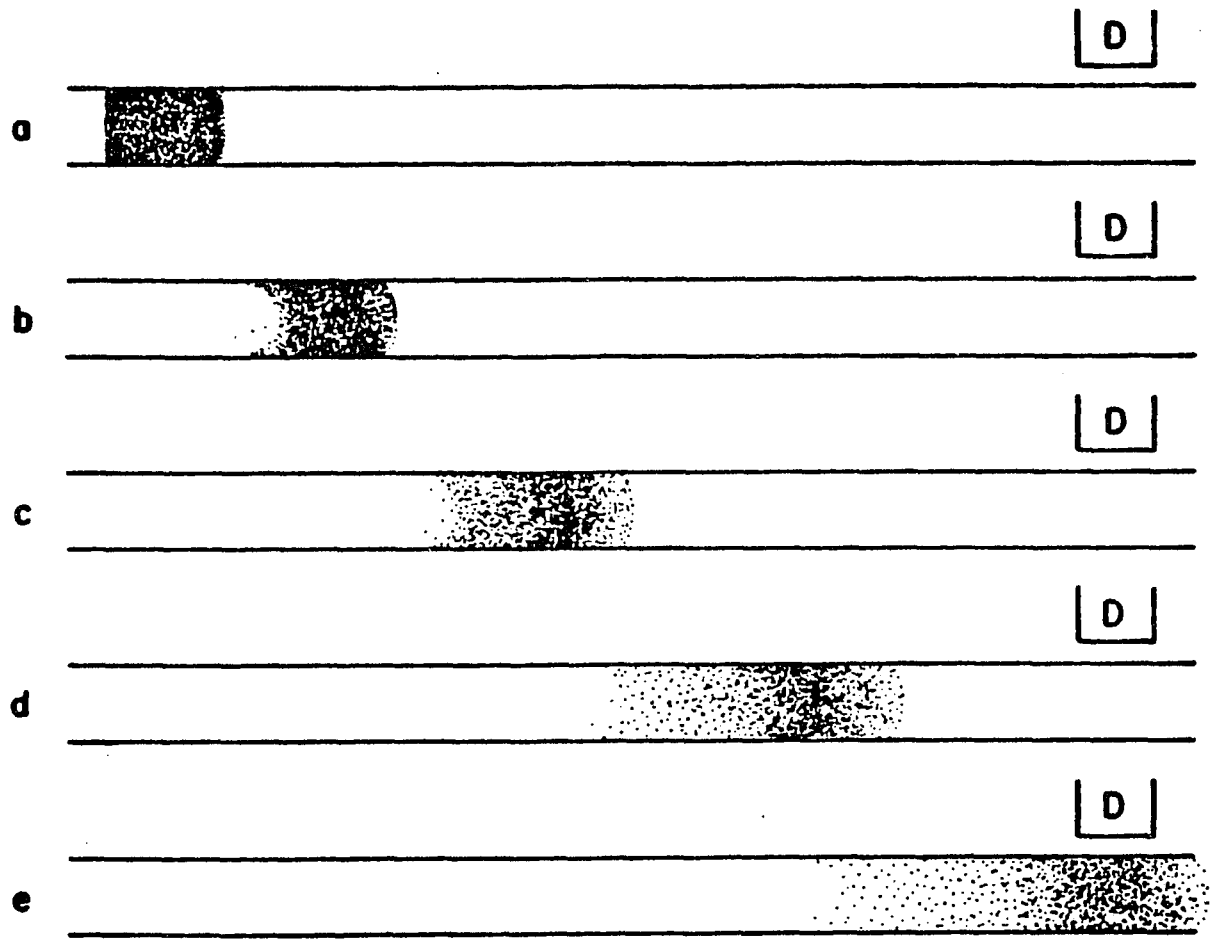

Figure 2-3. Dispersion of sample slug from injection (a) as the flowing stream proceeds down the tubing and past the detector D.

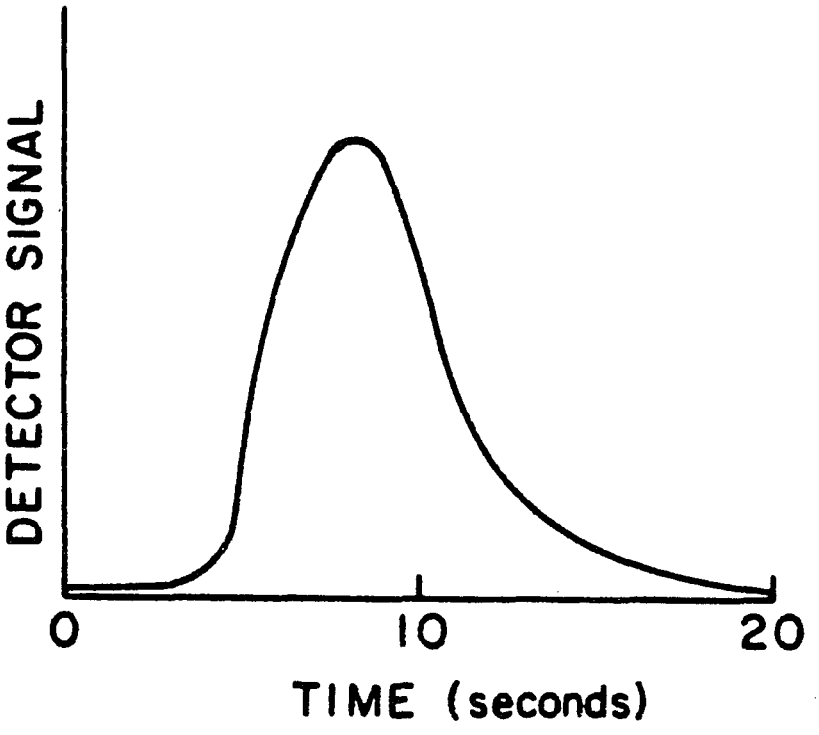

Figure 2-4. Typical FIA signal. The tailing is common for these systems.

sample volume, flow rate, tubing diameter and length, and the reaction kinetics. Larger sample volumes may give greater sensitivity because there is less dilution of the center of the sample zone, but will reduce sample throughput. Small diameter tubing (typically 0.5 to 0.8 mm i.d.) is preferred to enhance radial diffusion by reducing the distance between the wall and the center of the tubing. Faster flow rates lead to greater reagent consumption and cause a loss of sensitivity because of increased dispersion, but will increase sampling rate. Dispersion can be controlled by varying the length of tubing between injector and detector to allow adequate time for mixing, without causing excessive dilution.

The system must be designed such that there is adequate time for any chemical reaction to proceed far enough to produce a measureable amount of product by the time the sample zone passes the detector. The reaction need not go to completion, however. FIA is more useful for relatively fast reactions than for kinetically slow reactions. In order to obtain sufficiently long residence times for the reactants of slow reactions, one loses the main advantages of FIA, most notably the high sample throughput. Conventional air-segmented systems seem better suited for slow reactions.

The essential feature of FIA is reproducible and easily controlled dispersion. This is the reason FIA is so attractive to the analyst performing assays using CL. CL analyses require highly reproducible mixing in order to achieve adequate precision. FIA provides the reproducible mixing required, and offers the additional advantages of simple instrumentation, easy operation, high sample throughput, and adaptability to automation.

The interested reader is referred to several recent publications that describe FIA in more detail (63, 69, 68, 70). In particular, a recent book by Ruzicka and Hansen provides an excellent description of FIA (71). Many applications of FIA have been reported (68), including systems using CL detection (9, 10, 72).

Chromium(III) Determination

Introduction

The luminol reaction may be used to determine transition metal ions by reacting them with excess luminol and hydrogen peroxide (50, 51, 73, 74). This general approach, while quite sensitive, is not particularly selective. However, it is possible to design a system which is selective for chromium(III) by using ethylenediaminetetraacetic acid (EDTA) to eliminate interferences from other metal ions that also catalyze luminol CL (50, 51, 73, 74).

The complexes of EDTA with transition metal ions do not catalyze luminol CL. In particular, the Cr(III)-EDTA complex does not catalyze luminol CL, but it is kinetically slow to form (51). By adding EDTA to the sample, interfering metal ions are complexed quickly, while Cr(III) is still temporarily free to catalyze luminol CL. The CL intensity can then be related to Cr(III) concentration. Heating the sample after EDTA addition will accelerate formation of the Cr(III)-EDTA complex. This solution can then be used as a blank. Interfering ions that EDTA does not completely eliminate are Co(II), Fe(II) and Fe(III) (50). These interferences can be accounted for by the blank since CL catalyzed by Co(II), Fe(II) and Fe(III) is not affected by heating the EDTA-treated sample (50). Chromium(VI) does not catalyze luminol CL, so this method is specific for Cr in the 3+ oxidation state. This approach has been

applied in the analysis of biological and environmental samples (50, 51, 73, 74).

It should be pointed out that the work on a CL-FIA system for Cr(III) determination reported here was undertaken with a very specific application in mind. The limited experimentation was aimed at solving the problem at hand, rather than at characterizing the analytical performance of the CL-FIA system for Cr(III) determination in general.

It has been reported (75) that dichromate solutions are reduced to Cr(III) by irradiation with gamma rays. In acidic solutions, the yield of Cr(III) as a function of radiation flux is quite reproducible. This fact has been used as the basis for a proposed radiation dosimeter (75, 76) containing potassium dichromate in sulfuric acid solution. The amount of Cr(III) formed in the dosimeter solution after irradiation would be related to radiation dosage. Cr(III) concentration should increase linearly with dose (76). For such a dosimeter to be useful, there must be a method for measuring Cr(III) in the presence of Cr(VI). The project discussed here was undertaken to determine whether CL-FIA would be a feasible approach to this problem.

Experimental

Instrumentation. While the instrumental arrangement is somewhat similar to previously reported systems (51, 74), the use of FIA methodology offers the advantages of greater simplicity, versatility, high sampling rates, and small sample size requirements. The system used for this work is diagrammed in Figure 2-5. A Harvard Apparatus Compact Infusion Pump, model 975, drove the 50mL plastic syringes which contained reagents. Separate syringes for luminol and hydrogen

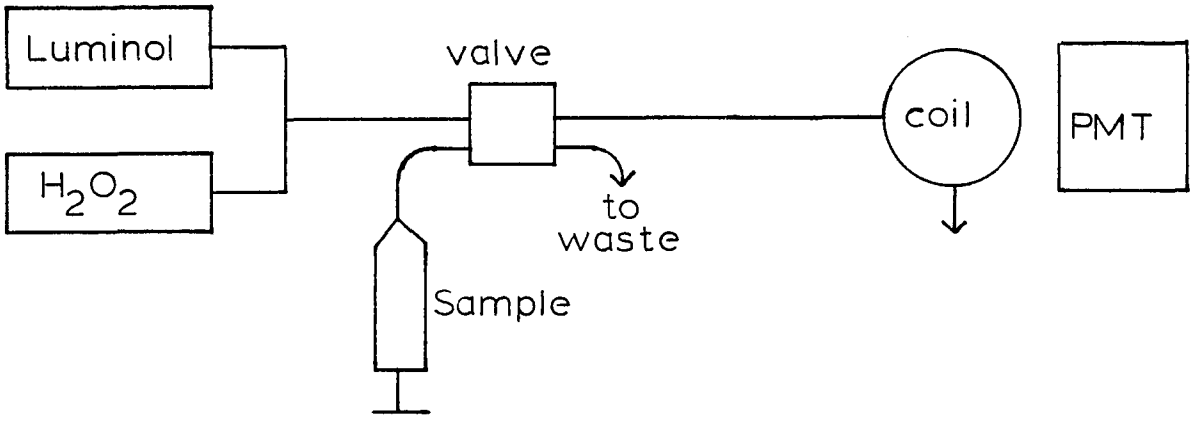

Figure 2-5. FIA system for Cr(III) determination. A two-channel peristaltic pump drove the luminol and H_2O_2 syringes. A separate, single-channel pump drove the sample syringe. The coil of Teflon tubing was mounted flat against the face of the photomultiplier.

peroxide were necessary because a single luminol-peroxide solution would not be stable. Teflon tubing, 0.79 mm i.d., was used throughout the system. Sample was injected by a pneumatically actuated Altex slider valve. The volume of the sample loop was approximately 107 uL, which represents a significant reduction in sample size compared to previously reported systems for CL determination of Cr(III) (51). The exact sample volume is not as important as reproducibility.

CL emission was detected by an EMI photomultiplier tube (PMT) operated at -1800 volts from a Fluke 412B high voltage power supply. A Keithly 610C electrometer was coupled to a Heath model 204 strip chart recorder for data readout. An Orion Research model 501 pH meter was used for all pH measurements.

Reagents. Luminol was used as received from Aldrich. Boric acid and KOH for preparation of 0.01 M borate buffers, pH 10.8, were obtained from Baker. Reagent grade sulfuric acid, Ultrex high purity sulfuric acid and potassium dichromate were also obtained from Baker. Hydrogen peroxide solutions were prepared by dilution of a 30% solution. Chromium(III) solutions were prepared from a stock solution of chromium nitrate. Deionized/distilled water was prepared in a Barnstead still. Disodium EDTA (Baker) was added to the borate-buffered luminol and peroxide solutions to complex any trace metal impurities that may have been present in the reagents.

Procedure. To a 10.0 mL aliquot of sample solution was added 1.0mL of 0.2M EDTA. Smaller samples may be used, in which case EDTA addition should be reduced accordingly. This solution was mixed, and aliquots were injected into the flow system for CL determination of Cr(III)

within about five minutes of EDTA addition. This length of time should not permit the formation of significant amounts of Cr(III)-EDTA complex (51). Blanks were prepared by heating the EDTA-treated sample before injection. Highly acidic samples were titrated with aproximately 1 M KOH to pH 3-4 before injection. The volume of the added base was recorded and accounted for in calculations of Cr(III) concentration.

Luminol concentration was 0.4 mM for all experiments and hydrogen peroxide concentration was 0.01M. A flow rate of 8.6 mL/min/syringe was employed.

Results and Discussion

Because this project was not concerned with complete characterization of the CL-FIA system for Cr(III) determination, many of the operating parameters were adapted from previous work with similar systems (10, 51). Reagent concentrations stated in the experimental section gave satisfactory results and therefore were not further optimized. The flow rate chosen provided good sensitivity and good precision for replicate injections.

The goal of the project was to measure Cr(III) concentration in several samples containing 70 μM potassium dichromate and either 0.4 M or 0.04 M sulfuric acid. These samples, prepared by Dr. K. G. McLaren of the Australian Atomic Energy Commission (AAEC), had been subjected to gamma ray doses ranging from 0 to 1000 rads. Irradiated samples were expected to contain 83 pM to 0.83 μM Cr(III) (76). The high concentrations of sulfuric acid were required for reliable yields of Cr(III) upon irradiation (76).

The high acid concentration was a problem, as might be expected, since the luminol reaction is most efficient at pH 10.4 to 10.8. When

standard samples of Cr(III) containing either 0.4 or 0.04 M sulfuric acid were injected into the flow system (after EDTA addition to each sample), three closely spaced peaks of unequal magnitude were observed. An example of this is shown in Figure 2-6. Precision of replicates was extremely poor, presumably because of pH gradient effects. The pH of the samples had to be raised in order to obtain single, well-shaped peaks. Standard Cr(III) solutions in 0.4 M sulfuric acid were titrated to a pH between 3 and 4 before injection. This modification resulted in smooth, single peaks with no shoulders. It is believed that the reduction in pH gradient effects resulting from adjusting sample pH closer to reagent pH eliminated the multiple peaks observed with no pH adjustment. Figure 2-7 shows some typical data.

Great difficulty was encountered in constructing calibration curves, even after the pH effect discussed above was controlled. Blank values from solutions with no added Cr(III) were high and nonreproducible. Heating these solutions after EDTA addition caused a loss of CL emission. It was therefore concluded that some source of interfering Cr(III) was present.

Experiments were undertaken to determine the source of the Cr(III) contamination (Table 2-1). Acid washed glassware was used for making solutions. Plastic ware was avoided because of the potential for reduction of dichromate in acidic solution in contact with polymers of the plastic (76). As can be seen from Table 2-1, reagent grade sulfuric acid contained significant amounts of Cr(III). The Ultrex acid was sufficiently low in Cr(III) to eliminate this problem. It can be seen from Table 2-1 that all components individually were interference free. Only when sulfuric acid and potassium dichromate were mixed was there an

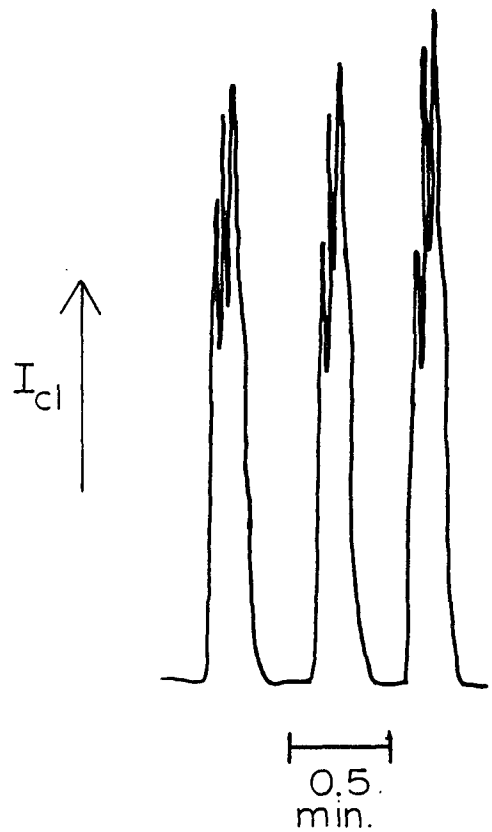

Figure 2-6. Signals for replicate injections of 10^{-6} M Cr^{3+} in 0.4 M H_2O_2, untitrated.

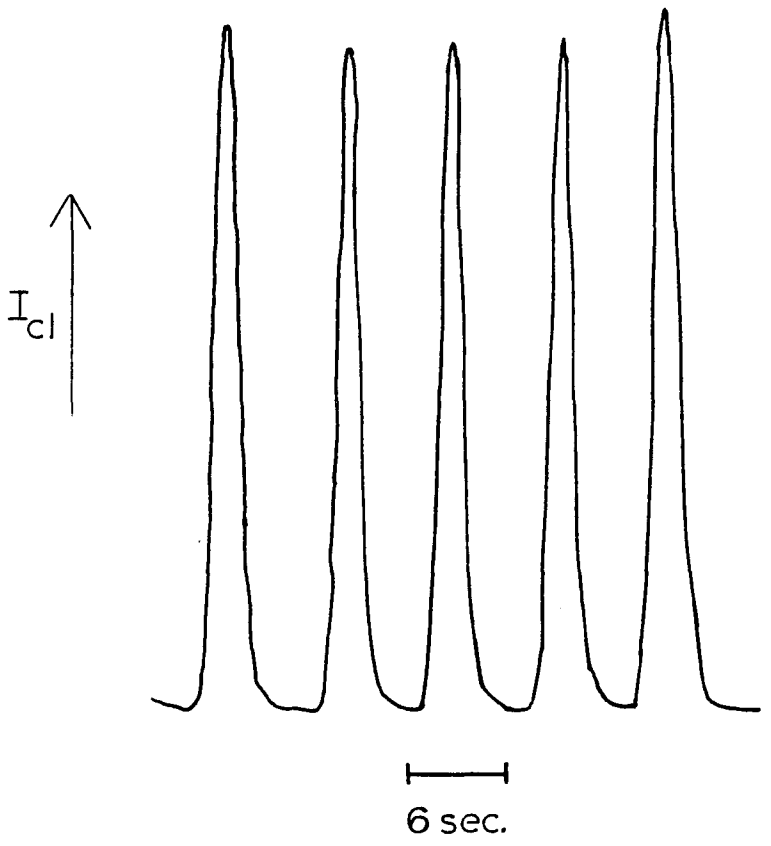

Figure 2-7. Replicate injections of 10^{-6} M Cr^{3+} in 0.4 M H_2O_2, titrated with KOH to pH 3-4.

Table 2-1

Results of experiments designed to identify the source of interference in the CL-FIA system for Cr(III) determination.

Standard Solution Contents	Interference Present
d/d water	no
KOH solution in d/d H_2O	no
d/d H_2O + H_2SO4, to pH 3-4	yes
d/d H_2O + Ultrex H_2SO_4, to pH 3-4	no
d/d H_2O + $K_2Cr_2O_7$	no
d/d H_2O + EDTA	no
d/d H_2O + Ultrex H_2SO_4 + $K_2Cr_2O_7$	yes

No interference means that no significant CL signal was observed with that given combination of components. d/d H_2O = deionized/distilled water.

interference problem. This interference was eliminated by heating the samples after EDTA addition, indicating the presence of Cr(III) as the interference. Apparently, some Cr(VI) was being reduced in the acid solutions.

The samples from the AAEC were treated with EDTA and base to pH 3-4 and injected into the CL-FIA system to see if any meaningful results would be obtained. All samples containing dichromate and either concentration of sulfuric acid (0.04 or 0.4 M) gave extremely high CL intensities, even solutions that had not been irradiated and were expected to contain no Cr(III). Meaningful quantitation of Cr(III) was not possible with any of the samples. Blank solutions containing no dichromate were included with the AAEC samples. These were simply solutions of sulfuric acid. These solutions with no dichromate were the only samples that did not give inordinately high CL signals.

Work on this project was suspended when it was realized that none of the samples received from the AAEC would give meaningful results in the CL-FIA system. Apparently, interaction between dichromate and sulfuric acid caused some reduction of Cr(VI). Although the CL-FIA method was not successful in this situation and was not characterized further, it is expected that it will perform quite well for many other less confounding sample matrices. Compared to previous methods for CL determination of Cr(III), this method offers the advantages of much simpler instrumentation, the ability to use significantly smaller sample sizes, and high sample throughput (on the order of 240 samples/hour). Provisions could easily be made for having EDTA mix with the sample in the flow system, eliminating the need for manual addition and mixing of EDTA. This would also offer more reproducible timing between EDTA

addition and CL detection. The detection limit was estimated to be on the order of 1 nM Cr(III).

Glucose Determination Using FIA with CL Detection

Introduction

The determination of blood glucose is one of the most frequently performed clinical assays (77), accounting for more than 20% of all chemical analyses performed by the average hospital lab (77). In addition, many more are performed in clinics and independent labs. It should be obvious, therefore, that clinical laboratories seek methods for glucose that not only offer a high degree of accuracy and precision, but also high sample throughput, easy operation and maintanance, and reliability.

This section describes a method for glucose that offers several advantages. The method, which is based on the use of FIA with CL detection, exhibits the high sensitivity and wide linear dynamic range typical of CL analyses and the high sample throughput, easy operation, and adaptability to automation characteristic of FIA. Instrumental requirements and cost are minimal. In addition, the method offers the specificity of an enzyme reaction.

Principles

The enzymatic oxidation of glucose by glucose oxidase (E.C.1.1.3.4) can be coupled to the luminol reaction, generating CL that will be proportional to glucose concentration. The glucose oxidase reaction produces gluconic acid and hydrogen peroxide. The enzyme is highly specific for β-D-glucose. The hydrogen peroxide generated by the enzyme reaction is allowed to react with an excess of luminol and catalyst/

cooxidant to produce CL. The overall scheme of the coupled system is shown in Figure 2-8.

Some methods based on this principle have been reported (78, 47, 52, 53, 49, 54), although none have made use of FIA. The method of Auses, Cook and Maloy (53) suffers most noticeably from a lack of convenience, since samples must be injected individually into a photometer, resulting in low sample throughput. The flow system described by Bostick and Hercules (49) uses an immobilized enzyme column and has been optimized in terms of reagent concentrations and other conditions, but is slower and more complicated than the FIA system. A recently reported method (78) takes an interesting approach by using a pressure gradient between reagent and analyte solutions to induce diffusion of reagent into the analyte solution, but seems to offer no advantages over the FIA method.

Combining the glucose oxidase-luminol coupled reaction with FIA results in a system that is simple, inexpensive (in terms of both equipment and reagent costs), reliable, easy to operate, and could easily be automated. FIA provides the controlled dispersion important for CL methods. Finally, immobilization of the glucose oxidase allows reuse of the enzyme for many analyses, significantly reducing reagent costs. Increased enzyme stability is another important advantage of immobilized enzymes. Tethering enzymes to Controlled Porosity Glass beads and packing them in a column permits easy utilization in a flow system. It would be an especially convenient arrangement for adaptation to automation.

Experimental

Apparatus. The flow system used for some early experiments with

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{G.O.}} \beta\text{-D-gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{luminol} \xrightarrow[\text{base}]{\text{catalyst}} \text{C.L.}$$

Figure 2-8. Reaction scheme for glucose determination using luminol chemiluminescence. G.O. = Glucose Oxidase.

the Cu(II) catalyzed luminol reaction is diagrammed in Figure 2-9. Two 50 mL plastic syringes (A) held the reagent solution which contained 0.2 mM luminol, 0.03 M Cu(II) and approximately 1.2 M NH_3. The solutions were buffered to pH 10.4. The reagent syringes were mounted in a Harvard Apparatus model 975 compact infusion pump. A reagent flow rate of 8.6 mL/min/syringe provided the best sensitivity and precision. The sample syringe was mounted in a Sage model 255-1 infusion pump operated at 3.2 mL/min. Teflon tubing, 0.79 mm i.d., was used throughout the flow system. The glucose oxidase column packed length was approximately 8.5 cm, in 3 mm i.d. plastic tubing. A pneumatically actuated Altex slider valve was used for sample injection into a 107 uL sample loop.

The instrumental arrangement for the ferricyanide catalyzed system is illustrated in Figure 2-10. A three channel Cole-Parmer peristaltic pump (model 7545) fitted with model 7014 pump heads was used to pump the luminol, ferricyanide and carrier lines. Cole-Parmer - Tygon tubing 6408-43 was used in the pumps; all other conduits were 0.79 mm i.d. Teflon tubing obtained from Altex. Sample flow from 50 mL plastic syringes was provided by a Sage model 255-1 infusion pump. The glucose oxidase column was approximately 6.5 cm long (packed length) in 3 mm i.d. plastic tubing. Glass wool plugs kept the column packing in place. Sample was injected from a sample loop by a pneumatically actuated Rheodyne 4-way rotary valve. The volume of the sample loop was approximately 100 μL.

A mixing coil was included to insure adequate mixing of the luminol solution with the cooxidant solution. These reagents were mixed in the flow system because a single solution containing both luminol and the cooxidant is not stable. The length of the mixing coil was approximately

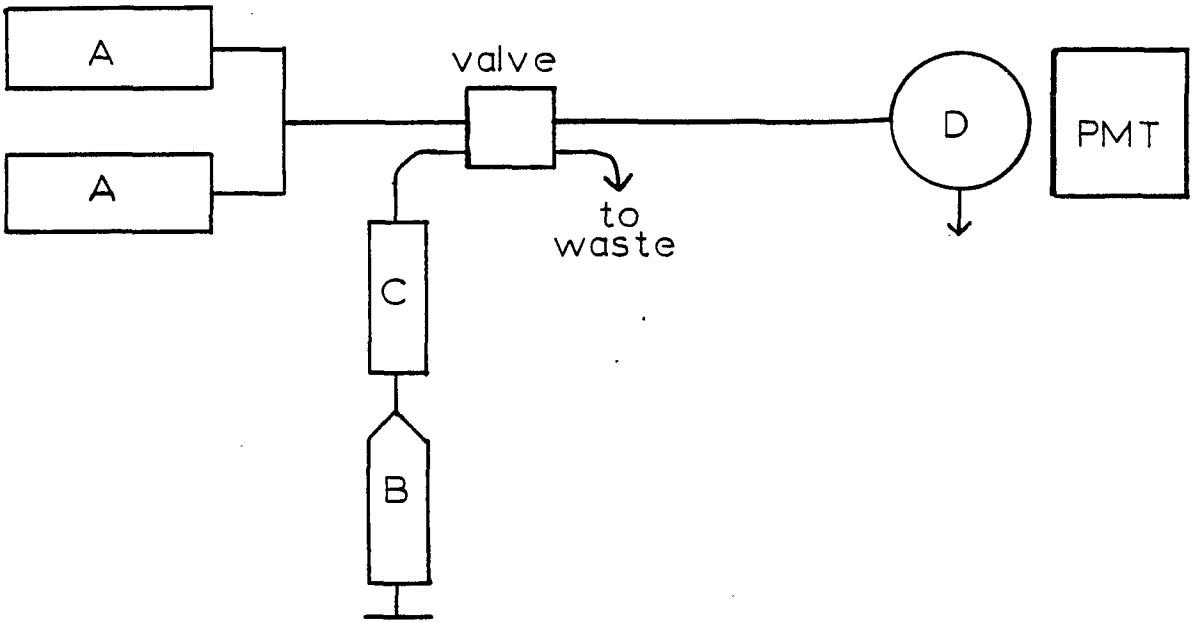

Figure 2-9. FIA system for Cu(II) catalyzed luminol system for glucose determination. A: luminol-Cu(II)-NH_3 solution; B: sample solution; C: immobilized glucose oxidase column; D: tubing coil mounted in front of photomultiplier.

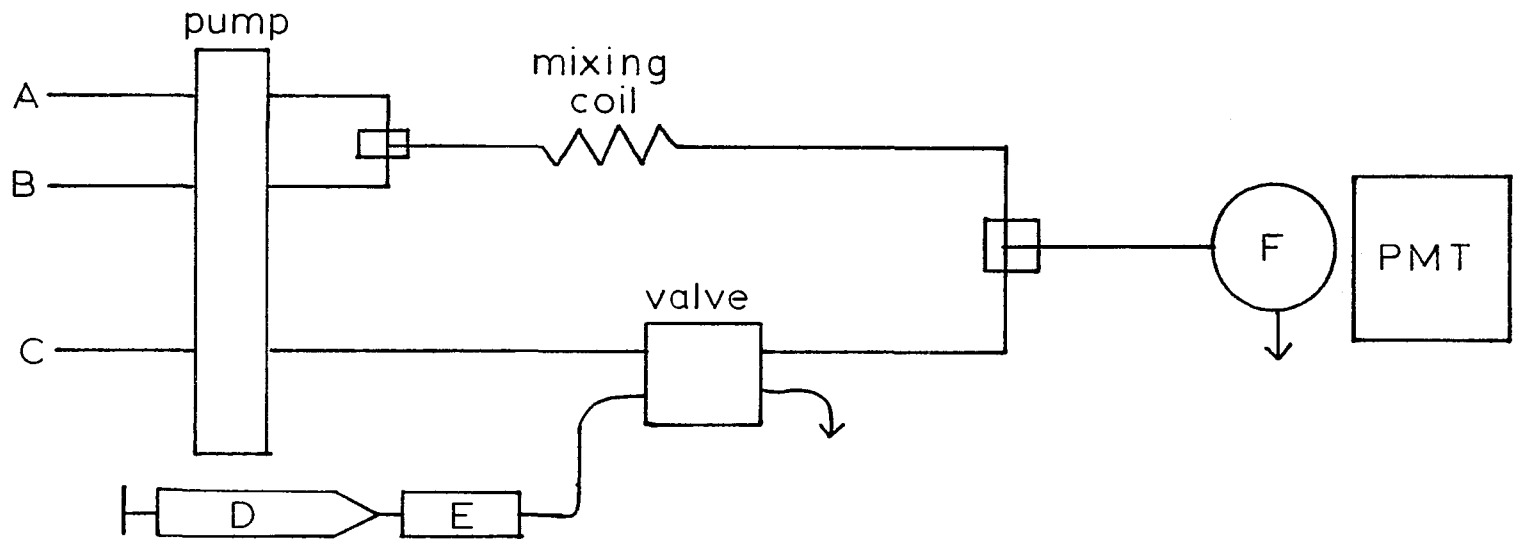

Figure 2-10. FIA system for $Fe(CN)_6^{3-}$-catalyzed system for glucose determination. A: ferricyanide solution; B: luminol in boric acid buffer; C: acetate carrier buffer; D: sample solution; E: immobilized glucose oxidase column; F: tubing coil mounted in front of photomultipier. The pump for solutions A, B, and C is a 3-channel peristaltic pump. The sample (D) was pumped by a single-channel infusion pump.

38 cm. The length of tubing from the sample injection valve to the T-fitting where sample and reagent solution joined was 2.3 cm. The length of the tubing coil in front of the PMT was about 38 cm. The PMT, electronics, and recorder were the same as used for the Cr(III) determinations.

Reagents. Luminol was used as received from Aldrich. Working solutions of luminol in 0.1M boric acid/KOH (Baker) buffer, pH 10.8, were prepared from luminol powder. Luminol solutions were allowed to stand for at least 24 hours prior to use. In some initial experiments an ammonia buffer was used instead of the borate buffer.

Potassium ferricyanide solutions (0.01M) were prepared immediately before use by dissolving crystals (Baker) in deionized/distilled water. Copper(II) solutions were prepared by dissolving copper nitrate crystal (Baker).

The carrier solution was 0.01M acetate buffer, pH 5.5, which is the pH of optimum glucose oxidase activity (79). The 0.01M acetate buffer also served as diluent for the preparation of standards and samples. The acetate buffers contained 0.1% sodium benzoate to inhibit microbial growth.

Glucose oxidase from Aspergillus Niger was obtained from Sigma. The enzyme was covalently immobilized on aryl-amine derivatized controlled porosity glass (CPG) beads, 20-80 mesh, obtained from Electronucleonics.

β-D-Glucose (Baker) was used to prepare stock solutions of approximately 100 mg/dL glucose. Standards were prepared by dilution of the stock solutions with 0.01M acetate buffer.

Human plasma samples containing sodium fluoride and potassium oxalate were obtained from the chemistry laboratory of Wentworth-Douglas Hospital in Dover, NH. Fluoride ion helps stabilize blood glucose concentration by preventing glycolysis; oxalate is used as an anticoagulant.

Distilled/deionized water was obtained by passing house deionized water through a mixed bed ion exchange column, followed by distillation in a Corning MP-1 still.

Procedures. Aliquots of glucose stock solutions were diluted with 0.01M acetate buffer for the preparation of standard curves. Plasma or serum samples were diluted 500-fold for the Cu(II) catalyzed systems and 1000-fold for ferricyanide systems, with acetate buffer as diluent. Samples and standards were placed in 50 mL plastic syringes forpumping through the glucose oxidase column and subsequent injection.

Flow time of the sample pump was timed; at the end of the appropriate time interval (which was determined by flow rate), the sample pump was stopped and sample was immediately injected into the flowing carrier stream. The carrier and reagent lines were allowed to flow continuously.

As soon as the CL signal was recorded, the valve was manually reset to the "load" position and sample pump flow was resumed. Replicate injections could be made as frequently as every 15 seconds. Changing samples merely required changing syringes in the sample pump and connecting the new syringe to the flow system. The glucose oxidase column was rinsed with acetate buffer for 1-2 minutes between samples.

The procedure for immobilization of glucose oxidase was adapted from Weetal and Filbert (80). Aryl-amine was converted to a diazonium

chloride, followed by covalent attachment of glucose oxidase through the diazo linkage. The diazonium salts will react with phenolic compounds and other heterocycles (80). The derivatized CPG is bright orange, reflecting the presence of the diazo chromophore.

The diazotization was carried out in an ice bath. Five mL of 2N HCl and 120 mg of solid sodium nitrite were added to a 25 mL Erlenmeyer flask containing 500 mg of aryl-amine CPG. The flask, in an ice-filled beaker, was placed in a vacuum dessicator and aspirated for about 20 minutes to remove air and dissolved gasses from the pores of the CPG.

The diazotized beads were recovered by filtration, washed with about 250 mL of cold deionized/distilled (d/d) water, and placed in a 25 mL Erlenmeyer flask containing 10 mL of a 5 mg/mL glucose oxidase solution adjusted to pH 8-9. The flask was then refrigerated. Incubation, with occasional swirling, was continued for at least 2 hours.

After incubation, the enzyme-containing CPG was recovered by filtration, washed with at least 250 mL of cold d/d water and stored at 4^{o} C in 0.1 M phosphate buffer, pH 7, or d/d water.

The columns were packed as a slurry and then stored at 4^{o} C. When in use, however, they were often at room temperature for as long as 10 hours at a time. Packed columns were stored with syringes on both ends to prevent the entry of air. Immobilized glucose oxidase proved to be quite stable. In spite of being left at room temperature for many hours at a time, columns could still be used for analyses 2.5 years after their preparation.

Results and Discussion

Choice of catalyst/cooxidant. Initial work was undertaken using a Cu(II)-NH_3 catalyst. This system, buffered with ammonia to pH 10.4, was similar to that used by Seitz and Rule for the determination of hydrogen peroxide in a FIA system (10). Reagent and sample flow rates were optimized, but the results obtained for blood glucose analysis with this system were not encouraging. Although calibration curves constructed with aqueous glucose standards over a limited concentration range (1 ug/mL to 6 ug/mL) were reasonably linear, results obtained for blood glucose determinations did not agree well with reported concentrations of glucose in the serum samples used for correlation studies. These sera were analyzed by the United Technologies Clinical Lab, East Hartford, CT. CL-FIA results were roughly 50% of the reported values, as shown by some typical data in Table 2-2. As a check on the values reported for the sera, glucose determinations using Sigma glucose determination kit 15-UV were performed. It should be stressed that no replicates were run, but the results did agree more closely with the reported values than with the CL-FIA results.

Some brief experiments provided evidence that uric acid was apparently an interference in the Cu(II) catalyzed luminol system for glucose. Three identical standard solutions of 1.7 μg/mL glucose in 0.01 M acetate buffer were prepared. To one solution was added uric acid to a final concentration of 0.045 μg/mL. Sodium chloride was added to the second glucose solution. The third glucose solution served as a control. These concentrations represent typical serum values for the various species assuming a 500-fold dilution for each sample. The results, summarized in Table 2-3, clearly show the detrimental effect of

Table 2-2

Comparison of results of serum glucose determination for the CL-FIA method, the results reported by the United Technologies Clinical Lab and results obtained using Sigma glucose kit #15-UV which is based on formation of NADH and its measurement by absorbance at 340 nm.

[Glucose] CL-FIA	[Glucose] "reported"	[Glucose] A_{340}
50 mg/dL	94 mg/dL	97 mg/dL
35	79	63
45	91	86
46	90	89
40	91	66
34	69	61

The CL-FIA data are from the Cu(II)-catalyzed luminol system.

Table 2-3

Results showing uric acid interference in the Cu(II)-catalyzed reaction for glucose determination in the CL-FIA system.

[Glucose]	[Uric Acid]	[NaCl]	Peak Height
1.7 ug/ml	0	0	5.1 cm
1.7	0.045 ug/ml	0	2.9
1.7	0	3% w/v*	5.2

* before 1000-fold dilution

adding uric acid. The signal from the uric acid-containing solution is about 57% of the signals for samples without uric acid which is approximately the same percentage difference seen between reported serum glucose levels and CL-FIA results.

It was decided that it would be more fruitful to try a different catalyst/cooxidant than to investigate the interfence problem further. The Cu(II) system was abandoned in favor of a ferricyanide catalyzed system. Bostick and Hercules present a discussion of the problems associated with Cu(II) catalysis for luminol-based analyses (49).

Ferricyanide was chosen as the catalyst/cooxidant for subsequent work because of its demonstrated behavior in luminol-based CL analytical systems (5, 47, 49, 53, 54) Ferricyanide systems show linear response to hydrogen peroxide over a wide range of peroxide concentrations, provide good sensitivity, and have sufficiently fast kinetics to be useful in FIA. Reagents and reaction products are readily soluble. The high level of background emission due to the reaction of ferricyanide, luminol and oxygen is sufficiently stable so that it can be reliably subtracted.

Choice of buffer system. Initial experiments with the ferricyanide catalyzed luminol reaction were carried out using an ammonia buffer at pH 9.5 as diluent for the reagents. These conditions were adapted from the work of Auses, Cook and Maloy (53). Luminol concentration was 2mM in a 0.3 M ammonia buffer. Ferricyanide concentration was 1mM. A 0.3M ammonia solution was used as the carrier in the third channel of the pump. Acetate buffer was used as diluent for samples and standards.

Some standard additions experiments were conducted using the ammonia-buffered luminol reagent. Serum samples were diluted 0.003 to 1.

Aliquots of an aqueous glucose standard were used to make the standard additions.

Typical results for a standard additions experiment are plotted in Figure 2-11. The linear least squares fit of the lines is good, but there is a large difference in the slopes, indicating the presence of an interference in the serum matrix. The exact nature of the interference was not investigated, and the ammonia buffered system was abandoned in favor of a borate buffered system.

All work discussed in the remainder of this chapter was carried out using 0.1 M borate buffers for dissolution of the luminol reagent.

pH. The luminol-ferricyanide reaction exhibits fairly constant response with pH variations between 10.4 and 10.8 (49). A pH of 10.8 provided satisfactory results and was used for all analyses. The high end of the pH plateau was chosen because the pH will decrease slightly when the luminol-ferricyanide reagent mixes with the acetate buffer.

Luminol concentration. It has been shown that CL intensity increases dramatically with luminol concentration using the ferricyanide system (49). There is an increase in CL response over the range 10 μM to 0.01 μM luminol, but this occurs at the expense of reproducibility. For the FIA system, a luminol concentration of 27 mg/L was found to give the most reproducible, least noisy results. This concentration was used for the glucose determinations. Higher luminol concentrations resulted in a higher and noisier baseline and poor precision for replicate injections of standard glucose solutions.

Reagent flow rate. CL response for a standard glucose solution was recorded for a series of reagent flow rates between 7 and 24 mL/min/

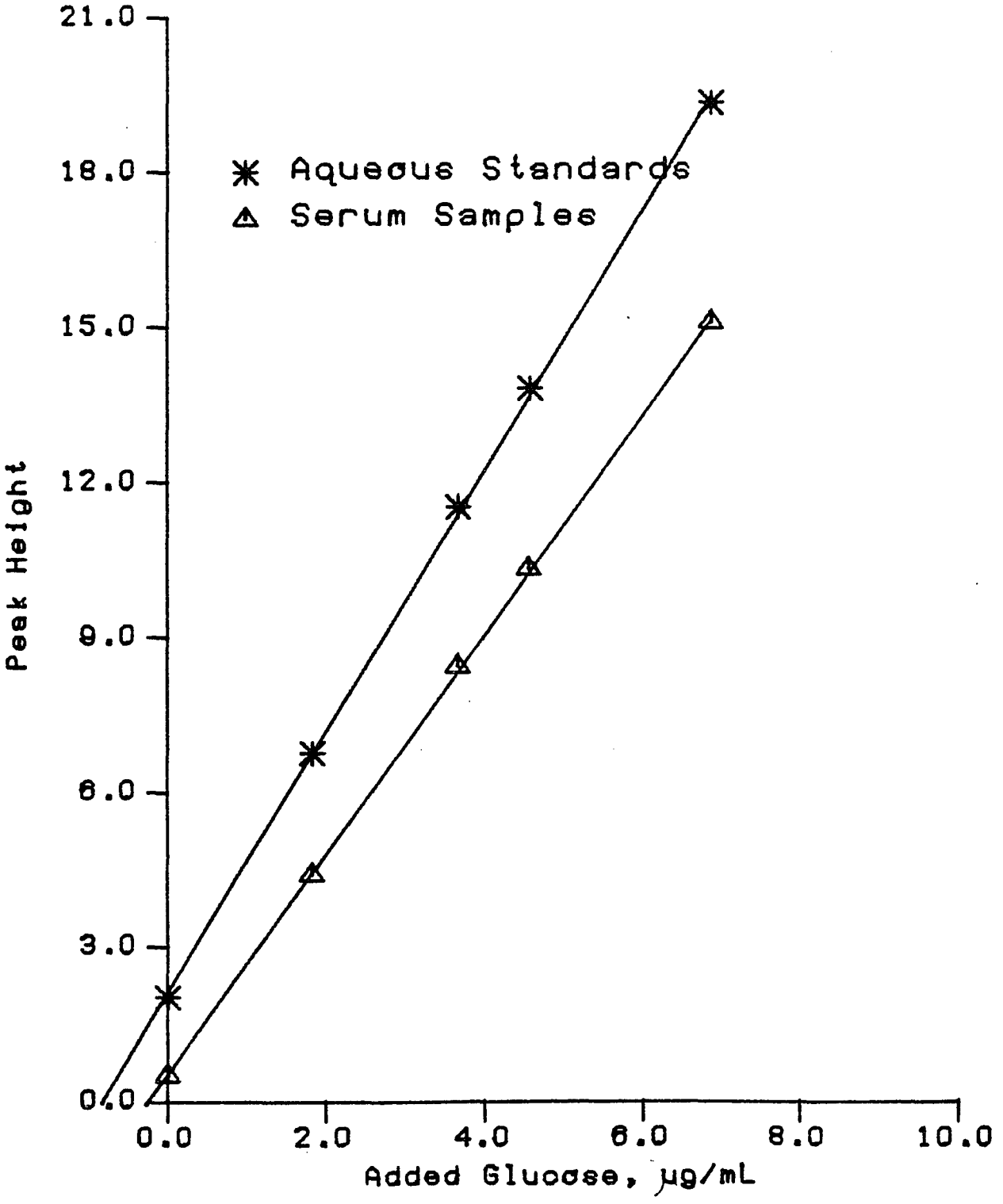

Figure 2-11. Standard additions curves for aqueous glucose solutions and serum standards. Difference in slopes of the lines indicates presence of an interference.

channel. Reagent flow rates between 12 and 24 mL/min/channel showed no significant differences in either signal intensity or reproducibility. Below 12mL/min/channel, the peaks were smaller and more poorly shaped, and sample throughput was reduced considerably because the peaks were excessively wide. A reagent flow rate of 12 mL/min/channel was chosen for the glucose analysis. Above that flow rate there were no gains in sensitivity, sample throughput or precision to justify the added reagent consumption.

The flow rates used here are considerably higher than those usually employed in FIA systems. This reflects the unique nature of CL detection which measures light from the reaction as it occurs. With CL detection, it is not necessary to allow the analytical reaction to go to completion. This differs from other forms of FIA that rely on concentration sensitive detectors.

Sample flow rate. Studies were conducted to determine the effect of varying the sample flow rate through the immobilized enzyme column and the length of time the sample was allowed to flow through the column. Sample flow times below 15 seconds were too short to provide useful results without resorting to excessively high flow rates that would drastically increase sample consumption. The sampling rate of the method was determined by the sample flow rate. The measurement of CL in the flow system was fast relative to sample flow time in the column. Flow rates of 2.3 mL/min for 20 seconds and 3.4 mL/min for 15 sec provided good sensitivity and precision, while keeping sample consumption within reason. Some typical data are shown in Table 2-4. A flow rate of 2.3 mL/min provided a good compromise between sensitivity and sampling rate, and was used for many of the plasma analyses.

Table 2-4

Intensity and precision for glucose determination at different sample flow rates through glucose oxidase column.

Sample Flow Rate	Sample Flow Time	Mean Peak Height	RSD
3.4 ml/min	15 sec	9.5 cm	0.8%
2.8	20	10.0	0.8
2.8	30	9.7	1.6
2.3	30	10.2	-
2.3	20	10.2	1.0
2.3	15	9.8	0.7

However, good results can still be obtained using a flow rate of 3.4 mL/min for 15 seconds, with a gain in sample throughput.

A set of experiments at sample flow rates between 2.3 and 3.4 mL/min with standard glucose and hydrogen peroxide solutions indicated that approximately 64% of the glucose originally added to the standard solution was converted to peroxide in the glucose oxidase column. When dissolved in aqueous solution, β-D-glucose will form an equilibrium mixture of approx 64% β-D-glucose and 36% α-D-glucose (81). Glucose oxidase is specific for the β form of D-glucose, so the experiments indicate essentially complete conversion of the β-D-glucose present in the equilibrium mixture to peroxide. The equilibrium of the glucose anomers will be disturbed as β-D-glucose is converted to gluconic acid, but apparently this is not an important effect at these flow rates.

Effect of the age of the reagents. Ferricyanide solutions were prepared immediately before use. Because ferricyanide solutions are unstable when exposed to bright light, 125 mL portions were dispensed into the ferricyanide reagent reservoir as necessary. The bulk of the solution was stored in the dark to prevent any decomposition during the course on an experiment.

Acetate buffers at pH 5.5 are highly susceptible to bacterial growth. These solutions were preserved with 0.1% sodium benzoate and used within 48 hours of preparation. Bacterial contamination could cause consumption of the glucose in the samples, and have detrimental effects on the column.

Luminol solutions were allowed to stand for at least 24 hours before use. Fresh luminol solutions show higher response to peroxide, but also have higher and noisier background emission in the absence of

peroxide. The precision of replicate injections of glucose standards was not as good for fresh luminol solutions as it was for aged solutions. Intensity due to peroxide may be lower, but the baseline is much more stable and precision of replicates improves considerably. After the first 24 hours, emission from luminol solutions is quite stable with time for over a week. This behavior is common for luminol solutions (53,82).

Loss of signal intensity with time. A decrease in CL intensity with time was sometimes observed if a given glucose sample was run several times during the course of an experiment as a control (Table 2-5). The signal loss was not observed within the time span of a set of as many as 12 replicate injections, however. Because of this effect, it was especially important to run samples randomly, and include frequent injections of a control standard. Typically, a control was run after every four or five samples. The reason for this signal loss over time is not completely understood. Luminol solutions would not appear to be at fault, since only aged (but not excessively old) solutions were used. The problem appears to occur in the glucose oxidase column. Washing the column with 0.01 M acetate buffer for 1 to 2 minutes between samples seems to reduce the effect. This effect repesents the only major problem with the technique at present.

First peak anomaly. In all cases it was observed that the first injection of a set of replicates resulted in a considerably lower intensity than the average for all the other replicates in a given set. This phenomenon is attributed to conditioning effects in the enzyme column. Carryover from rinse solutions in the column, sample loop, and

Table 2-5

Signal loss with time during Glucose determination in the ferricyanide catalyzed CL-FIA system

Sample	Mean Peak Height
control	5.81 cm
7 different samples, 5 rep's each	-------
control	5.77
8 samples, 5 rep's each	-------
control	5.45

Samples and controls were run in the order shown in the table. This data actually represents one of the smallest decays observed. In all cases the glucose oxidase column was rinsed with acetate buffer for about two minutes between each sample. All samples and controls were run with 5 replicates each. The control solution was a 100 µg/mL glucose standard solution made in acetate buffer.

valve may also contribute. No efforts were made to identify or eliminate the cause of the effect, since it appeared to cause no problems. As a matter of convention, first injections were never used for the calculation of any results. An example of this effect can be seen in Figure 2-12.

CL Determination of Blood Glucose

Glucose standard solutions in 0.01 M acetate buffer, pH 5.5, were used to construct a calibration curve. Because of the reproducible mixing characteristics of FIA, the peak height could be related directly to concentration. No integration was necessary. Figure 2-13 shows a typical set of replicate injections of a standard glucose solution. Figure 2-13a, obtained using a faster than normal chart speed, shows the smooth shape of the CL emission peak in the FIA system. Typical width of the peaks at half height was 1.2 seconds. The slight tailing is typical of FIA signals because of the nature of the dispersion in the flowing stream.

For routine measurements, the chart recorder was run at 0.5 in/min, resulting in the narrow spikes shown in Figure 2-13b.

Precision of replicate injections of any given glucose standard solution was typically 0.8 to 1.2% RSD, using the pneumatically actuated Rheodyne valve. Precision was not this good with the Altex slider valve, hence its replacement with the Rheodyne valve. Glucose concentrations below about 0.75 mg/mL tended to have slightly higher standard deviations among replicates than the more concentrated samples. The detection limit for glucose is estimated to be approximately 0.05 µg/mL (using calculations based on three times the standard deviation of the blank signal). The system exhibits linear response to glucose

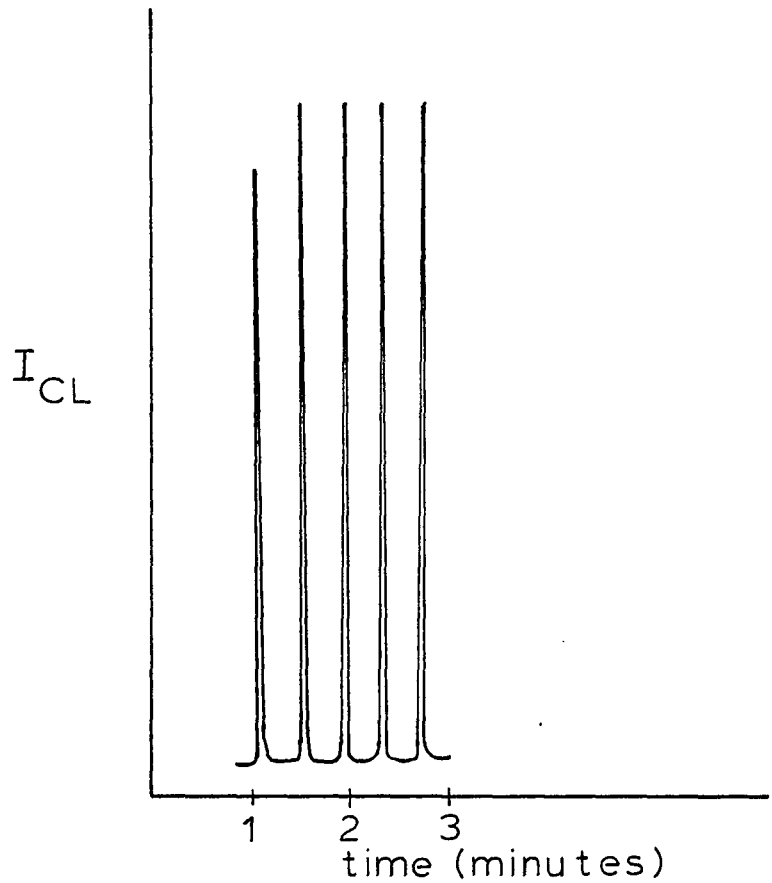

Figure 2-12. Typical CL-FIA data showing "first peak anomoly" (see text).

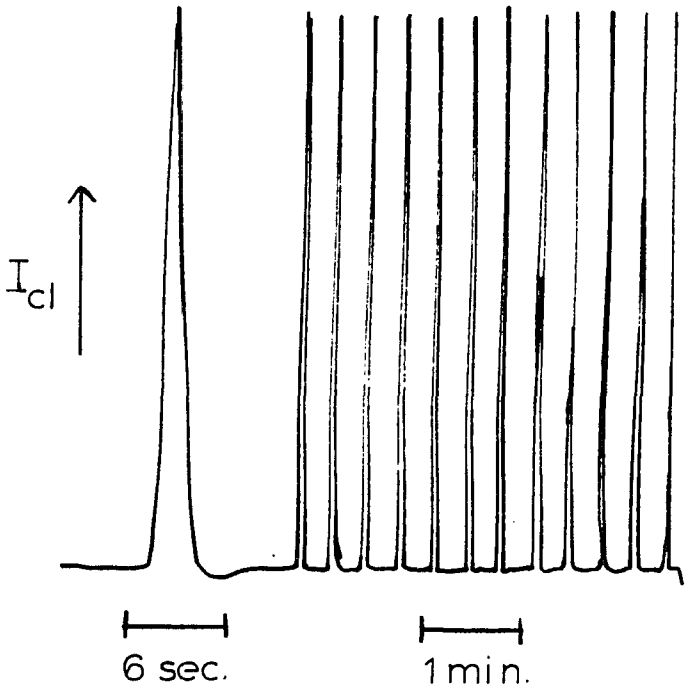

Figure 2-13. Typical data for glucose determination. Data shown are replicate injectons of a 0.85 μg/mL glucose solution. Intensity is on an arbitrary scale. The first peak was run at a high chart speed to show peak shape more clearly. The second set shows the type of data normally taken for analysis of peak height.

concentration from the detection limit to at least 5 μg/mL. This may seem like a small linear range, but based on the 1000-fold dilution used for blood samples, this range corresponds to a blood glucose range of roughly 40 mg/dL to 500 mg/dL which easily covers the normal range of blood glucose levels of 60 to 100 mg/dL (77), and includes a wider range of abnormal values than is usually encountered clinically. A typical standard curve is shown in Figure 2-14.

A correlation study with human plasma samples was performed to compare the accuracy of the CL-FIA method for glucose determination to that of a commercially available glucose assay kit. Plasma samples, previously treated with sodium fluoride and potassium oxalate, were diluted by a factor of 1000 with 0.01 M acetate buffer, pH 5.5. No other pretreatment was employed. Glucose concentrations were calculated from the standard curve prepared with the aqueous glucose standard solutions. At least five successive replicate injections were made for each sample to evaluate the precision of the measurement step of the procedure.

Reference values for the glucose concentration of the plasma samples were obtained using a Sigma glucose determination kit number 15-UV. This method is based on the use of hexokinase and glucose-6-phosphate dehydrogenase, as outlined in Figure 2-15. The NADH produced by these reactions has a high molar absorptivity at 340 nm and was determined spectrophotometrically in a Shimadzu 200-UV spectrophotometer. The procedure is described in the instruction bulletin furnished with the kit (83). Most samples were analyzed in triplicate; some samples were run in quadruplicate. Average precision was approximately 5% RSD.

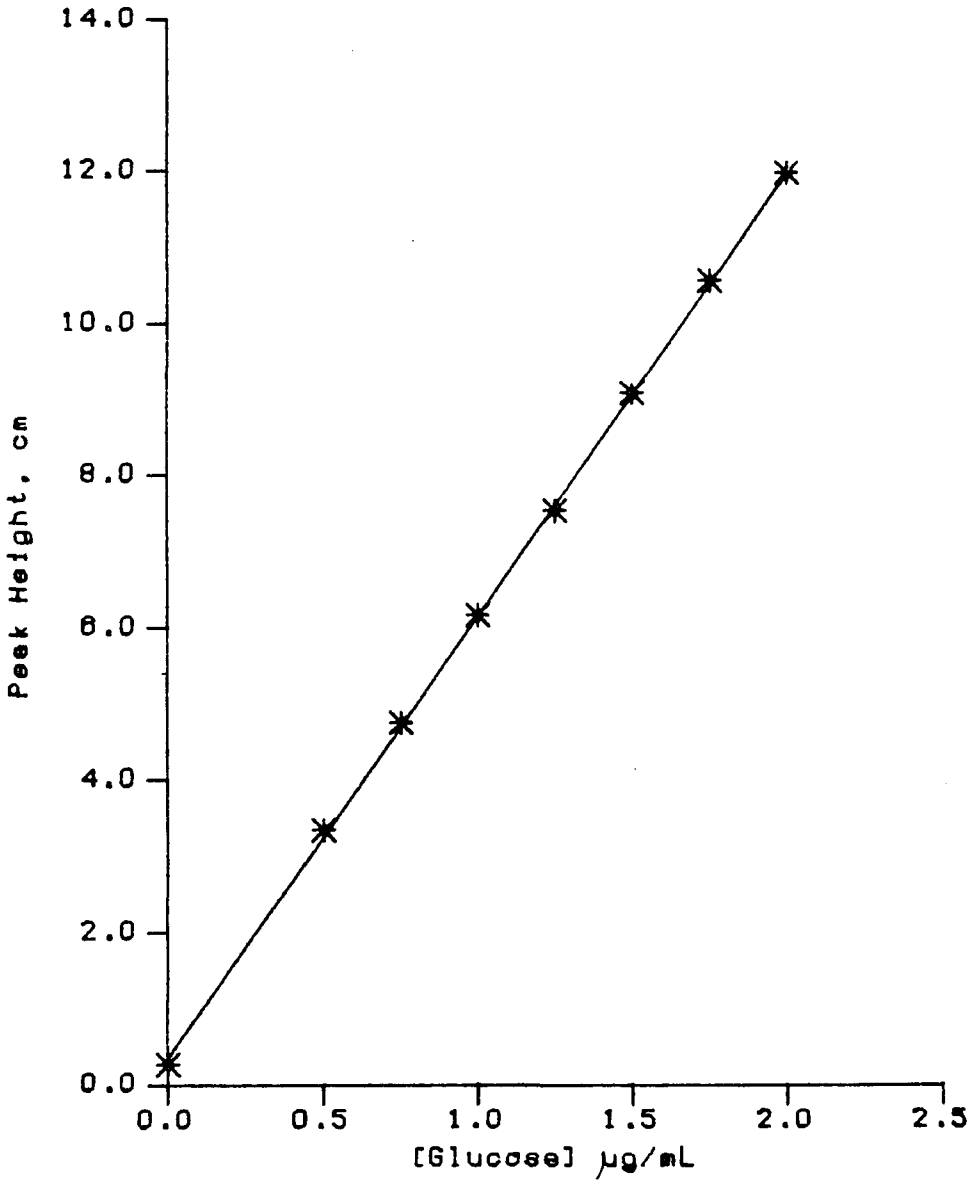

Figure 2-14. Standard curve for glucose in CL-FIA system.

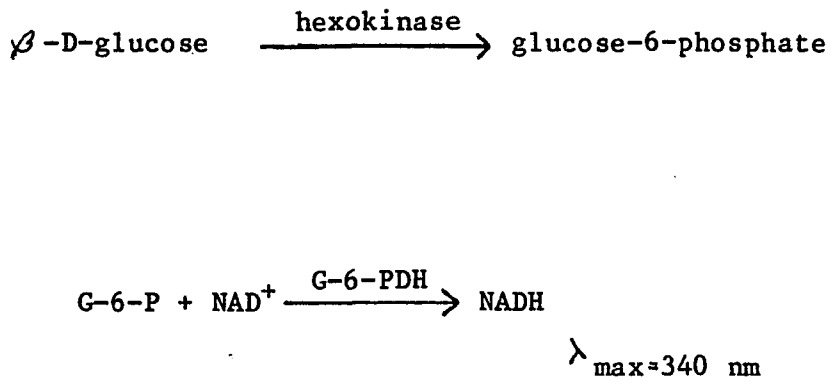

Figure 2-15. Reaction scheme for glucose determination method used as reference method in correlation studies (Sigma Kit 15-UV). G-6-PDH = glucose-6-phosphate dehydrogenase.

The results of the correlation study are shown in Table 2-6 and they are plotted in Figure 2-16. The line drawn is a linear least squares fit. There seems to be no consistent bias of the CL results high or low relative to the spectrophotometric results. In general, the two methods agree reasonably well. The precision for replicate injections of plasma samples in the CL method was typically 0.8 to 2.0% RSD. On the average, the two methods agree within about 5%; in no case do the methods differ by more than 12%. A t-value of 2.0 can be calculated by using the difference between each of the paired measurements. The tabulated critical value of t for 10 degrees of freedom and 95% confidence is 2.228. Therefore, at the 95% confidence level, there is no difference between the values obtained by the two methods (84).

These results are better than some previously reported work (53) based on the use of glucose-water standards. It is possible that the results from the CL-FIA method could be improved somewhat if clinical standards were used instead of the glucose-water standards. Adequate rinsing of the glucose oxidase column is important, especially in reducing the tendency toward loss of signal intensity over time. Deproteination of blood samples would probably help eliminate some problems by reducing column fouling due to the presence of blood proteins. Bostick and Hercules (49) used a deproteination step that improved their results, but this added step was inconvenient.

Conclusions

This work has shown that the CL-FIA method has the potential to be quite useful and convenient for the routine determination of blood glucose. The method has a low detection limit and a sufficiently wide

Table 2-6

Correlation Study: Blood Glucose Determination

Plasma	[Glucose] Hexokinase	[Glucose] CL-FIA*
1	131 mg/dL	136 mg/dL
2	148	144
3	71	74
4	120	125
5	99	87
6	102	92
7	99	91
8	138	134
9	131	127
10	77	70
11	81	79

* Results obtained using the ferricyanide-catalyzed system.

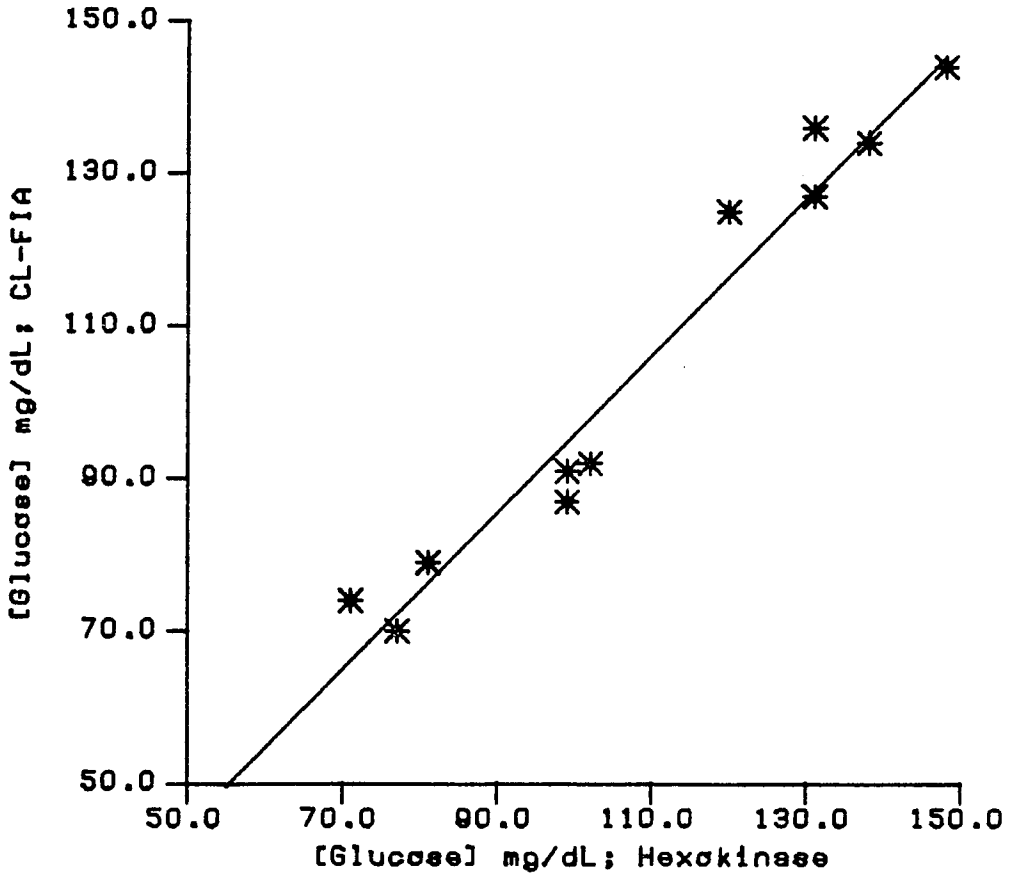

Figure 2-16. Results of a correlation study comparing results for blood glucose determination by CL-FIA and a reference method (Sigma glucose determination kit 15-UV).

linear range to accomodate the entire range of blood glucose concentrations normally encountered clinically. Sample preparation is simple, involving only dilution and perhaps deproteination. The large dilution factor helps reduce the effects of some interferences present in blood, such as reducing species like uric acid. The method is highly specific for β-D-glucose because of the specificity of glucose oxidase. Immobilization of glucose oxidase permits reuse of the enzyme for many analyses, and the enzyme is stabilized by the immobilization. The immobilization procedure is simple, and because the enzyme columns remain active for years, reagent costs are very low.

Instrumental requirements are minimal, and the system could easily be automated. There are few moving parts, and because of the simplicity of the apparatus, trouble-shooting and repair should be easily and quickly accomplished, resulting in less down-time. A deproteination step could be added to an automated system, perhaps as a precolumn before the glucose oxidase column. Sampling rates are as high as 240 samples/hour.

The CL-FIA system is at least as simple, fast, and easy to operate as previously described systems based on luminol CL (78, 53, 49). Results for glucose determination, based on the use of glucose-water standards, are comparable.

To increase the versatility of the CL-FIA approach, it should be possible to construct a system with more than one sample injection valve and thus increase the sampling rate. In the present system, sample throughput is limited by the time required for the sample to go through the column, not by the FIA measurement step. With a multi-valve system it would also be possible to perform more than one type of assay at a

time in the same flow system. For example, a second column containing an immobilized enzyme that catalyzes peroxide production from some other clinically important analyte could be incorporated into the CL-FIA system. The peroxide produced in this second column would then be measured in the flow system the same way the peroxide from the glucose oxidase reaction was measured. Systems combining the CL-FIA approach with automated sampling and data handling would seem to hold considerable promise as useful clinical techniques.

CHAPTER 3

TWO PHASE CHEMILUMINESCENCE

Introduction

The subject of this chapter is a two phase flow system in which the CL reagents and the analyte are in solutions separated from each other by a membrane. Although some work with two phase CL systems has appeared (2, 18, 78, 85), it is a fairly new approach to CL analysis. In a heterogeneous system, the CL reagent or enzymes catalyzing CL are immobilized by entrapment behind a membrane permeable to analyte but not to the larger CL reagents or enzymes. Analyte solution is delivered to the opposite side of the membrane by a flow system from whence it diffuses across the membrane into the reagent phase where it can take part in the CL reaction. The system is outlined schematically in Figure 3-1. This two phase flow system is an extension of the FIA approach to CL-based assays since the reagent phase can serve as a specific detector of compounds generated in the flow injection system.

A two phase system offers several advantages over the single phase FIA described in earlier sections, especially for the firefly and bacterial BL systems, while retaining many of the advantages of FIA. One of the advantages of the two phase system is that the immobilized reagent can be reused for many analyses. This is an important advantage when using the enzymes necessary for bacterial or firefly BL. Immobilization by entrapment behind a membrane is not only convenient but also allows the reuse of enzymes that are difficult to immobilize chemically without severe losses in activity.

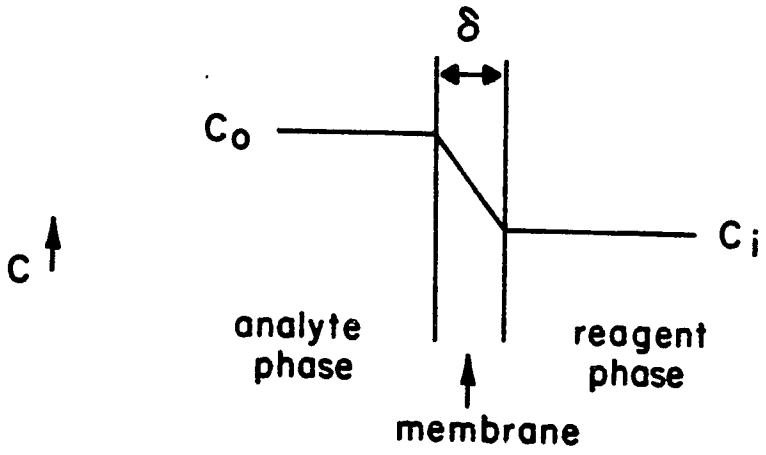

Figure 3-1. Schematic showing analyte concentration profile in ideal two phase flow cell. Co is analyte concentration in the bulk analyte phase. Ci is analyte concentration in the reagent phase.

The importance of mixing in CL analyses has been pointed out in Chapter 1. Mixing in the two phase system is controlled by analyte mass transfer across the interface and into the reagent phase. Under the conditions employed, the measured signal reaches a steady state and thus is less sensitive to variations in mixing.

Another potential advantage of the two phase system is the possible ability to exclude interferences from the reagent phase. For example, the protein component of biological samples is excluded by the dialysis membrane.

The system should be useful both for continuous measurement of analyte or for analysis of small, discrete samples in a flow system. Steady state measurements may be made, or small sample slugs may be injected as in single phase FIA, resulting in higher sample throughputs.

The purpose of the work in this chapter was to extend the possibilities of FIA by incorporating the advantages of a two phase CL cell with a flow system. A theoretical model of a simple two phase system was developed. A cell for experimental two-phase measurements was constructed. The performance of this cell was evaluated and compared with the theory for several reactions.

Theory

CL intensity is proportional to the amount of substrate reacting with the reagents per unit time. In a two phase system, CL intensity will depend on the rate at which analyte reaches the phase boundary, crosses the interface and mixes in the reagent phase, as well as on the reaction kinetics.

The two phase CL reaction cell consisted of a flowing stream of analyte solution separated from the reagent phase by a dialysis

membrane. The reagent phase contained enzymes that catalyzed CL. The reagent cell was made of clear Plexiglas and was mounted with its polished face toward the photocathode of a PMT. The reagent phase was stirred magnetically. A simplified outline of the system is shown in Figure 3-2.

In the model for the system shown in Figure 3-1, the variable C_o is the concentration of analyte flowing through the system. C_i is the concentration of analyte on the reagent side of the membrane. Analyte passes through the membrane to the reagent phase under a concentration gradient. Convection is the major means of mass transfer in the stirred reagent phase.

Several assumptions will be made in order to develop a theoretical treatment. It will be assumed that there is no significant depletion of analyte on the analyte side of the dialysis membrane. This is reasonable because the flow system can continuously pump analyte through the cell to the phase interface, although this assumption becomes less true at very slow flow rates. It will also be assumed that the convective mass transfer in the reagent phase is efficient so that the reagent phase will be completely mixed and analyte concentration will be uniform throughout this phase.

These assumptions result in the following expression for R_e, the analyte flux into the reagent phase:

$$R_e(\text{moles/cm}^2\text{/sec}) = D/d \times (C_o - C_i) \qquad (3.1)$$

where D is the diffusion coeffecient of analyte in the membrane (cm^2/sec) and d is the thickness of the membrane.

In practice, these assumptions represent approximations that make

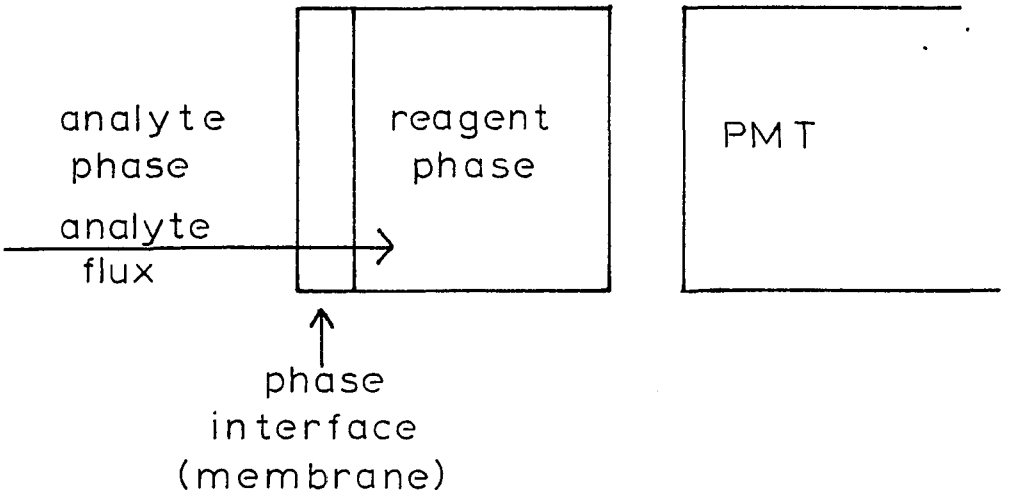

Figure 3-2. Simplified outline of two phase cell.

it possible to develop a simple mathematical description of the response of the system. The assumption of a homogeneous reagent phase is not strictly true because of the unavoidable presence of pockets of poorly stirred reagent solution because of the design of the cell. However, it is still true that mass transfer will be proportional to C_o-C_i

$$R_e = K(C_o-C_i) \qquad (3.2)$$

The difference between D/d and experimental values of K is an indicator of how closely the two phase cell conforms to the model of Figure 3-1.

If it is assumed that the CL reaction is first order in analyte, then

$$dC_i/dt = K_1 \times C_i \qquad (3.3)$$

where K_1 is the rate constant of the CL reaction. An expression for the response of the two phase system can now be readily derived.

Initially, there is no analyte present in the system, and C_i=0. At this time, t=0, analyte solution at concentration C_o is introduced into the flow system. Analyte will diffuse across the membrane into the reagent phase and concentration in the reagent phase will increase until a steady state is reached. The rate of change of analyte concentration inside the reagent phase will equal the rate at which analyte enters the cell minus the rate at which it reacts:

$$dC_i/dt = K(C_o-C_i) - K_1 \times C_i \qquad (3.4)$$

Equation 3.4 can be solved (see appendix A) to give the following expression for the concentration of analyte in the reagent phase at any time t:

$$C_i = \frac{KC_o}{K+K_1}(1-e^{-(K+K_1)t}) \qquad (3.5)$$

The CL intensity observed will be determined by C_i. The term $e^{-(K+K_1)t}$ determines the response time of the system. As shown in equation 3.5, the overall response time depends on both the rate of analyte entering the reagent phase (K) and the rate constant of the CL reaction (K_1).

When a steady state is reached, at large values of t, equation 3.5 becomes

$$C_{i(ss)} = \frac{KC_o}{K+K_1} \qquad (3.6)$$

At steady state, the rate at which analyte enters the reagent phase equals the rate at which it is consumed.

If the CL reaction is slow relative to analyte diffusion, then K_1 is much less than K and the response time term becomes e^{-Kt}. Such a situation will cause C_i to build up and will therefore result in maximum sensitivity, but the response time will become unacceptably long.

Ideally, the approach to achieve rapid response is to work under conditions where the analytical reaction is rapid. This is not always feasible, however. The firefly reaction is an example of a very slow CL reaction. An alternative means of achieving rapid response is to introduce a degrading reaction that will consume excess analyte. This reaction will then determine K_1. If the degrading reaction is enzyme catalyzed, this enzyme can be co-immobilized with the CL enzyme, and thus not affect the bulk analyte solution. The CL intensity will be proportional to $K_2 \times C_i$, where K_2 is a proportionality constant for the CL reaction. K_2 will be much less than K.

As K_1 is increased, response time will be shortened. The improvement in response time will come at the expense of sensitivity,

however, because C_i will not build up as much. If a degrading reaction is being used, less analyte will be avaliable to the CL reaction because it will have been consumed by the degrading reaction.

Recall that at steady state,

$$C_i = \frac{K}{K+K_1} C_o \qquad (3.6)$$

If K_1 is much greater than K (fast CL reaction), then equation 3.6 will become

$$C_i = \frac{K}{K_1} C_o \qquad (3.7)$$

It can be seen from this theoretical treatment that the response time of the two phase system will be determined by both the rate of analyte mass transfer into the reagent phase and the kinetics of the reaction(s) occuring inside the cell. This theory can serve as a model to predict the response characteristics of the two phase system for reactions with different kinetics or slightly different physical characteristics (such as interface thickness or area). It is useful for obtaining an understanding of why the system behaves as it does in any given situation. It also gives the analyst clues about how to modify the system to provide an acceptable tradeoff between resonse time and sensitivity if the analytical reaction is slow.

Experimental

Apparatus

The two phase flow cell assembly is diagrammed in Figure 3-3. The components of the cell itself were constructed of Plexiglas. The bottom of the reagent compartment and outside face of the cell were polished

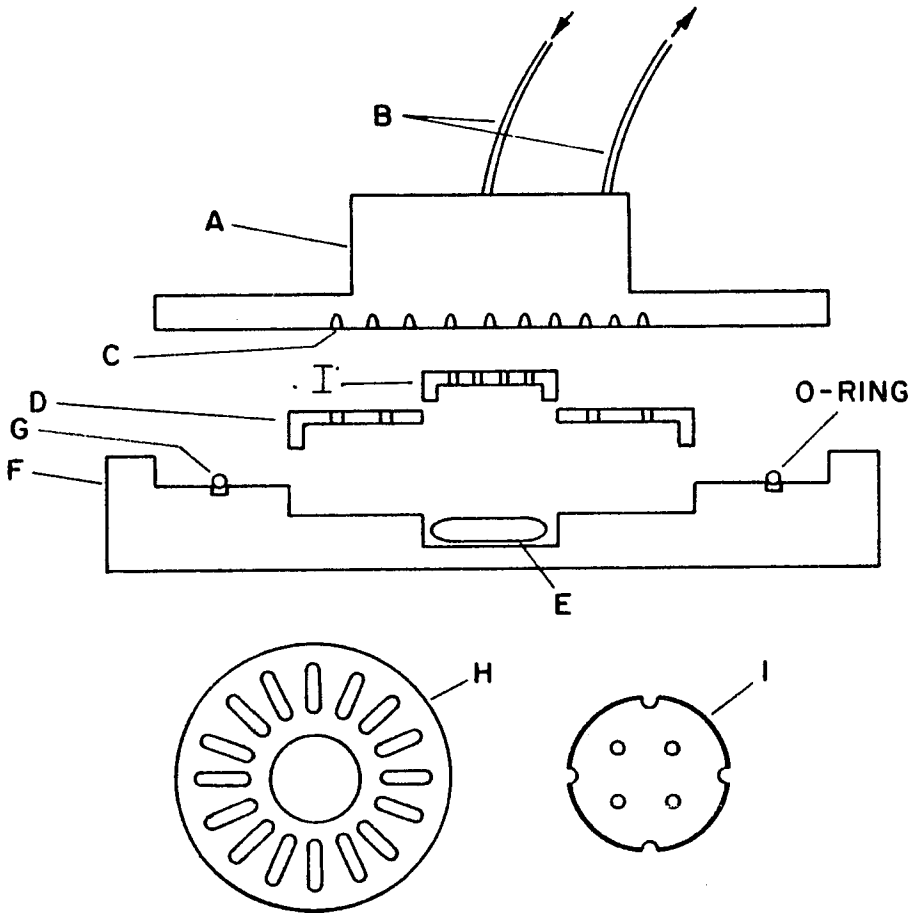

Figure 3-3. Two phase flow cell. A: aluminum spiral channel piece; B: Teflon tubing; C: spiral groove channel for analyte; D: plexiglas retainer for supporting membrane; E: magnetic stir bar; F: plexiglas base of cell; G: rubber o-ring for leak prevention; H: top view of piece D, showing arrangement of slots that allow analyte to diffuse into reagent compartment; I: plexiglas cap for holding stir bar in place.

clear to allow maximum light transmission. A 2 mm x 7 mm Teflon stir bar fits into the well in the reagent compartment. The volume of the reagent compartment was approximately 1.5 mL (not including the volume of the stir bar). A rubber o-ring mounted in a circular groove in the cell helped prevent leaks.

A piece of dialysis membrane was cut to fit over the top of the reagent compartment. The slots and holes in the two top pieces of the cell allowed diffusion of molecules into and out of the cell, but kept the stirring bar in place. These top pieces were also very important for keeping the membrane tight against the spiral channel piece. Without adequate tension on the spiral piece, the flow of sample would not be confined to the spiral channel and the cell would be useless. Several earlier cell designs suffered from this problem and could not be used. The area over which diffusion can occur (i.e., the area of the slots and holes) is approximately 1.3 square cm.

Providing adequate tension on the spiral channel without severely reducing the area over which solution species can diffuse was a major problem encountered in developing a functional cell. The cell was designed primarily to provide a system which offered relatively trouble-free plumbing. In addition, there must be provisions so that sample solution can be easily delivered to and pumped away from the phase interface. The current cell represents a compromise between tractable plumbing and optimum performance. The performance will be compromised by the necessary reduction in the interfacial area and mixing efficiency in the reagent phase. The physical limitations of the cell will make some of the assumptions made in the previous section less valid. For example, it was assumed that the reagent phase was homogeneously mixed;

this will not be entirely true, because there are zones of unstirred solution in the grooves and holes of the top pieces of the cell. However, one can still assume the existence of a concentration gradient between the analyte and the reagent phases. Mixing in the bulk of the reagent phase will still be more efficient than if it was strictly diffusion controlled. The validity of the assumptions made above with the current cell were evaluated by a series of experiments to be described shortly.

The sample flow channel is a spiral groove approximately 1 mm deep, machined into an aluminum disk which fit tightly into the top of the reagent cell. Two holes were drilled through the disk, one at each end of the spiral channel. On the back side of the disk, Teflon tubing, 0.79 mm i.d., was glued into the holes to allow connection of the spiral channel to external plumbing. "Super Glue" was used to secure the tubing to the aluminum. Improved adhesion was obtained by roughening the outside of the tubing before applying glue. An aluminum housing, threaded on one end, was the mount for the cell and spiral channel disk. The cell and disk were screwed into the housing rather tightly to prevent leaking. The two tubes to the spiral channel were fed through two small holes in the housing. Once assembled, the entire assembly could be handled with no danger of leakage.

The threaded end of the cell housing was screwed to the end of the PMT housing so that the bottom of the Plexiglas cell was directly in front of the photocathode of an end-on PMT which was covered with black cloth to reduce the effect of light leaks.

The Plexiglas reagent cell, and several earlier models along its evolutionary path, were made to order by the UNH Physics Machine Shop,

as were the PMT housing and cell housing. The spiral groove disk was made by Granite State Tool and Die Company, Dover, New Hampshire.

The PMT, recorder, and electronics were the same as those used for the glucose work. Interchangable PMT end-housings made it easy to switch from the two phase cell to the housing containing the simple coil of Teflon tubing used for the FIA experiments.

A Cole-Parmer model 7545-10 peristaltic pump, equiped with 7013 pump heads and 6408-41 tubing was used for most experiments. For some experiments, a Harvard infusion pump was used. Flow lines other than those in the pump heads were 0.79 mm i.d. Teflon tubing (Altex). The various tubing fittings were also obtained from Altex.

Magnetic stirring plates from Fisher were used. An Orion model 501 pH meter was used for pH measurements.

Chemicals

Reagent grade potassium dihydrogen phosphate, boric acid, KOH, sodium acetate, acetic acid, and fluorescein were obtained from Baker. HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid) buffer and luminol were obtained from Aldrich.

Horseradish peroxidase (E.C.1.11.1.7), apyrase (E.C.3.6.1.3), NADH, FMN, synthetic firefly luciferin, Tris buffer, and decanal were obtained from Sigma.

BL enzyme reagents were obtained from LUMAC. LUMASE is a highly purified and stabilized bacterial luciferase - NADH-FMN oxidoreductase preparation. LUMIT-PM is a highly purified and stabilized preparation of firefly luciferase and luciferin.

The dialysis tubing used was Spectrapore 6, 10 000 molecular weight cutoff (MWCO), 20 um thick, obtained from Spectrum Medical Industries.

It was slit before use and used as a single layer membrane.

Procedures

An excess volume of enzyme-containing solution was placed in the well of the Plexiglas cell. The stir bar was set in place and the plastic cover pieces of the cell were carefully fitted into the well. Any air bubbles trapped in the cavity were eliminated by using an eye dropper and extra filling solution. The rubber o-ring was placed in its groove, and the wet dialysis membrane was then placed over the top of the cell, care being taken to avoid trapping air bubbles under the membrane.

With the membrane in place, the plexiglas cell was mated to the spiral channel sample flow disk which had been previously mounted inside the cell housing. The cell was screwed to the housing, and the entire assembly was screwed to the PMT housing.

The PMT was mounted face down. Thus, the plexiglas cell was oriented such that it was essentially upside down, i.e., smooth face of cell on top, spiral channel disk below. The assembly was held in place over a magnetic stirrer. Magnetic coupling through space and the cell components was sufficient to stir the reagent.

Sample and blank solutions contained all necessary reagents that could diffuse out of the reagent compartment so that there would be no depletion inside the cell. Reagent concentrations were the same in the sample and blank as they were in the original reagent solution. All solutions in a given experiment were buffered to the same pH and ionic strength.

Solution containing non-immobilized reagents and buffer was pumped through the spiral channel to establish a baseline. Flow was switched

manually to the sample solution to record the response of the system to analyte. A few experiments were performed in which a slug of sample was injected into the flowing background stream by a pneumatically actuated Rheodyne 4-way valve.

Results and Discussion

Fluorescein Diffusion Experiments

Initial experiments with the two phase flow cell were conducted using fluorescein solutions to characterize the mass transfer of the model analyte across the membrane. These data allow calculation of an experimental value of K (equation 3.2). The difference between the measured K and the value of D/d indicates how closely the system conforms to the simple model illustrated in Figure 3-1.

For all of these experiments, 1 mM fluorescein solutions in 0.1 M borate buffer, pH 9.0, were used. Blank solutions were 0.1 M borate buffers with no added fluorescein. Two types of experiments were performed. The fluorescein solution was either used to fill the reagent chamber while buffer was pumped through the spiral, or buffer was placed in the cell and fluorescein was pumped through the spiral. The experiments were performed at a number of flow rates with the reagent compartment stirred in all experiments.

For the case in which fluorescein solution was initially in the cell, buffer was pumped through the system and the effluent was collected in timed fractions. The timing was determined by the flow rate and was chosen such that 3 to 5 mL would be collected per fraction. Absorbance of the effluent fractions at 490nm was measured on a Shimadzu Spectronic 200-UV spectrophotometer. The molar absorptivity of the fluorescein dianion at 490 nm in moderately basic solution is 88 000

(86). Fluorescein concentrations in the effluent fractions were calculated from the absorbances. From these effluent concentrations and the flow rate, the rate of mass transfer of fluorescein out from the cell could be calculated. Once the rate of diffusion and interfacial area are known, an experimental K can be calculated from equation 3.2.

Results for this series of experiments are summarized in Table 3-1. It should be pointed out that the values in the table for fluorescein diffusion are average values. There was a tendency for the rate of diffusion to decrease somewhat with succesive fractions, probably because of depletion of the fluorescein in the cell. As can be seen in Table 3-1, with the exception of the slowest flow rate, the values for fluorescein diffusion out of the reagent cell are roughly comparable. The experimental K's are much smaller than the calculated value of D/d. These values, and the implications of their difference, will be examined after the next set of experiments is considered.

A second set of experiments in which buffer was trapped in the reagent compartment while 1 mM fluorescein was flowing through the spiral was performed. In these experiments, the fluorescein was allowed to flow at a given flow rate for a measured length of time. At the end of the specified time interval, air was pumped through the spiral so that fluorescein solution would not remain stationary in the spiral. The cell was disassembled and the absorbance of the reagent compartment solution (buffer solution plus the fluorescein that diffused in) was measured in a microcuvette. Measurement of absorbance on the flow stream effluent by difference would of course be highly imprecise, given the high concentration of fluorescein still present in that solution.

Using this method, one essentially integrates the fluorescein flux

Table 3-1

Results from diffusion experiment in which 10^{-3} M fluorescein was in the reagent compartment and 0.1 M borate buffer was flowing in sample channel of the two phase flow cell.

Flow Rate	Flr Diffusion[a]	Re	expt'al K
0.4 mL/min	4×10^{-11} moles sec^{-1}	3.1×10^{-11} moles/cm^2-sec	3.1×10^{-8} cm sec^{-1}
1.2	6.7×10^{-11}	5.2×10^{-11}	3.1×10^{-8}
4.5	7.5×10^{-11}	5.8×10^{-11}	5.2×10^{-8}
8.8	7×10^{-11}	5.4×10^{-11}	5.4×10^{-8}

[a] These are average values from several fractions (see text).

into the cell over the length of time that the fluorescein was flowing. The results are compiled in Table 3-2.

From equation 3.2, the experimental K for fluorescein diffusion into the cell can be calculated from the rate of fluorescein diffusion and the interfacial area. The initial conditions are such that C_i, the concentration of analyte (fluorescein) inside the cell is zero at t=0. As time passes, C_i will increase, but at short times will still be much less than C_o. This is confirmed in the present case by the low concentrations of fluorescein observed diffusng into the cell. Thus, Co>>Ci, and it can be assumed that $R_e=KC_o$. The rate at which fluorescein enters the cell can be taken from Table 3-2. The concentration of fluorescein on the sample side is 1 mM. The experimentally determined values of K are also listed in Table 3-2. These values are apparently independent of flow rate.

The fluorescein diffusion experiments confirm that diffusion of molecules across the membrane does indeed occur in the flow cell. However, the results show that some of the assumptions made earlier are not strictly correct. The evidence for this is given by a comparison of the experimentally determined mass transfer constants (K) with the calculated value of D/d.

Using an approximate value of $5x10^{-6}$ cm^2/sec for the diffusion coefficient of fluorescein, and the membrane thickness of 20 um (reported by the manufacturer), D/d equals $2.5x10^{-3}$ cm/sec. Comparison with the data in Tables 3-1 and 3-2 show that the experimental K's are much smaller than this value.

The reasons for this disagreement lie in the assumptions that were made concerning analyte depletion and mixing efficiency in the reagent

Table 3-2

Data for fluorescein diffusion from sample channel into reagent phase in two phase flow cell.

Flow Rate	Diffusion Rate	Re	expt'al K
0.4 mL/min	5.3×10^{-11} moles sec^{-1}	4.1×10^{-11} moles/cm^2 sec	4.1×10^{-8} cm sec^{-1}
1.2	5×10^{-11}	3.9×10^{-11}	3.9×10^{-8}
4.5	4.7×10^{-11}	3.6×10^{-11}	3.6×10^{-8}
8.8	6.1×10^{-11}	4.7×10^{-11}	4.7×10^{-8}

0.1 M borate buffer, pH 9, was in the reagent chamber. Stirrer was on.
Spectrapore 6 membrane, 10 000 MWCO, 20 um thick, was used as interface material.

phase. If these assumptions hold, then K=D/d. There may also be inaccuracies in the reported membrane thickness. It was assumed that there was no analyte depletion on the analyte side of the membrane. This is probably not strictly true because while analyte is diffusing into the reagent phase, it is not getting immediately replenished from the bulk analyte solution because the solution at the membrane surface is almost static under the conditions of laminar flow. Therefore, analyte depletion at the membrane surface extends the diffusion zone into the analyte phase somewhat.

Probably the most important reason for disagreement between K and D/d is due to the reagent phase not being homogeneously mixed. Because of the limitations on cell design imposed by plumbing considerations, there are unavoidably some pockets of reagent solution that are essentially unstirred, as was mentioned in the experimental section of this chapter. These unstirred zones are located in the slots and holes cut through the two top pieces of the cell that hold the membrane against the spiral channel. These unstirred zones effectively increase the distance over which analyte must diffuse resulting in an effective d that is much larger than the actual membrane thickness. This increase in d apparently accounts for the small values of K observed.

Although the assumptions made to develop the theory do not seem to hold strictly for the present cell, the theory is useful for predicting the general response characteristics and for suggesting ways to improve the design. The most important conclusion to be drawn is that substantially improved performance should be possible if a cell that minimizes the effective diffusion distance can be designed.

Luminol-Peroxidase System

The purpose of the next set of experiments was to characterize the two phase flow cell using a CL reaction that has easily adjustable kinetics and predictable characteristics. The reader is referred back to Chapter 1 for an introduction to the luminol reaction. Peroxidase was used as the catalyst in these studies because it can be immobilized by entrapment behind the membrane and beause it is easy to change the kinetics of the CL reaction by changing peroxidase concentration or pH. This system is therefore analogous to the bacterial and firefly BL reactions which will be investigated later, but is more easily manipulated and requires much less expensive reagents.

In these experiments, a borate buffer solution containing peroxidase and luminol was used to fill the reagent compartment of the two phase flow cell. Spectrapore 6 dialysis membrane, 10000 MWCO, 20um thick, separated the reagent compartment from the sample spiral. The sample used for these experiments was a solution of hydrogen peroxide and luminol buffered to the same pH as the reagent solution. Buffer concentration and luminol concentration in the samples and blanks were identical to those in the original reagent solution so that there would be no effects due to luminol depletion in the cell or pH or ionic strength gradients between the sample and reagent sides of the membrane.

The apparatus for this set of experiments was quite simple. A single channel of the peristaltic pump provided the flow. A buffer solution containing luminol was pumped through the system as a blank to establish a baseline. Once a steady baseline had been achieved, the tubing to the pump was switched from the blank solution to the sample solution reservoir. This process resulted in the introduction of a

small air bubble between the blank and the sample, which prevented them from mixing.

The sample solution was pumped through the system until a steady state response was reached. Return to the baseline was achieved by switching back to the blank solution.

It proved to be more efficient to vary the kinetics of light emission by changing peroxidase concentration than by changing pH, so most experiments were carried out at pH 9.0 using different peroxidase concentrations. It was very difficult to get a steady baseline with solutions at pH 10; the baselines were very noisy and exhibited a steady rise in intensity with time.

The response of the two phase flow system to reactions with different kinetics was compared to the response observed in a single phase experiment in which sample solution was injected directly into a cuvette containing reagent solution. An Aminco Chem-Glow photometer was used for the single phase experiments.

Fast kinetics. Fast kinetics were observed at a peroxidase concentration of 1 mg/mL in a solution buffered to pH 9.0. The kinetics of the reaction under these conditions were verified by injecting 10 uL of 1 uM hydrogen peroxide solution via syringe into a cuvette containing 100 uL of the peroxidase-luminol reagent solution. The cuvette had been mounted in the photometer before injection; the syringe needle was inserted through a rubber septum. It was assumed that the force of injection would be sufficient to insure adequate mixing, but because of variations in this injection-induced mixing, precision of replicate injections was extremely poor. The purpose of the experiment, however, was to observe the rate of light emission, and the data were sufficient

for this purpose. While mixing effects would affect the observed CL as a function of time (i.e., the observed rate), this did not seem to be a problem here since the necessary kinetic information was unambiguous.

The rise to maximum intensity was essentially instantaneous, followed by a very rapid return to the baseline. The maximum intensity was reached less than 1 sec after injection; return to the baseline took an additional 5 sec or so. A typical intensity-time curve is shown in Figure 3-4. More accurate measurement of rise and fall times was not possible with the instruments used, but the results are sufficient to confirm that the reaction under these conditions is quite fast.

The results of a series of experiments to investigate the response of the two phase flow cell to a fast CL reaction are summarized in Table 3-3. In all cases, 1 mg/mL peroxidase and 1 mM luminol in 0.1 M borate, pH 9, was used as the reagent solution. A 1 μM hydrogen peroxide solution flowed in the sample spiral at the flow rates indicated in Table 3-3. Figure 3-5 shows typical steady state response curves for the flow cell with the fast CL reaction.

Several observations can be made from the results in Table 3-3. With a small analyte like hydrogen peroxide and a fast CL reaction, the response of the flow cell is quite rapid, with 90% rise times for most flow rates of about 7 sec. This is a significant improvement in response time compared with a somewhat similar two phase system previously reported (18, 85). The rise time seems to be independent of flow rate from 1 mL/min to 10 mL/min, but a flow rate of 0.5 mL/min is sufficiently slow to cause an increase in response time. This could be due to a depletion of analyte at the surface of the membrane as a result of the slow flow rate. This effect would not have shown up in the

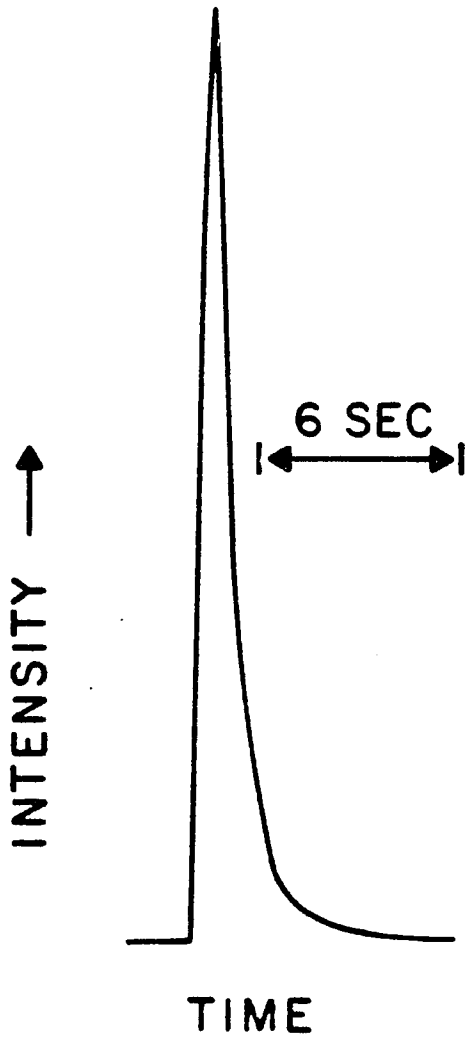

Figure 3-4. Intensity-time response for injection of H_2O_2 into the photometer, with fast kinetics luminol-peroxidase system.

Table 3-3

Response characteristics for fast kinetics luminol-POD reaction in two phase flow cell

Flow Rate	Mean 90% Rise Time	Mean SS Intensity	Mean 90% Fall Time
0.5 mL/min	12 sec	34 mv	25 sec
1	7	44	15
5	7	79	8
10	7	104	14

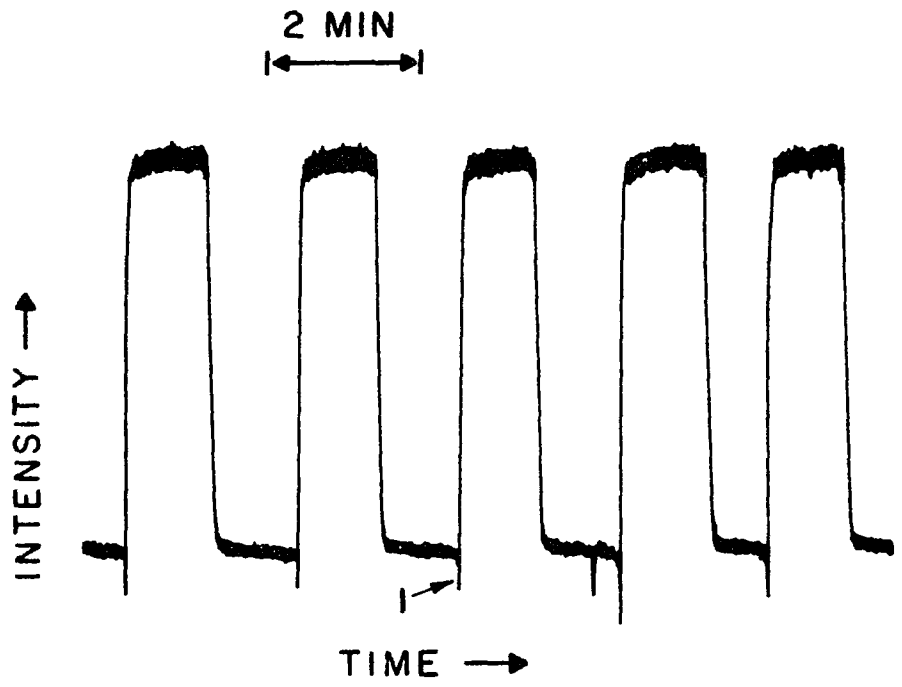

Figure 3-5. Steady state response for 10^{-6} M H_2O_2 in the two phase flow system, using fast kinetics luminol-peroxidase reaction. Flow rate = 5 mL/min.

fluorescein experiments because those experiments dealt with integrated fluxes.

The steady state intensity increased considerably as flow rate was increased. At first this is surprising, since the results in Table 3-2 show that analyte diffusion rate seems to be constant regardless of flow rate. The fluorescein results, however, do not account for the effect of analyte consumption in the reagent phase, since the fluorescein concentration would build up in the cell and thus change the rate of diffusion in. Hydrogen peroxide, on the other hand, is consumed quite rapidly in the reagent cell and can not build up as the fluorescein did. The ability of the higher flow rates to bring more analyte to the phase interface per unit time may account for the increased intensities at higher flow rates.

There was some fluctuation of the baseline in all these experiments. As can be seen in Figure 3-5, the slow chart speed caused these fluctuation to appear as a thick baseline. Average thickness of the baseline (i.e., noise variation) was about 2.4 mv. The average noise level at steady state intensity levels was about 3.5 mv. All measurements of steady state intensities were taken from the top of the tracings, which were relatively flat. The noise fluctuations were about the same whether the peristaltic pump or the infusion pump was used, ruling out pulses from the peristaltic pump as the cause of the fluctuation. The noise apparently came from fluctuations in background emission from the CL reagents in the absence of peroxide or from detector noise.

Some short experiments were performed to evaluate the performance of the system in a flow injection mode. The Rheodyne injection valve

was used to inject a 200 μL slug of 1uM hydrogen peroxide into the flowing buffer-luminol background stream. In this case, the FIA technique is used merely to inject the sample and transport it to the reagent cell. Typical intensity-time plots are shown in Figure 3-6. In this experiment, steady state is never reached, but since sample size and dispersion are controlled, the peak height of the results is quite reproducible. The intensity for the 200 μL samples was about half of the steady state intensity at a given flow rate. The results of this experiment are collected in Table 3-4. The flow rate of 0.5 mL/min is too slow to be useful for injection analysis. There was a much noisier baseline, the intensity was much lower, and the peaks were excessively wide. The best results were observed at a flow rate of 5 mL/min; the peaks were symmetrical, of relatively high intensity, and only 10 sec wide at the base. These experiments suggest that the two phase flow cell could be useful for analyses using small samples at relatively high sampling rates. This would reduce the amount of sample needed significantly and eliminate the need for reaching the steady state. This approach would seem to be most useful for reactions that have fast kinetics.

Slow kinetics. Initially, attempts were made to slow down the CL reaction by increasing the pH while keeping peroxidase concentration at the relatively high level of 1 mg/ml. At pH 10, the reaction was still fast, and it was very difficult to get a stable baseline in the flow system. Peroxidase concentrations of 0.5 and 0.1 mg/mL were likewise not useful in the flow system because of erratic and noisy behavior. The use of pH's higher than 9 was discontinued because of this.

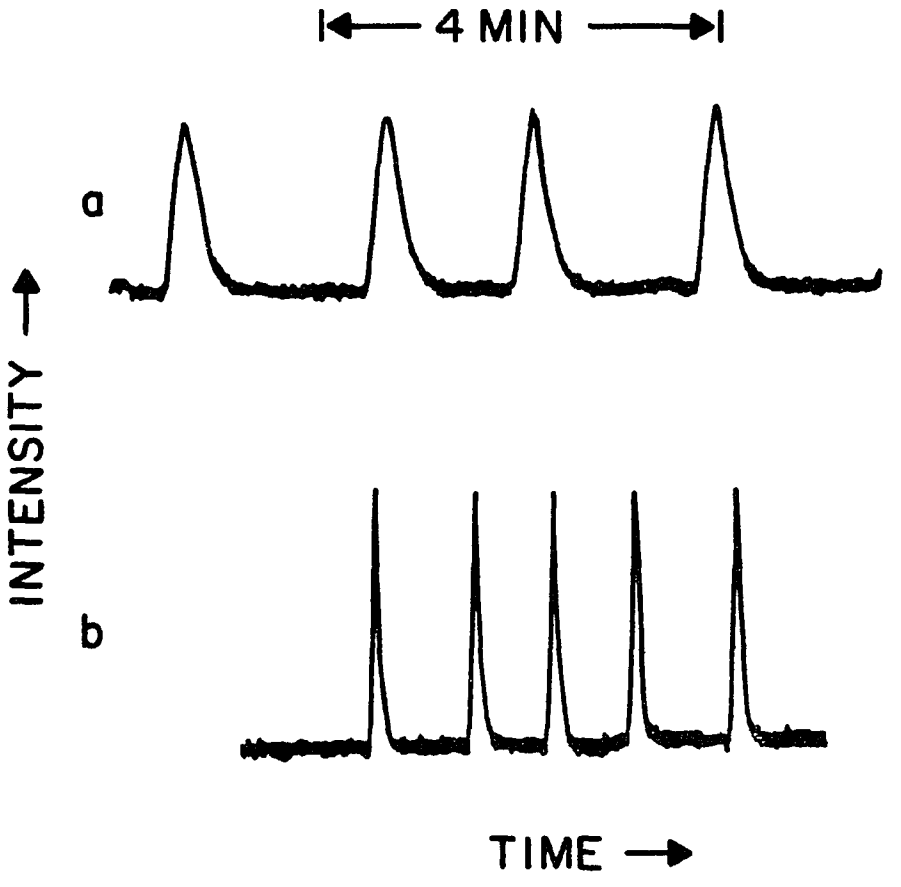

Figure 3-6. Replicate injections of 10^{-6} M H_2O_2 into two phase flow system operated in a flow injection mode with fast kinetics luminol-peroxidase system. Sample slug size was 200 µL. (a) flow rate = 1 mL/min; (b) flow rate = 4.5 mL/min.

Table 3-4

Data for 1 μM H_2O_2 using the two phase flow cell in a flow injection mode.

Flow Rate	Mean Peak Intensity	Steady State Intensity	RSD, peak int.	mean w_b
0.5 mL/min	23 mv	34 mv	5%	80 sec
1	25	54	7	50
5	42	79	2.2	10

Sample loop volume was 200 μL.

Note: the intensities reported in this table cannot be directly compared to those reported in table 3-3, because different batches of luminol were used in the two experiments from which these data were taken.

The CL reaction at pH 9.0 was slowed by reducing the peroxidase concentration. Peroxidase at 0.001 mg/mL with 1 mM luminol in 0.1 M borate buffer was used for the slow kinetics experiments.

When 10 uM hydrogen peroxide was injected into the photometer via syringe, there was a gradual increase in intensity, rising to a broad maximum about 28 sec after injection. The maximum was followed by a very slow decrease in intensity, returning to the baseline about 9 min after injection. The general shape of the slow kinetics intensity-time curve is shown in Figure 3-7. The 90% rise times averaged approximately 11 sec.

As expected, the two phase flow system exhibited slower response with the slower CL reaction. Experiments were carried out in the same manner as were the fast kinetics experiments; only the peroxidase concentration and peroxide concentration (10 μM) were different. The higher peroxide concentration was needed to obtain adequate intensity on the photometer. Because the reaction was slow, emission was much longer lived, but intensities were considerably lower. Results for the slow kinetics reaction in the flow system are collected in Table 3-5. A typical response curve is shown in Figure 3-8.

Only two flow rates were used for this set of experiments. A flow rate of 0.5 mL/min was judged to be too slow to be useful for the slow CL reaction, while 10 mL/min did not seem to offer any advantages to justify the added solution requirements and more erratic behavior.

It can be seen from Table 3-5 that the response of the system was considerably slower than it was for the faster CL reaction. The intensities of the steady state signals in Tables 3-3 and 3-5 can not be compared directly because different peroxide concentrations were used.

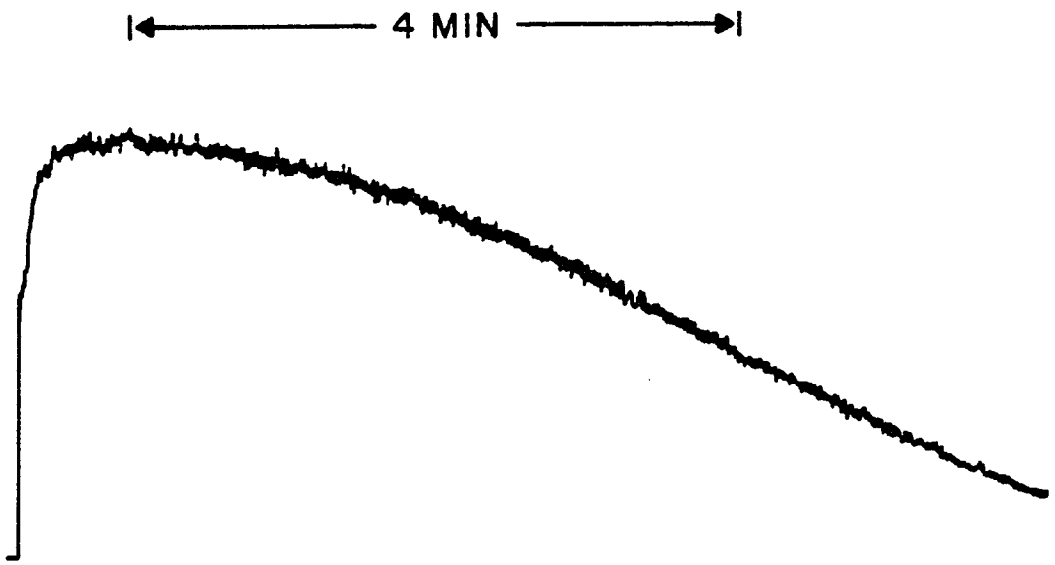

Figure 3-7. Photometer injection of 50 μL of 10^{-5} M H_2O_2 into 200 μL of slower kinetics luminol-peroxidase reagent.

Table 3-5

Results for luminol-peroxidase reaction with slow kinetics (0.001 mg/mL POD) in the two phase flow cell

Flow Rate	Mean 90% Rise Time	Mean I_{ss}	Mean 90% Fall Time
1 mL/min	4 min	930 mv	3.7 min
5	3.5	1150	4.2

mean I_{ss} = mean intensity at steady state

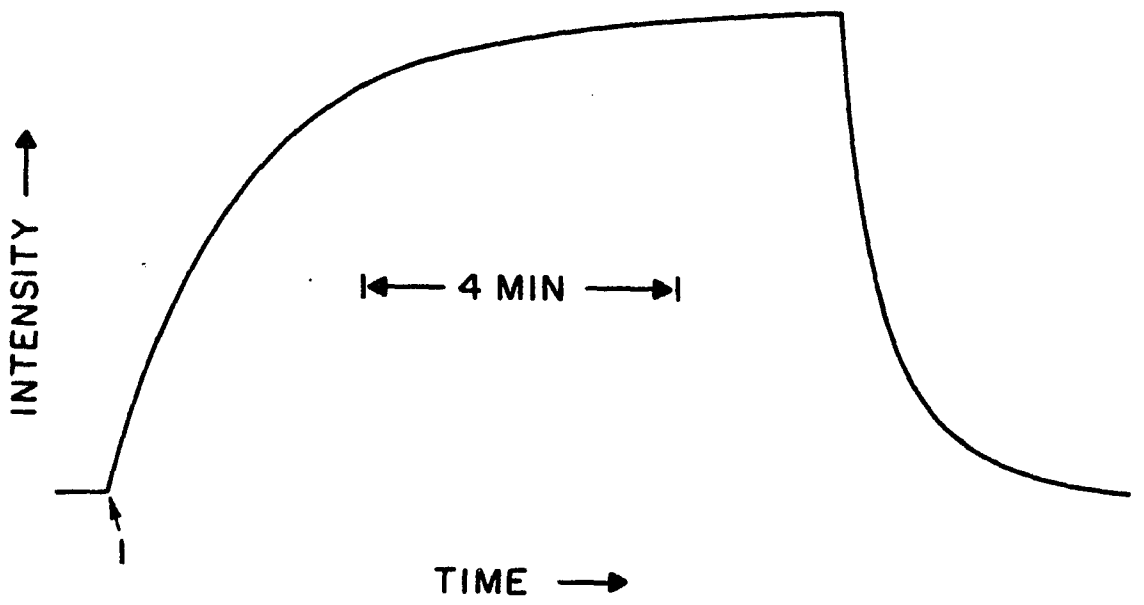

Figure 3-8. Steady state response of 10^{-5} M H_2O_2 in slower kinetics luminol-peroxidase reaction in two phse flow system. Flow rate = 1 mL/min.

However, a later experiment in which 10 μM peroxide was used in the fast CL system gave a steady state intensity comparable to that observed for the slow CL system (1.06 v for fast CL vs. 0.93 v for slow CL).

Steady state intensities for the slow CL system showed a much smaller dependence on flow rate than did those for the fast CL system. The fast CL results showed a marked increase in intensity with increasing flow rate. This probably occurs because the effects of the broadened diffusion zone are less exaggerated than they are in the case of a fast analytical reaction. Analyte is consumed less rapidly in a slower reaction, and therefore depletion zones are less likely to develop to the point where they cause the effects seen in the fast CL cases. The kinetics of the reaction are no longer very much faster than the rate of analyte mass transfer into the reagent phase.

The flow system seemed to behave best at flow rates of 1 or 5 mL/min. At slower flow rates, there were considerable problems in getting a stable baseline.

Experiments with the luminol-peroxidase reactions confirm that the two phase flow system was performing more or less as expected. Results from single phase sample injection experiments can be used to predict the response characteristics of the flow system. A flow system can be used for analysis much more easily and reliably than can a photometer with single phase discrete sample injection using no flow system. The photometer method suffers from a severe lack of reproducibility while the flow system offers much better precision for replicates as well as simpler sample handling and more rapid sampling rates.

Firefly and Bacterial BL

Once the two phase flow cell was shown to perform satisfactorily,

if somewhat inefficiently, using the luminol-peroxidase chemistry, work was undertaken to evaluate the performance of the system using the more complicated firefly and bacterial BL reactions. It was hoped that the flow cell would be useful for analyses based on these reactions, since the cell allows expensive enzymes to be reused without the difficulties associated with chemical immobilization procedures. The advantages of the flow cell for sample handling and precision compared to single injection techniques have already been cited. The system also offers the advantages of higher throughput and more reproducible mixing when compared to the immobilized luciferase techniques described by DeLuca (11, 16, 17). In addition, it should be possible to manipulate the response characteristics of the systems by using analyte degrading enzymes, allowing the analyst to make trade-offs between response time and signal intensity.

Firefly BL

Introduction. A limited number of experiments were performed with the firefly BL system to compare the performance of the present flow cell to one described earlier by Freeman (85), and to see whether it could be useful for ATP analysis.

Although the firefly reaction is one of the most efficient CL reactions known, it is also kinetically slow, an obvious disadvantage if the reaction is to be used for analytical purposes in the flow cell because it will lead to poor response times. This was the case in the work described by Freeman (85) in a two phase cell that did not offer convective mass transfer in the reagent phase.

It is possible, however, to modify the observed kinetics of the firefly reaction by adding an ATP-consuming enzyme such as apyrase.

Apyrase will consume ATP fairly rapidly, so that any ATP that does not bind to luciferase quickly will not get to take part in the BL reaction. In the two phase flow cell, this means that ATP concentration in the reagent phase will not build up to the same extent. Response times will be shortened, but the emission intensity will be reduced because less ATP will be available to the BL reaction. This behavior is predicted by the theory developed earlier in this chapter.

Because the firefly reaction was known to be kinetically slow, all experiments described in this section employed reagent solutions that contained apyrase. The products of the apyrase-catalyzed reaction are ADP, AMP, and phosphate:

$$ATP \longrightarrow ADP + P \longrightarrow AMP + 2P$$

Experimental. Most of the conditions used in the firefly experiments described here were adapted from previous work with a similar, though less sophisticated, two phase flow cell (85). No attempt was made to define optimum conditions. The main purposes of these experiments were to evaluate the new cell's performance with the firefly chemistry and to observe the effects of adjusting reaction kinetics by adding an analyte consuming enzyme.

For all firefly experiments, LUMIT-PM with added Mg(II) was used as reagent. One vial of LUMIT was reconstituted with 5 mL of 0.025 M HEPES (N-2 - hydroxyethylpiperazine - N' - 2 - ethane sulfonic acid) buffer, pH 7.5. The HEPES buffer was made 0.01 M in Mg(II). Previous work (85) indicated that added Mg(II) at 0.01 M improved system response to ATP. The 0.025 M HEPES buffer at pH 7.5 was recommended by LUMAC for optimum performance of the LUMIT-PM reagent.

The LUMIT reagent is highly specific for ATP. It contains low amounts of endogenous ATP, so that low blank values are expected. LUMIT does not contain contaminating enzymes that could consume ATP. Reconstituted LUMIT is expected to show no significant loss of activity for at least 12 hours at 20° C (87). Crude firefly extracts do not offer the purity and stability advantages of LUMIT.

A 0.025 M HEPES buffer, pH 7.5, containing 0.01 M Mg(II) and 1 mg/mL luciferin was used as a blank solution and also as diluent for making ATP sample solutions. Magnesium(II) and luciferin were necessary in all solutions to prevent depletion of these species in the reagent phase, since both could diffuse out through the membrane.

The ATP experiments were performed in a similar fashion to the luminol-peroxidase experiments discussed earlier. LUMIT reagent was loaded into the reagent compartment of the cell, and blank or sample solutions were pumped through the spiral channel by the peristaltic pump. Switching from blank to sample and vice-versa was again accomplished by inserting the pump tubing into the appropriate reservoir. Air bubbles separated the samples and blanks. A few trials in a flow injection mode were run, using sample slugs inserted into the flowing background stream by a manually switched Rheodyne valve. For comparison, BL response was observed for samples injected by syringe into a reagent-filled cuvette mounted in the Aminco photometer.

All firefly experiments in the flow system were run using a flow rate of 1 mL/min. This flow rate was chosen because it gave good results with the luminol-peroxidase system and would conserve reagents without severe losses in performance. Because both background and sample solutions contained expensive luciferin, it was desirable to keep

solution consumption to a minimum. Although no attempts were made to define optimum flow rate for the firefly system, the results of the luminol-peroxidase experiments suggest that 1 mL/min is a good compromise in terms of performance and sample consumption.

No temperature control was used, nor was the effect of temperature on response investigated. Although temperature variations could have some effect on the response characterisics, it would not be expected to be a large effect. The maximum reaction rate for LUMIT reagent is observed at room temperature, and there is no appreciable change in reaction rate between 16 and 24 degrees C (87), although temperatures above 30° C should be avoided. Likewise, the mass transfer rates were not expected to vary significantly over the range of ambient tmperatures encountered.

Results and Discussion

1 mg/mL Apyrase Experiments

The first set of firefly experiments was run using LUMIT reagent containing 1 mg/mL of added apyrase. The reported activity of the apyrase was approximately 0.42 units/mg protein for ATP.

The reproducibility of results for injection of 25 µL portions of 1 uM ATP into 100 µL of LUMIT containing 1 mg/mL apyrase in the photometer was poor, but the following observations can be made regarding the kinetics of this chemical system: the observed 90% rise times averaged about 5 seconds, although some replicates were much slower, and the 90% fall times were generally in the 3 to 4 minute range. Figure 3-9 shows the general shape of photometer injection intensity-time curve for these conditions.

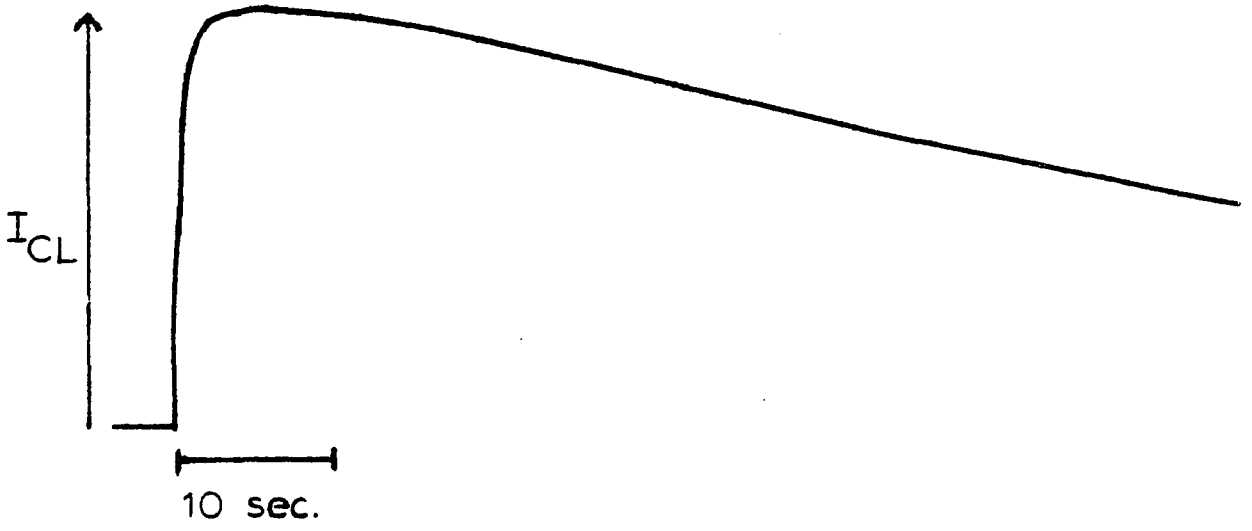

Figure 3-9. Photometer injection of 25 µL of 10^{-6} M ATP into 100 µL of LUMIT firefly reagent with 1 mg/mL of added apyrase.

Typical response from the two phase flow cell in a steady state measurement mode is shown in Figure 3-10. The baseline was very stable, and background emission was low. The 90% rise times to the steady state averaged approximately 1.5 minutes, while the 90% fall times were closer to 1.8 minutes. This is considerably better than the 4 minute 90% rise times reported for Freeman's two phase cell (85). The results are summarized in Table 3-6.

The system showed good sensitivity to ATP. Most experiments concerned with response times were done with 1 μM ATP. Although it was not investigated in detail, there was no evidence that response time was dependent on ATP concentration over the range 0.01 μM to 1 μM. Based on limited experimentation, the relationship between steady state intensity and ATP concentration seemed to be linear between 0.01 μM and 1 μM. The detection limit for ATP is estimated be slightly below 10 nM.

The steady state signal was very stable for any given experimental trial. The steady state intensity for a 1 μM ATP solution was observed to remain unchanged for as long as 14 minutes, the longest period investigated. There was a noticeable loss of intensity, however, from one replicate sample to the next. This effect is illustrated by the data in Table 3-7 and Figure 3-11. In some experiments the losses between consecutive trials were as high as 15%.

This loss of intensity was puzzling, since the steady state intensity for any given trial was quite stable. Some possible explanations for this behavior will be considered following a discussion of experiments in which different apyrase concentrations were used.

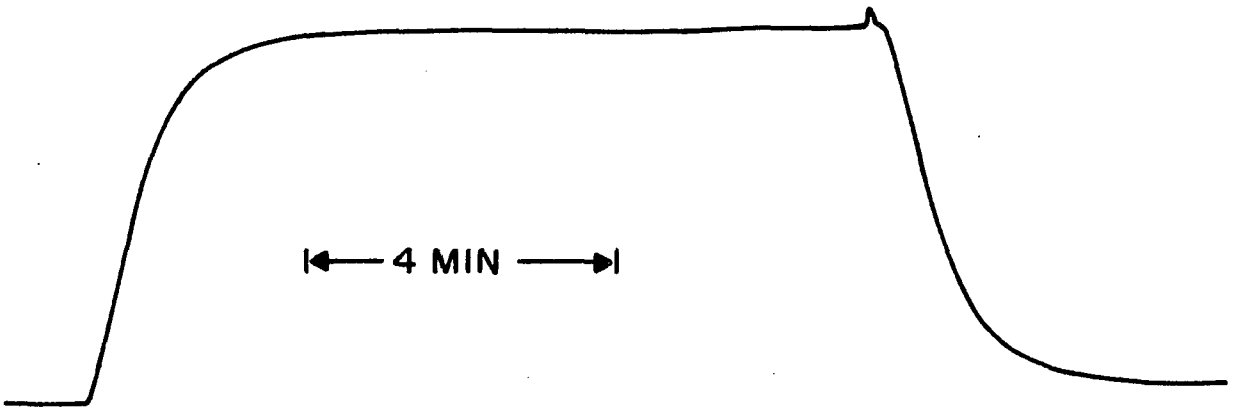

Figure 3-10. Steady state response of 1.6×10^{-6} M ATP in two phase flow system. LUMIT reagent in the reagent cell contained 1 mg/mL added apyrase. Flow rate =1 mL/min.

Table 3-6

Performance characteristics of two phase flow system using the firefly reaction. Also shown are results from photometer injection experiments.

[apyrase] in Lumit Reagent	Photometer Results		2 Phase Flow System			Approximate Detection Limit in Flow System
	90% Rise Time	90% Fall Time	90% Rise Time	90% Fall Time	I_{ss}*	
1 mg/mL	5 sec	3-4 min	90 sec	110 sec	500 mv	0.01 μM
10 mg/mL	1 sec	16 sec	40 sec	50 sec	50 mv	0.1 μM

* steady state intensities compared are approximate averages for replicate determinations using 1 μM ATP in the two phase flow system

Table 3-7

This table shows the percent decrease in the steady state intensity for replicate samples of 1 μM ATP using firefly reagent with 1 mg/ml apyrase

Trial #	% Decrease in I_{ss} From Trial n-1 to Trial n
1	--
2	-7.8
3	-5.4
4	-4.9
5	-3.8
6	-4.0
7	-1.8
8	-1.9
9	-1.9
10	-2.9

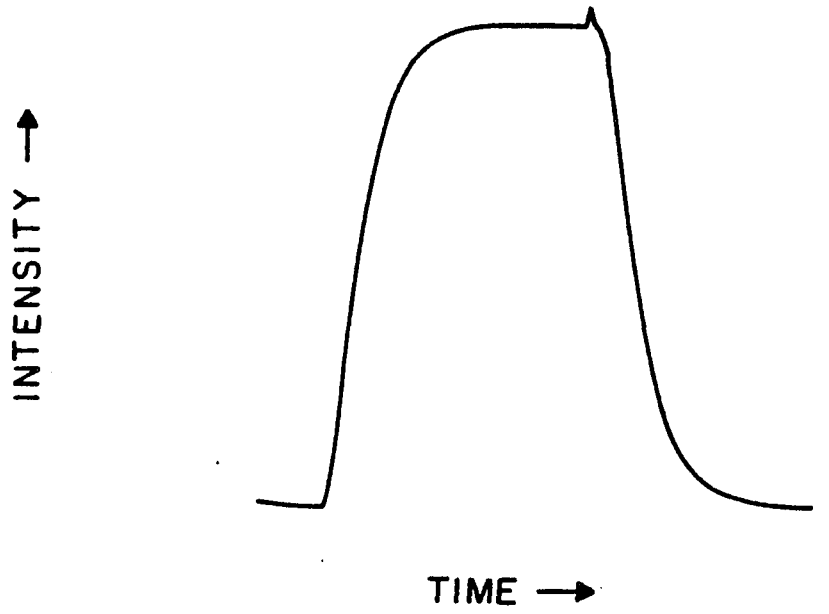

Figure 3-11. Steady state response for replicates of 10^{-6} M ATP in two phase flow system with 1 mg/mL apyrase. Note the decrease in I_{ss} from one replicate to the next. Flow rate = 1 mL/min.

10 mg/mL Apyrase Experiments

The use of a LUMIT reagent with 10 mg/mL of added apyrase resulted in a rapid reaction that exhibited 90% rise times in the photometer that were typically less than 1 second. The fall times were usually 15 seconds or less. These values can be compared to those of the previous experiments in Table 3-6. Figure 3-12 shows the shape of a typical intensity-time curve. The short fall times confirm that any ATP that does not bind quickly to luciferase is consumed by apyrase, thus reducing the amount of ATP available to the BL reaction. This reduces the overall light output for a given amount of injected ATP, while concurrently inceasing the observed rate.

In the flow system, the addition of 10 μg/mL of apyrase to the LUMIT reagent cut the response time roughly in half compared to the 1 mg/mL case, but the sensitivity to ATP suffered, as expected. A typical intensity-time curve for steady state measurement of ATP is shown in Figure 3-13. The 90% rise times averaged approximately 40 seconds; the 90% fall times were typically about 50 seconds (see Table 3-6).

The steady state intensities observed with the 10 ug/mL apyrase reagent for 1 μM ATP were about ten times lower than those seen in the 1 μg/mL apyrase case. This reduction in signal intensity is predicted by the theory, which states that as K_1 (rate constant of CL reaction) is increased, C_i (concentration of analyte in reagent phase) will decrease. Table 3-6 clearly shows the tradeoff between response time and steady state intensity.

Because of the increased consumption of ATP by apyrase in the 10 mg/mL reagent, the signal intensity suffered to the point that the detection limit with this reagent was estimated to be roughly 0.1 μM

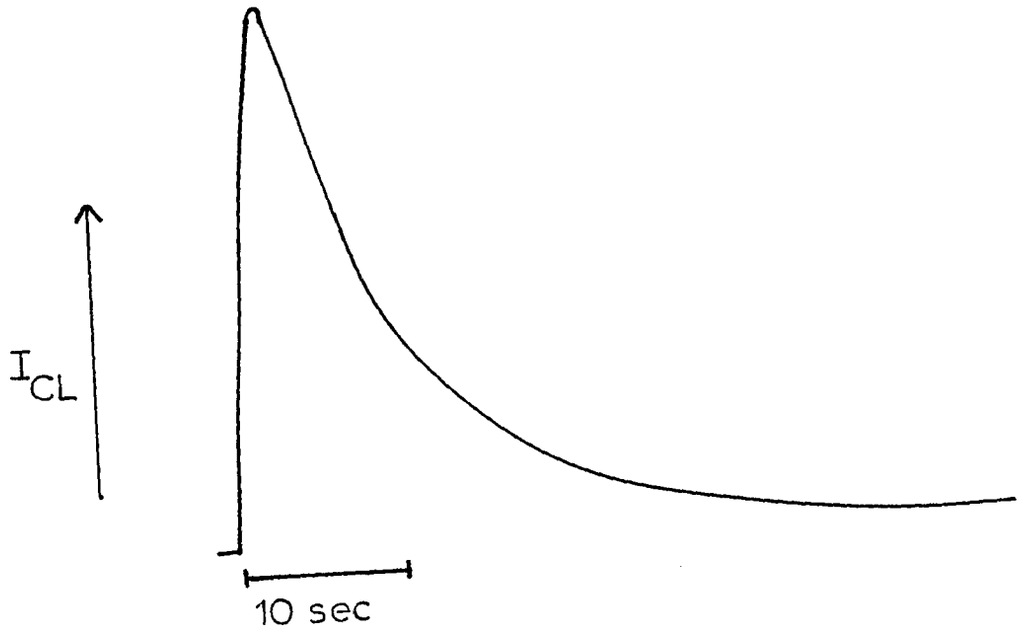

Figure 3-12. Photometer injection of 10^{-6} M ATP using LUMIT reagent containing 10 mg/mL added apyrase.

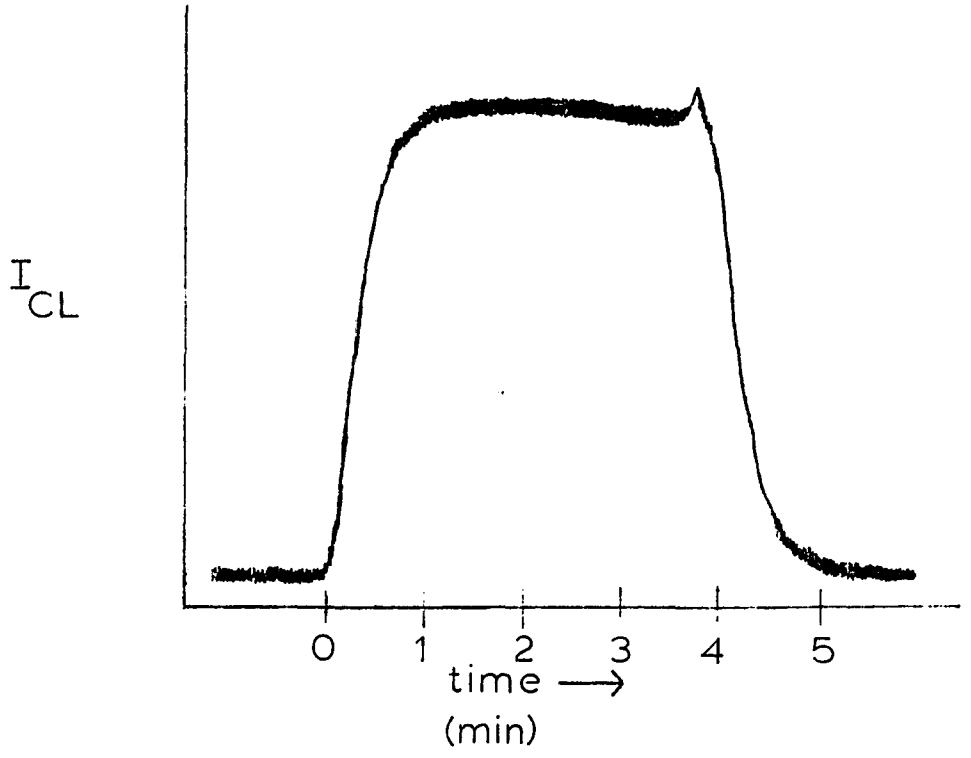

Figure 3-13. Steady state response of firefly system in two phase flow system with 10 mg/mL added apyrase. Flow rate = 1 mL/min.

ATP. This limit was imposed by background and detector noise that made it difficult to resolve the weaker ATP signals. It should be noted that it was difficult to report a true mean steady state intensity for either apyrase level because of the decreases in intensity from one replicate trial to the next.

The loss of steady state intensity with consecutive trials was a problem with the 10 mg/mL apyrase reagent. The magnitude of the loss, as a percent, seemed to be slightly greater at this higher apyrase level (approx. 3-4% vs. 2% for 1 mg/mL), although this was not investigated thoroughly.

Flow Injection Experiments

A short series of experiments was performed in a flow injection mode similar to that described for the luminol-peroxidase system. The ATP-containing sample solution was injected into the flowing stream of background solution (containing buffer, Mg(II) and luciferin). Because the analytical reaction in this case was slower than the luminol-peroxidase reaction, and because some analyte was consumed by the degrading reaction, a larger sample size was necessary in order to obtain an adequate signal. The 1.0 mL sample loop was manually actuated.

Peaks obtained in the flow injection experiments were reasonably smooth and slightly tailed, as illustrated in Figures 3-14 and 3-15. Representative data are collected in Table 3-8. Obviously, the faster analytical reaction gives a faster rise to the peak maximum, but the most noticable difference is in the peak width at the base, or the time required for passage of the entire peak which will ultimately determine sampling rate. Thus, higher sample throughput would be possible with

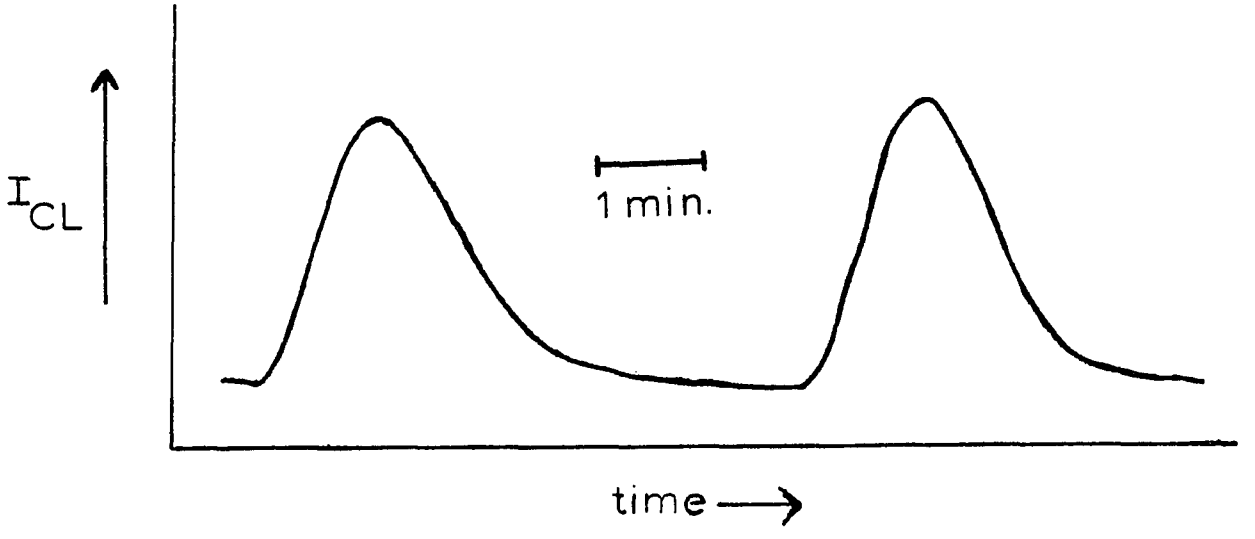

Figure 3-14. Replicate injections of 10^{-6} M ATP into two phase flow system operated in a flow injection mode. Reagent contained 1 mg/mL apyrase. Sample slug size was 1.0 mL. Flow rate = 1 mL/min.

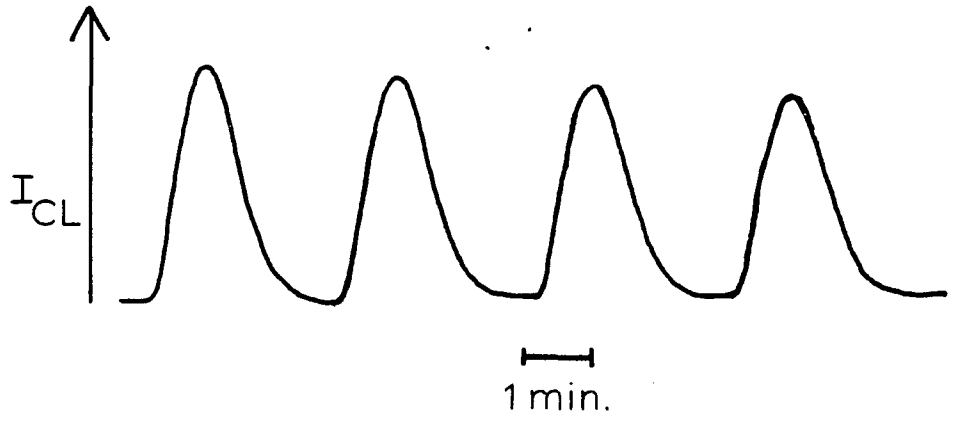

Figure 3-15. Replicate injections of 10^{-6} M ATP into two phase flow system operated in flow injection mode. Reagent contained 10 mg/ml apyrase. Sample size was 1.0 mL. Flow rate = 1 mL/min.

Table 3-8

Performance of firefly reaction in two phase flow system operating in a flow injection mode, with different apyrase levels

[apyrase] in Lumit Reagent	Mean Time to Reach Peak Maximum	Mean $w_{1/2}$	Mean w_b	Mean Peak Height	Sampling Rate
1 mg/mL	66 sec	84 sec	4.0 min	375 mv	15 samples hr^{-1}
10 mg/mL	52 sec	64 sec	2.6 min	47 mv	24 samples hr^{-1}

Data are for 1 μM ATP samples. Volume of the sample loop was 1.0 mL. Flow rate was 1 mL/min. $w_{1/2}$ = peak width at half-height; w_b = peak width at the base.

the 10 mg/mL apyrase reagent. There is a tradeoff, however, because the 1 mg/mL apyrase reagent is considerably more sensitive to ATP.

It was mentioned above that the faster reaction exhibited intensities roughly ten times lower than those of the slower reaction. The difference in intensity is not as pronounced in the flow injection mode because the flow injection experiment does not reach steady state. Because the two reactions proceed at different rates, they reach a different percentage of the steady state with any given amount of sample solution. Thus, the 10 mg/mL apyrase system, being faster, gets closer to its steady state intensity than does the slower reaction.

The flow injection experiments showed a signal loss from one injection to the next, similar to that seen in the steady state experiments. This fact tends to rule out the air bubbles present between all steady state replicates as the cause of the decrease, since there were no air bubbles in the flow system during the flow injection experiments.

Discussion

Reasons for the signal decay observed with consecutive trials are not clearly understood. Decomposition of ATP in sample solutions was ruled out as a cause by a series of experiments in which fresh 1 μM ATP standards were made periodically during the course of an experimental run by dilution of a refrigerated 0.1 mM stock solution prepared from solid ATP immediately prior to the start of the actual flow system experiments. Age of the ATP sample solution seemed to have no effect on the signal.

Although it has not been confirmed by detailed experimentation, it is suspected that signal decay is the result of three factors: thermal

denaturation of reagents, interaction between apyrase and the firefly reagents, and mechanical denaturation. Because of the proximity of the cell to the PMT and the lack of air circulation around the cell, the cell assembly can become rather warm after extended use Although LUMIT should be stable up to 30° C, the temperature in the cell may become warm enough that thermal denaturation of luciferase does become a factor. The increase in signal loss at higher apyrase levels could be an indication of some interaction between apyrase and luciferase that somehow reduces the activity of the luciferase. Perhaps there were some proteolytic enzymes or other interfering impurities in the apyrase preparation. Finally, part of the problem could be caused simply by mechanical denaturation of the proteins because of the action of the stirring bar in the cell.

Buildup of reaction products in the cell should not be a problem since they are free to diffuse out under a concentration gradient. Furthermore, the steady state, once established for a given trial, was seen to be stable for at least 14 minutes, suggesting that product inhibition is not likely to be a cause of decay.

The limited investigations into behavior of the two phase cell with the firefly reaction provided considerable information. The purpose of the experiments was not to optimize an analytical system for ATP, but to observe the performance of the cell with the complicated chemistry of the firefly reaction with kinetics modified by apyrase. The flow cell performed as predicted by theory. It was also demonstrated that addition of an analyte-consuming enzyme could effectively speed up slow analytical reactions, although there is a tradeoff between response time and sensitivity. The cell does represent a considerable improvement

over earlier designs of Freeman (85) and use of highly purified LUMIT reagent makes the system easier to work with and less noisy. Detection limits and linearity, at least for the reagent containing 1 mg/mL apyrase, are good, indicating potential for the development of an analytical system for ATP based on this approach.

The firefly reaction is somewhat slow for a flow injection approach with this cell. Large samples are required in order to obtain adequate intensity, but the flow injection approach does show promise as a feasible alternative to steady state measurement if the system were to be developed specifically for that purpose. Smaller sample sizes would result in narrower peaks, but also lower maximum intensities, so a compromise in these terms would have to be made. The effect of varying flow rate was not investigated, but could be a useful parameter to manipulate.

The two phase flow cell appears to hold promise as a useful approach to automated ATP analyses. Especially important are reproducible, mass transfer controlled mixing and the reuse of expensive enzymes. This approach seems preferable, for example, to the glass rod-bound enzyme system of DeLuca (11), especially in terms of sample handling and precision. The biggest problem with the flow cell method is signal decay from sample to sample. This problem needs to be solved before the system can be useful for ATP analysis.

Bacterial BL

The final experiments with the two phase flow cell were conducted using the bacterial BL reaction. Bacterial BL offers perhaps the greatest potential of any CL or BL system because of the large number of

enzymatic reactions that generate or consume NADH and could therefore be coupled to bacterial BL.

Instrumentation used for the bacterial BL experiments was essentially the same as was used in the firefly BL experiments. Lumase, a highly purified and stabilized preparation of bacterial luciferase and NADH-FMN oxidoreductase w s used as the BL reagent sensitive to NADH. The important features of the bacterial BL coupled reaction for NADH were discussed in Chapter 1. For early experiments, Lumase was reconstituted with 3.0 mL of 0.1 M phosphate buffer, pH 6.9. The phosphate buffer contained 4.6 μg/mL FMN and 0.017% decanal. Solutions containing decanal were made by diluting a stock solution of 1% decanal in methanol. This buffer with FMN and aldehyde was also used as diluent for NADH samples and as a blank solution in the flow system. This arrangement was changed in late experiments, however.

For the first experiments with bacterial BL, Lumase reagent (with added aldehyde and FMN) was loaded in the reagent cell while either buffer or NADH sample was pumped through the spiral channel. Because of the poor solubility of decanal in aqueous solutions, the buffer and sample solutions were quite turbid. Since decanal tended to float to the top of solution, the solutions were shaken occasionally.

Figure 3-16 shows a typical intensity vs time curve for injection of 100 μL of 0.1 mM NADH into 200 μL of Lumase reagent, measured on an Aminco photometer. Again, precision of replicate injections was poor, but the results were sufficient to give an idea of the speed of the reaction. Note in Figure 3-16 that the fairly rapid rise to maximum intensity (approximately 4 seconds) is followed by a rapid but short

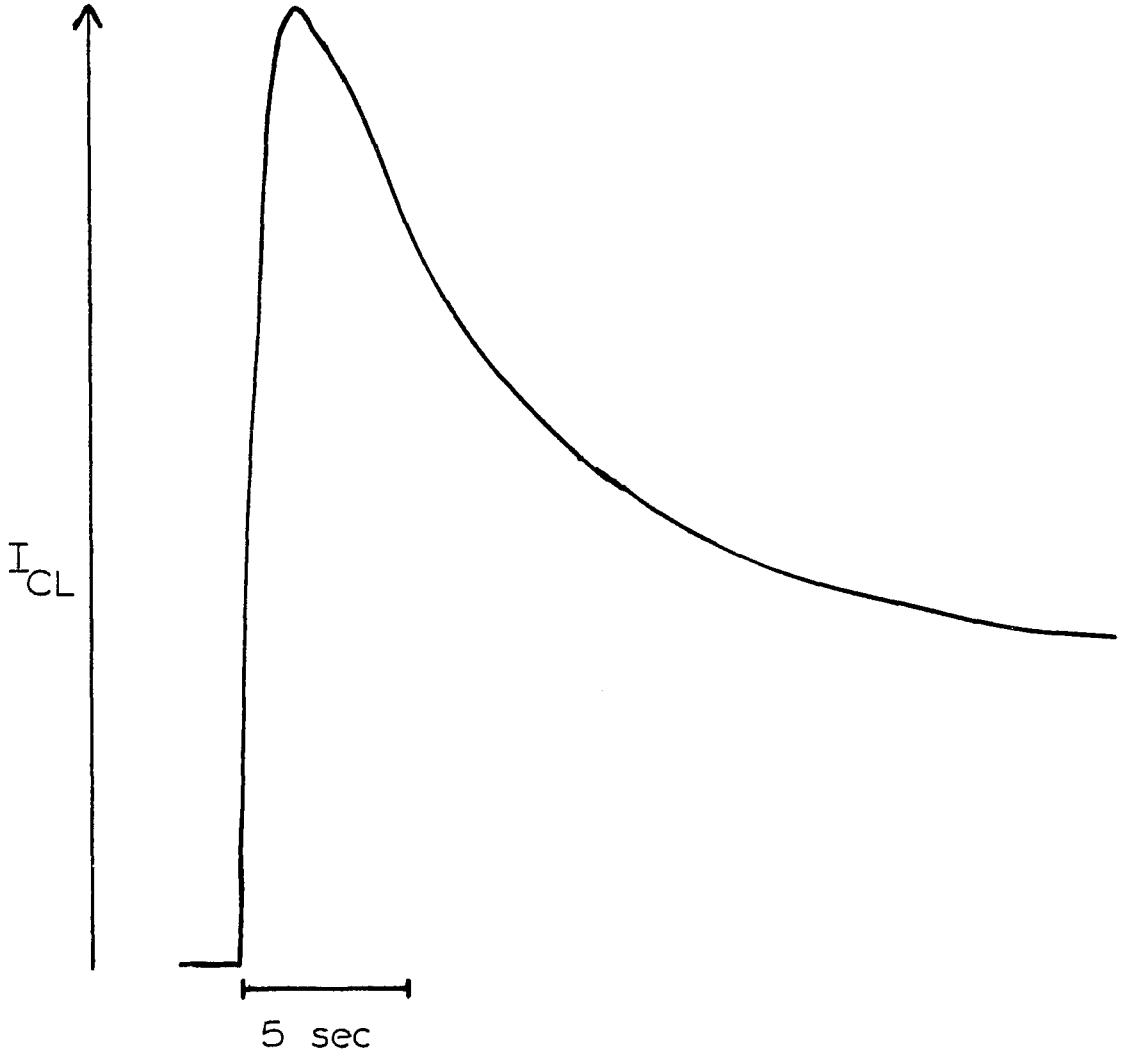

Figure 3-16. Photometer injection of approx. 100 µL of 10^{-5} M NADH into 100 µL LUMASE bacterial BL reagent.

decrease in intensity which in turn is followed by a long-lived plateau and very gradual decay to the baselines.

A representative scan for steady state measurements in the two phase flow system is shown in Figure 3-17. Several observations can be made from the steady state experiments. From Table 3-9 it is apparent that response time is a function of NADH concentration. Lower concentrations seem to give faster response times. This suggests that the overall kinetics of the process are not first order in NADH. The response times are encouraging, however, since the data are for Lumase reagent with no analyte-consuming enzyme. The bacterial system responds faster than the unmodified firefly system.

It is difficult to make any statements regarding the linearily of steady state intensity as a function of NADH concentration because of a severe signal intensity decay problem. This signal decay from one replicate to the next was similar to that observed with the firefly system, though more pronounced. The loss of signal between consecutive replicates was often on the order of 10% or more (Table 3-10). In addition, during some experimental runs the steady state intensity for a given trial tended to decay. This latter effect was not observed in the firefly system.

The system was somewhat less sensitive to NADH than had been hoped, with a detection limit for NADH estimated to be only slightly below 1 μM using fresh sample and reagent solutions.

The fact that response time seemed to be concentration dependent was disappointing, but the biggest problem encountered was the rather rapid decay of signal intensity from replicate to replicate. Reconstituted Lumase should be stable for one working day (88), so even

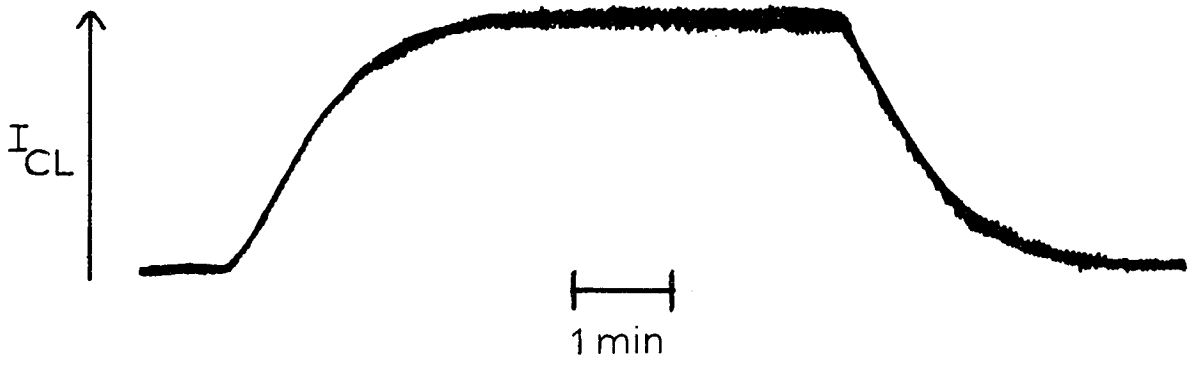

Figure 3-17. Steady state response of bacterial BL in two phase flow system. Sample was 10^{-5} M NADH. Flow rate = 1 mL/min.

Table 3-9

90% rise and fall times as a function of NADH concentration for bacterial BL in two phase flow system oeprated at 1 mL/minute. Sample contained FMN and decanal.

[NADH]	90% Rise Time	90% Fall Time
10^{-4} M	2.7 min	2.7 min
10^{-5} M	2 min	2.2 min
10^{-6} M	1 min	1 min

Table 3-10

Signal decay in two phase flow system using bacterial BL with a single mixed-reagent reservoir

Number	I_{ss} mv	Loss in Intensity From One Replicate to the Next, Expressed as % of First of a Pair
1	82	-
2	71	-13%
3	69	- 3
4	65	- 6
5	61	- 6
6	56	- 7
7	48	-16
8	41	-15
9	36	-10

All data for 10^{-5} M NADH (fresh solution).

with warm temperatures inside the cell and mechanical stirring, such a large decay would not be expected. Initially, it was thought that NADH decomposition in the sample solution could be one of the problems, since NADH is not particularly stable in solution. Preparation and immediate use of fresh 10 uM NADH from a refrigerated stock solution did not change the magnitude of the decay. The observed rate of decomposition of a single buffered NADH solution as measured spectrophotometrically at 340 nm is plotted in Figure 3-18. Such a rate, which corresponds to a lowering of NADH concentration of about 2% per hour, does not account for the large decays seen in the BL flow system.

Visual observation of the sample and buffer solutions gave a clue to a possible cause of the signal loss problem. As mentioned previously, the buffer solution (and, therefore, the sample solution which was diluted with buffer) contained FMN and decanal and was quite turbid because of the decanal. After an hour or so of experimentation, however, it was noticed that these solutions became increasingly clear. The longer the solution was allowed to stand, the greater the degree of clarification. Shaking or stirring did not restore the turbidity. Solutions stored in the dark seemed to clarify more slowly than those exposed to ambient light, indicating a possible photochemical reaction. This loss of turbidity suggested that something was happening in the solution to consume the decanal. The concentrations of NADH and FMN could also have been changing, although this obviously could not be determined simply from observation. The reasons for the apparent consumption of decanal were unknown.

A very short experiment provided further evidence that consumption of decanal was contributing to the signal loss problem. Toward the end

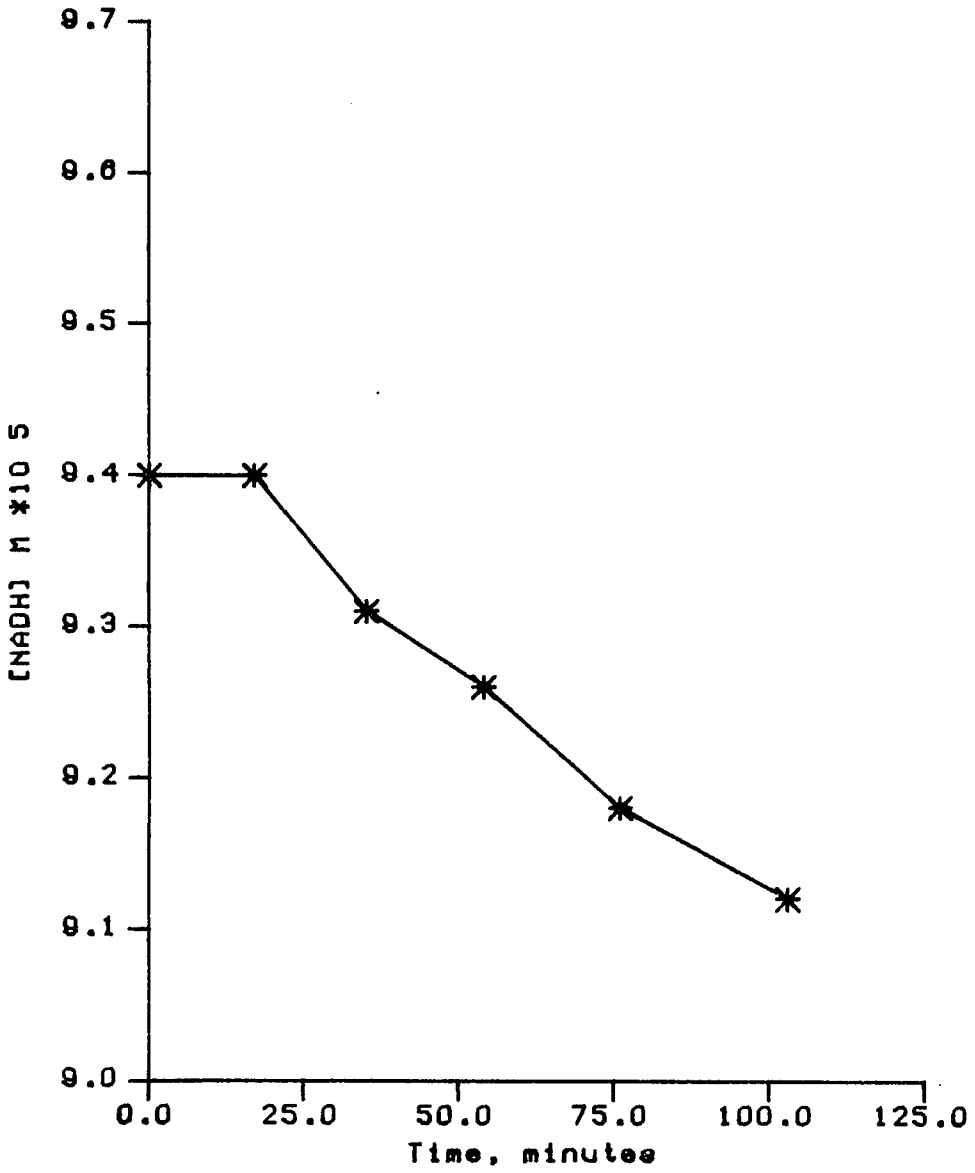

Figure 3-18. Plot showing degradation of NADH over time, as measured by absorbance at 340 nm.

of a steady state experiment in which the signal intensity was decreasing by about 10% for each consecutive aliquot of sample, 600 µL aliquots of 1% decanal in methanol were added to the sample solution before each trial. This increased the turbidity and resulted in signals that did not show the decrease in intensity from one replicate to the next as had previous steady state measurements. Because the fresh additions of decanal seemed to significantly reduce the signal decay problem it was apparent that somehow decanal was decomposing or being consumed when solutions were allowed to stand.

Although the nature of decanal consumption was not understood, it was suspected that there may have been some interaction between the various components of the sample solution that was responsible for decanal decomposition, as well as possible consumption of FMN and/or NADH. To determine if there was indeed an interaction between solution components, a new flow system was designed (Figure 3-19). The modifications allowed FMN, decanal, and NADH to be stored in separate vessels and mixed in the flow system. This way, components would not be mixed until immediately before they were needed. Hopefully, any destructive interaction between components would be slow enough to be unnoticible with the rapid mixing and subsequent use in the BL reaction. The decanal reservoir was magnetically stirred to maintain a homogeneous dispersion of decanal in the aqueous solution. The FMN reservoir was loosely covered with brown paper to reduce ambient light incident on the solution.

In this system, Cole-Parmer 7013 pump heads with 6408-41 tubing were used on a Cole-Parmer peristaltic pump motor. The FMN concentration was 16-20 mg/L and decanal concentration was approximately

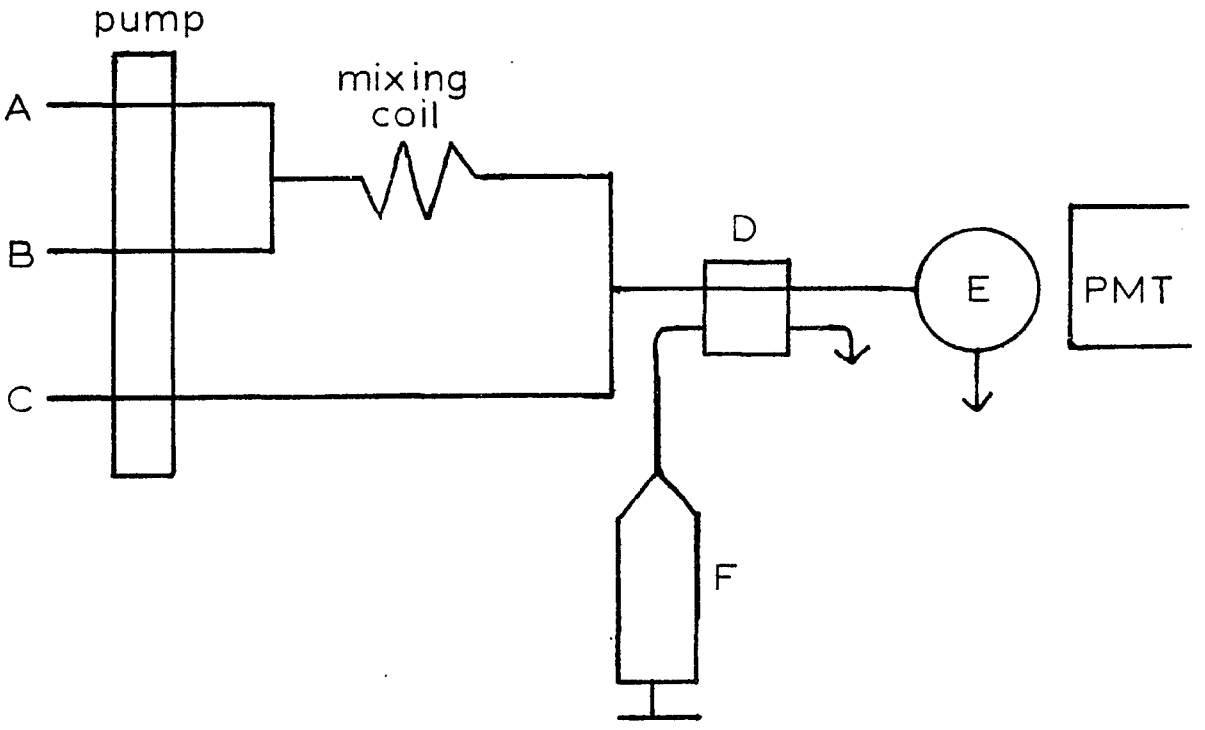

Figure 3-19. Final two phase flow system for bacterial BL. A: FMN solution; B: stirred decanal solution; C: NADH sample solution (or blank buffer); D: Rheodyne 4-way injection valve (for flow injection experiments); E: two phase flow cell; F: syringe for injection of sample (flow injection mode).

0.068% v/v. Both solutions were made with 0.1 M phosphate buffer, pH 6.9. This flow system will produce an FMN concentration of 4 mg/ML and decanal concentration of 0.017% at the cell interface. A flow rate of about 0.8 mL/minute per channel was used to give a total flow rate through the cell of about 2.4 mL/minute. This was the slowest flow rate that could be used with 7013 pump heads and 6408-41 tubing without causing overheating of the pump motor.

For steady state measurements with the three channel flow system, switching from background to sample solution was simply accomplished by inserting the third channel's tubing into the appropriate reservoir, as in previous experiments. The sample solutions were refrigerated between trials to minimize any problems associated with NADH degradation.

Mixing the components in the flow system provided much better results in terms of signal loss between replicates than did the experiments using one sample solution containing NADH, FMN, and decanal. Data for a representative set of replicates, collected in Table 3-11, illustrates this improvement. Signal decay was not a problem in these experiments until the system had been in use for three to four hours. After this length of time, the intensities start to drop from one replicate to the next. A loss of signal after this length of time is not too suprising, and could be caused by thermal denaturation of the oxidoreductase and/or luciferase enzymes. Both enzymes, though stabilized by other components of the Lumase reagent, are rather heat labile. After three hours of operation, the cell becomes quite warm, as mentioned in the firefly BL section. Mechanical denaturation of the enzymes by the magnetic stirring bar also could be partially responsible for the breakdown of the system after several hours.

Table 3-11

Replicate steady state measurements in two phase flow system using separate reagent and sample reservoirs. Data for 5×10^{-6} M NADH.

Replicate Number	Intensity of Steady State Signal
1	19.4 mv
2	20.6
3	20.6
4	20.6
5	20.6

With the three-channel system, as with the single channel system, response time seems to increase slightly with NADH concentration. Mean 90% rise times to the steady state as a function of concentration of NADH are collected in Table 3-12. The 90% fall times are slightly longer than the rise times.

The steady state intensity as a function of NADH concentration was only investigated over a limited range of NADH concentrations because of the limited useful lifetime of the Lumase reagent in the cell. The relationship between concentration and steady state intensity over the range studied is fairly linear, but it must be remembered that this is a small range of concentrations. Typical data are collected in Table 3-13 and plotted in Figure 3-20. The estimated detection limit for the system as described here is slightly below 10^{-6} M NADH in the sample solution. This corresponds to an NADH concentraton of about 3×10^{-7} M when the sample reaches the cell. Reproducibility of steady state intensities for successive replicates, as shown in Table 3-13, was adequate, but not great.

A few short experiments in a flow injection mode were performed, using the manually actuated Rheodyne valve positioned as shown in Figure 3-19. Buffer solution flowed in the sample channel while the other channels were as in the steady state experiments. The results were encouraging; some representative data for injection of 1×10^{-4} M NADH using a 500 μL sample loop are collected in Table 3-14. While the system as described does seem to work reasonably well in a flow injection mode, there are several factors that would seem to be contraindications to its routine use as a clinical method without some modifications. Fairly large samples are required and the peaks are

Table 3-12

Mean 90% rise times as a function of NADH concentration in two phase flow system using bacterial BL; separate reagent and sample reservoirs

[NADH]	Mean 90% Rise Time
1×10^{-6} M	1.5 min
5×10^{-6}	1.92
1×10^{-5}	1.95
1×10^{-5}	2.10
1×10^{-4}	2.6

Table 3-13

Steady state intensity as a function of NADH concentration; two phase flow cell using Lumase reagent

[NADH]	Mean I_{ss}	RSD for Replicates
1 μM	3.7 mv	6
5	20.4	3
10	39.2	5
50	177.3	5

mean I_{ss} = mean steady state intensity for a set of replicate

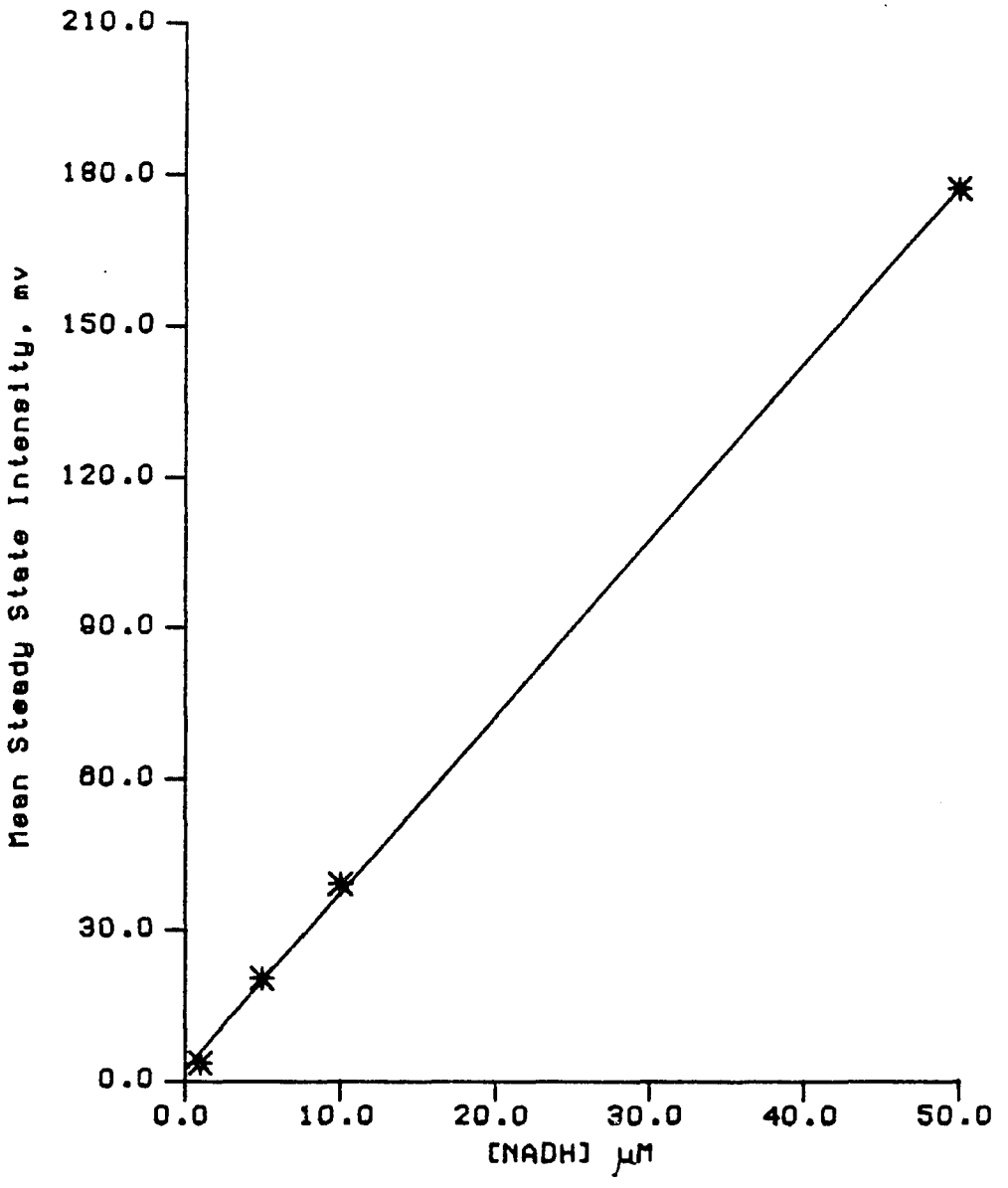

Figure 3-20. Standard curve for a limited number of NADH standards in the two phase flow system.

Table 3-14

Results from bacterial BL in two phase flow system operated in a flow injection mode

Replicate Number	Peak Height	$W_{\frac{1}{2}}$	W_{base}	Time to Reach Peak Maximum
1	284 mv	1.34 min	4.3 min	47 sec
2	267	1.20	4.3	45
3	276	1.34	5.0	43
4	272	1.34	4.7	43
5	276	1.30	4.7	43
means:	273	1.30	4.6	44
RSD:	2.7%	4.4%	7%	4%

$W_{\frac{1}{2}}$ = width at half-height

W_{base} = width at base of peak

rather wide (Table 3-14). Peak width would severely limit sampling rate. Since this system had not been designed specifically for flow injection methodology, and no attempts were made to optimize the system, it is not unreasonable to assume that a more efficient flow injection system could be designed to make use of the bacterial BL reaction in the two phase flow cell.

The results of the bacterial BL experiments in the two phase flow cell show that there is the potential to develop several interesting analytical systems based on the two phase CL approach. The results with bacterial BL were encouraging. It was not necessary to add an NADH-consuming enzyme, although such an enzyme could be used if faster response times were desired. The detection limit for NADH was not as low as had been hoped, especially in consideration of the ATP results. This would limit the effectiveness of adding an NADH-consuming enzyme to increase response time since it would necessarily involve a loss of signal.

Keeping the decanal, FMN, and NADH separate until they mix in the flow system seems to be the key to assuring stable response with time. The reasons this is so, presumably because of some interaction between FMN, aldehyde and possibly NADH, are not understood and were not further investigated.

It is clear from the results presented here that the two phase flow cell offers a feasible approach to analysis based on firefly and bacterial BL, although further investigation would be required in order to develop a routinely useful and efficient analytical method.

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APPENDIX

Solution of Expression for C_i

Equation 3.3 can be solved in the following manner to obtain the express for C_i given in equation 3.4.

$$\frac{dC_i}{dt} = K(C_o-C_i)-K_iC_i \qquad (3.3)$$

Equation 3.3 can be rearranged to

$$\frac{dC_i}{dt} = KC_o-(K+K_1)C_i \qquad (A.1)$$

or,

$$dC_i = [KC_o-(K+K_1)C_i]dt \qquad (A.2)$$

Equation A.2 can be solved by separating the variables in order to have terms involving C_i combined for the purposes of integration:

$$\frac{dC_i}{KC_o-(K+K_1)C_i} = dt \qquad (A.3)$$

letting $u = KC_o-(K-K_1)C_i$ (A.4)

then $du = -(K+K_1)dC_i\ C_i$ and $dC_i = \frac{-1}{K+K_1}\,du$

so that A.3 becomes $\frac{-1}{K+K_1}\,\frac{du}{u} = dt$ (A.5)

From A.4 and the initial conditions ($C_i = 0$ at $t = 0$), it can be shown that $u = KC_o-(K+K_1)\,C_i$ will have positive values, so that $u = u$ and A.5 then gives:

$$\frac{-1}{K+K_1} \ln u = t+C \qquad (A.6)$$

or,

$$\frac{-1}{K+K_1} \ln[KC_o-(K+K_1)C_i] = t+C \qquad (A.7)$$

The initial conditions allow evaluation of the constant of integration C:

$$c = \frac{-1}{K+K_1} \ln KC_o \qquad (A.8)$$

thus,

$$\frac{-1}{K+K_1} \ln[KC_o-(K+K_1)C_i] = t-\frac{1}{K+K_1} \ln KC_o \qquad (A.9)$$

Equation A.9 can be rearranged and solved for C_i:

$$\frac{1}{K+K_1}[\ln KC_o-\ln(KC_o-(K+K_1)C_i] = t \qquad (A.10)$$

$$\ln \frac{KC_o}{KC_o-(K+K_1)C_i} = (K+K_1)t \qquad (A.11)$$

$$\frac{KC_o}{KC_o-(K+K_1)C_i} = e^{(K+K_1)t} \qquad (A.12)$$

$$\frac{KC_o-(K+K_1)C_i}{KC_o} = e^{-(K+K_1)t} \qquad (A.13)$$

$$1-\frac{(K+K_1)}{KC_o} C_i = e^{-(K+K_1)t} \qquad (A.14)$$

$$C_i = \frac{KC_o}{K+K_1} (1-e^{-(K+K_1)t}) \qquad (3.4)$$