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CLINICAL APPLICATIONS OF CHEMILUMINESCENCE DETECTION

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CLINICAL APPLICATIONS OF CHEMILUMINESCENCE DETECTION

BY

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B.S., Kings College, 1974

DISSERTATION

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September 4, 1982
Date

DEDICATION

To my parents

ACKNOWLEDGEMENTS

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ABSTRACT

CLINICAL APPLICATIONS OF CHEMILUMINESCENCE ANALYSIS

by

MARY LYNN GRAYESKI

University of New Hampshire, December, 1982

This dissertation evaluates the potential of chemilumin-
escence analysis for two types of clinical applications:
(1) rapid analysis with a flow injection system which con-
sumes small reagent volumes (25 µL) and (2) a new approach
to chemiluminescence immunoassay.

The first application studied uses the firefly reaction
with a flow system to analyze for adenosine triphosphate (ATP).
Two flow configurations called the "valve within a valve" and
"merging zones" configurations were evaluated with respect to
effect of parameters such as flow rate and sample volume on
precision, sensitivity and sample throughput. With the
appropriate choice of parameters, both systems achieve pre-
cisions of 1-2% relative standard deviation and throughputs
of 5-10 measurements per minute. The merging zones configura-
tion consumes smaller volumes of sample.

The second project demonstrates a new approach to immuno-
assay based on chemical excitation of fluorophor labelled
immunochemicals using the peroxyoxalate reaction. The effects
of fluorophors, esters, and solvents, etc. on the

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chemiluminescence measurement were evaluated. The conditions for achieving the best precision (1-3% R.S.D.) with a high water component in the measurement were rhodamine B in Tris buffer adjusted to pH 8.00, 10^{-3} M bis(2,4,6-trichlorophenyl) oxalate (TCPO) in ethyl acetate and 10^{-3} M hydrogen peroxide in acetonitrile in the ratio 5:2:3. Under these conditions, the detection limit for rhodamine B is 10^{-9} M. The detection limit is established by variations in the background chemiluminescence.

The possibility of both homogeneous and heterogeneous immunoassays was evaluated. It was shown that the chemiluminescence excitation efficiency decreases when fluorophors were bound to albumin but increases when the fluorophor was coupled to folic acid. The feasibility of a homogeneous immunoassay was demonstrated by binding albumin to rhodamine labelled antiserum. It was shown that the chemiluminescence intensity of the fluorophor labelled antibody decreases with increasing concentrations of bound antigen. The heterogeneous chemiluminescence immunoassay was evaluated by comparison with a commercially available fluorescence immunoassay kit by Bio Rad. Both immunoassays are limited by imprecision of mixing of the solvent systems for the chemiluminescence measurement.

PART I

FLOW INJECTION ANALYSIS WITH CHEMILUMINESCENCE DETECTION

CHAPTER I

BACKGROUND

Chemiluminescence

Introduction

There is growing interest in chemiluminescence (CL) and
bioluminescence (BL) methods of analysis as reflected by
several recent reviews (Seitz, 1981; Seitz and Neary, 1976,
1977; Wehry, 1980, 1982; Gorus and Schram, 1979; Whitehead
et al., 1979; Isacsson and Wettermark, 1974). These methods
offer several advantages over conventional techniques: high
sensitivity, wide linear dynamic range, low cost per test
with relatively simple and inexpensive equipment. Because
of these advantages, chemiluminescence is expected to make
an impact on clinical analysis.

There are several reasons for the surge of interest in
CL and BL. One is the need for alternatives to radioisotope
labels in immunoassays. CL and BL are among the few methods
with detection limits low enough to compete with radioiso-
topes in many immunological assays. Another reason for
increased interest in CL and BL methods is that they have the
potential to increase sample throughput and reduce sample con-
sumption in clinical analyses. Finally, the recent commercial
availability of improved instrumentation and reagents has
made these methods more accessible to clinical laboratories.

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Theory

Chemiluminescence may be defined as the emission of
light accompanying chemical reactions. CL that occurs in a
living system or is derived from one is known as biolumin-
escence and may be considered a special case of CL. Three
requirements must be met for CL to occur: (1) The chemical
reaction must release enough energy to populate an excited
energy state; (2) The reaction pathway must favor the forma-
tion of an excited state product; and (3) The excited state
product must be capable of emitting a photon itself or trans-
ferring its energy to another molecule that can emit.

In principle, one molecule of a chemiluminescent reac-
tant can react to form one electronically excited molecule,
which in turn can emit one photon of light. Light yields can
therefore be defined in terms of photons of light emitted per
molecule of chemiluminescent reactant. This is the chemilu-
minescent quantum yield or efficiency and can be expressed as

$$\phi_{CL} = \frac{\text{\# of photons emitted}}{\text{\# of molecules reacting}}$$

The highest efficiencies are observed for BL such as the
firefly reaction which is reported to have a ϕ_{CL} of 88%
(Seliger and McElroy, 1960). Most reactions are inefficient
with ϕ_{CL} values on the order of 1% or much less.

The factors which comprise the CL efficiency, ϕ_{CL}, are
the efficiency of excited state production, ϕ_{EX}, and the
fluorescence efficiency, ϕ_F

$$\phi_{CL}\left(\frac{\text{\# photons emitted}}{\text{\# molecules reacting}}\right) = \phi_{EX}\left(\frac{\text{\# excited states}}{\text{\# molecules reacting}}\right) \phi_F\left(\frac{\text{\# photons emitted}}{\text{\# excited states}}\right) \quad (1)$$

Chemiluminescence reactions can be used for analysis by adjusting the concentrations so that the CL intensity, I_{CL}, is related to the concentration of the reactant to be determined. At any time, t, I_{CL} is given by

$$I_{CL}(t)\left(\frac{\text{photons}}{\text{sec}}\right) = \phi_{CL}\left(\frac{\text{photons}}{\text{molecules reacting}}\right)\frac{dC(t)}{dt}\left(\frac{\text{molecules reacting}}{\text{sec}}\right) \quad (2)$$

where $dC(t)/dt$ is the reaction rate for the starting chemiluminescent reagent. For a reaction first order in C, equation (2) reduces to

$$I_{CL}(t) = \phi_{CL}(t) \, K\left[C(t)\right] \quad (3)$$

where K is a rate constant.

I_{CL} can be measured at a specific time after mixing (kinetic analysis) or it can be integrated for the entire time of light emission (endpoint analysis). I_{CL} can also be integrated for a specified time period. Because CL intensity varies with time as reactants are consumed, it is crucial that CL reactions are initiated in a controlled and reproducible manner to achieve precise measurements. The most common method is to rapidly mix CL reactants and then to measure the CL intensity as a function of time after mixing. This approach has been called the "batch method" in a recent review article (Seitz, 1981). Most commercial instruments

use this method because it minimizes reagent consumption.

It is also possible to mix CL reagents continuously and measure steady-state light output. This is called the "continuous method" but is more commonly used in gas phase CL and will not be considered further here.

Method of Measurement

A typical intensity-time curve for the batch method is shown in Figure 1. The shape of this curve depends on the kinetics of the reaction and any change in ϕ_{CL} with time (ϕ_{CL} will vary if either reactant or product quenches light emission or if the light emission process involves energy transfer) (Seitz, 1981). The initial part of the intensity-time curve may also be affected by the mixing process. The initial part of the intensity-time curve may not be recorded if reactants are mixed externally before being placed in front of the detector. In this case the interval between mixing and measurement must be reproducible for precise results.

Instrumentation

The only apparatus required for CL analysis is a light detector, a system for mixing the reactants, and in some cases, a filter to resolve the CL of interest from other sources of radiation.

Detectors. Photoelectric detectors such as photomultiplier tubes are widely used for measuring CL. Because of the
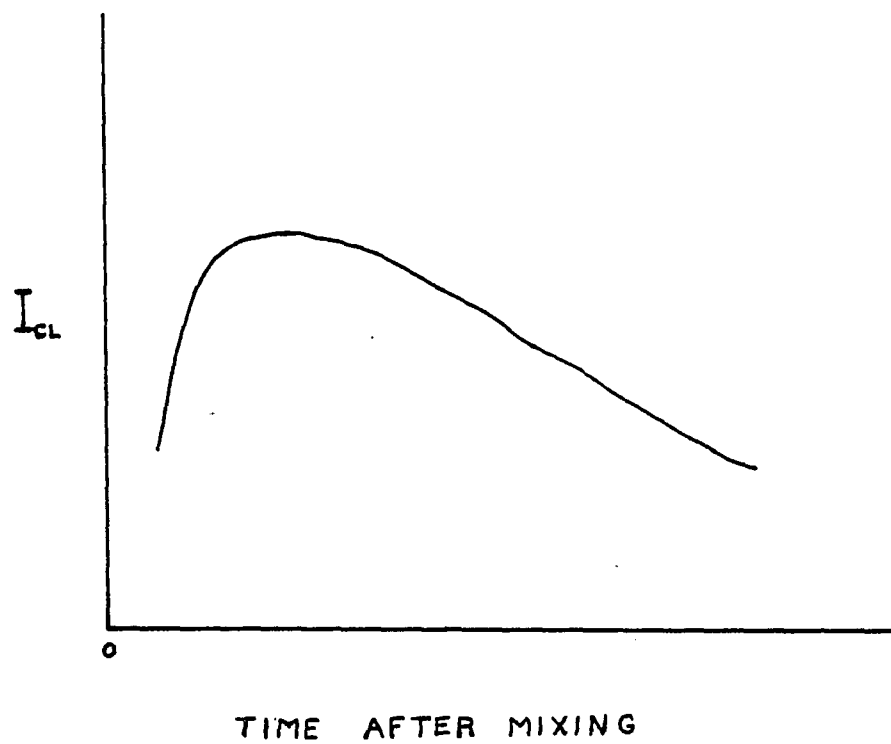
Figure 1. Hypothetical curve showing chemiluminescence
intensity as a function of time after the reagents
are mixed. This is characteristic response for the
batch method of chemiluminescence analysis.

excellent detectability of CL methods, the light detector
need not be particularly sensitive but should have adequate
spectral response at the CL wavelength maximum. Detection
limits are often blank limited rather than detector limited.

Cells. CL cells must be reproducible optically and
reproducibly positioned with respect to the detector. Many
instruments use disposable cells for speed and convenience
but this results in imprecision due to variations in optical
properties of the cells,

Mixing Methods. A variety of mixing methods have been
used. For batch analysis, the sample can be added gently to
the chemiluminescent reagents and then swirled or stirred.
Another approach is to add the sample and/or the reagents
forcibly enough to induce mixing without further stirring.
This can be done manually with a syringe or by using an auto-
matic injector. Some commercial instruments are equipped with
automatic injectors. In continuous analysis, mixing is
accomplished by natural hydrodynamic processes or by use of
special devices in the flowing stream of reagent and sample.

It should be noted that a given mixing method may not
work equally well for all reactions. An automatic injector
that has been designed for firefly ATP assays will be inade-
quate for mixed solvent systems. In general, reactions with
mixed solvent systems present a difficult mixing problem
which is not adequately handled by automated mixing devices
so mixing must be done manually.

An important consideration in the choice of mixing

methods is the kinetics of the analytical reaction. Ideally
a reaction with fast kinetics should be mixed in front of
the detector but this is not always possible. When the
reagents are mixed before placing in front of the detector,
much of the light intensity is not measured causing a loss
of sensitivity. Precision is also affected by kinetics.
Fast kinetics require fast mixing for good precision but this
is less critical for slow kinetics.

Advantages and Disadvantages of Chemiluminescence Assays

Detection Limits. One of the most important advantages
of CL methods is their high detectability. For example, as
compared with spectrophotometry, the BL assay of NADH is esti-
mated to be some 25,000 fold more sensitive (Stanley, 1974).
The reason for this high detectability is the fact that light
can be measured at very low levels. In CL methods, there are
no instrumental sources of background radiation. In practice,
detection limit is often limited by reagent purity rather
than light measuring capability. For many reactions the
background emission in the absence of analyte is fairly
intense so that detection limits are blank limited rather
than detector limited.

Linear Response Range. Since CL is an emission process
(as opposed to absorption), response is usually linearly pro-
portional to concentration from the lowest detectable concen-
tration up to the point where it is no longer possible to
maintain an excess of reactants relative to the analyte

(Whitehead et al., 1979).

Speed of Analysis. This depends on the kinetics of the chemiluminescent reaction, For some reactions, a rapid flash is observed, the peak height of which may be related to analyte concentration, In other cases, the light lasts for several minutes or even days. In this situation, an integral of the intensity-time curve is frequently measured, but this reduces the throughput and speed of the analysis.

Cost. The sensitivity of CL assays allows the use of very small reagent volumes, thereby reducing the cost per test. However, the cost of highly purified CL reagents, particularly enzymes used in BL, must be taken into account but the use of small volumes still makes the assay cost effective,

Specificity. Because they involve enzyme catalysis BL reactions are usually highly specific. CL reactions are less intrinsically specific but can be coupled to highly specific enzymatic processes.

Reactions. A limitation of CL and BL is that, although many reactions exhibit CL, few are efficient enough to be used for analytical purposes. However, the fact that these reactions can be coupled to many biochemical processes extends their range of applicability.

Toxicity and Stability. CL reagents (e.g. luminol) are stable and have been used as non-toxic, stable alternatives to radio and enzyme labels in immunoassay. BL reagents such as luciferases suffer from the same stability problems as

other enzymes.

Applications

Several CL reactions have been successfully applied to clinical analysis. These include (1) the firefly reaction which is used to determine adenosine triphosphate (ATP), (2) bacterial BL which is used to determine the reduced form of nicotinamide adenine dinucleotide (NADH), (3) the luminol reaction, often used to measure hydrogen peroxide, and (4) the peroxyoxalate reaction, also used to measure hydrogen peroxide. Two of these reactions will be discussed in this dissertation. The peroxyoxalate reaction will be discussed in Chapter III and the firefly reaction will be considered here.

Firefly Reaction

Mechanism and Analytical Characteristics. Numerous reviews deal with the firefly reaction (DeLuca and McElroy, 1974; Kishi et al., 1968; Lundin and Thore, 1975) and the proposed mechanism is shown in Figure 2. The initial reaction is the rapid conversion, in the presence of Mg(II) and ATP, of luciferin to luciferyl adenylate, which, in the presence of luciferase, combines with molecular oxygen to give an oxyluciferyl adenylate-enzyme complex in the excited state. After emission, the ground-state complex dissociates to form enzyme, AMP, oxyluciferin, and carbon dioxide, the last being derived from the carboxyl group of luciferin. The
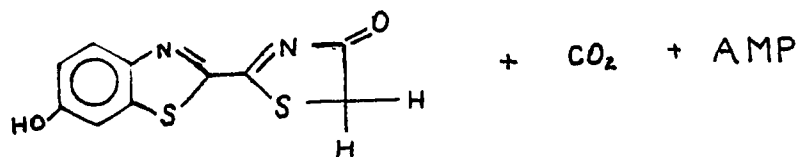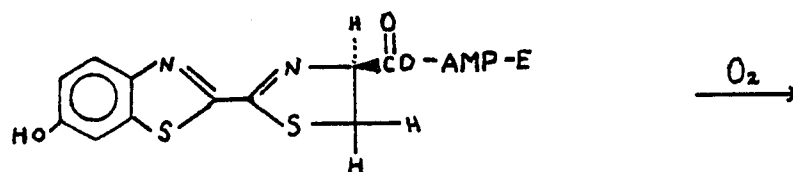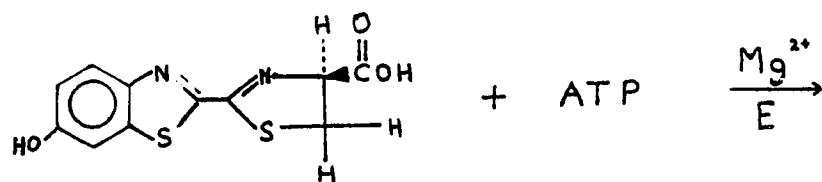
Figure 2. Proposed mechanism for the firefly reaction.

reaction is best carried out at pH 7-8 (Seliger and Morton, 1968). The color of the light emitted varies for different firefly species. This is thought to be due to inter-species differences in the structure of luciferase, because the structure of luciferin is identical for all species (Kishi et al., 1968). Intensity and wavelength of maximal emission are also altered by changes in pH, ionic strength, temperature, and the presence of chlorides of Zn(II) and Cd(II) (Seliger and Morton, 1968).

The analyte of major interest is ATP. Absolute detection limits generally fall in the range of 10^{-11} to 10^{-14} moles of ATP. The more highly purified the luciferase, the lower the detection limit. Stability of the enzyme is also affected by purity depending on the method of preparation. A luciferin-luciferase preparation which is stable for several days at room temperature is commercially available but expensive.

The kinetics of the firefly reaction have been studied extensively (DeLuca and McElroy, 1974). After a lag time of about 25 msec, there is a rapid increase in light intensity to a maximum followed by a gradual decrease caused by inhibition of the luciferase by pyrophosphate and oxyluciferin. The rising part of the curve can be used for ATP analysis but this adversely affects the precision.

Two types of interferences must be considered in the firefly ATP assay. One type is due to contaminating enzymes in the luciferase preparation which catalyze reactions

producing or consuming unwanted ATP. Purified luciferase reduces this problem (Lundin and Thore, 1975). The other type of interference is anion and ionic strength effects on the intensity-time curve (Denburg and McElroy, 1970). Anions such as citrate which bind Mg(II) inhibit the firefly reaction. Such anions may be present in the matrix to be studied and should be avoided in preparing buffers for the reaction. These interferences can be handled by the method of standard additions.

Applications. Most applications of the firefly ATP assay involve relating ATP concentrations to biomass, i.e. the total weight of living organisms, usually bacteria, in various types of samples. Other applications involve measuring ATP in erythrocytes, platelets, and pancreatic islets (Nakac et al., 1962).

Many analyses based on ATP allow the measurement of other biological compounds using enzymic coupled reactions. For example, creatine kinase (CK) activity can be determined by coupling the following reaction

$$ADP + creatine\ phosphate \underset{\longleftarrow}{\overset{CPK}{\longrightarrow}} ATP + creatine$$

to the firefly reaction. The CL reagents are added to the products of the above reaction and the light produced is a measure of CK activity. Other coupled assays are possible since many biological processes result in the formation or consumption of ATP (Lundin, 1979).

Flow Injection Analysis

Introduction

Flow Injection Analysis (FIA) allows simple, versatile, rapid, low-cost automated analysis. This method of sample handling was introduced independently by Ruzicka and Hansen (1975) in Copenhagen and by Stewart (1974, 1976) in Washington, D.C. It provides an alternative to methods based on segmented flow.

Segmented flow systems have been used extensively by clinical laboratories since their introduction by Skeggs in 1957. These systems are called continuous flow analysis and use flowing streams segmented by air to prevent carryover of sample (Betteridge, 1978). By contrast, FIA does not rely on air segmentation to prevent successive samples from interacting. Instead it minimizes sample spreading by controlling flow conditions. This simplifies the development of automated assays to a degree that is not possible with segmented flow. FIA has already achieved performance characteristics competitive with and in some cases superior to continuous flow. Consequently, it is likely that FIA techniques will play a significant role in clinical laboratories in the future.

Principles

The capabilities and limitations of FIA can best be understood by considering the principles of the technique. A simple flow injection system is represented in Figure 3.
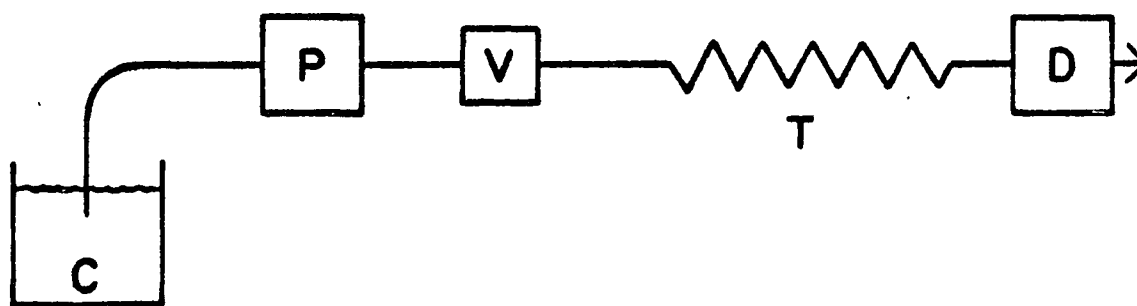
Figure 3. Simple system for flow injection analysis. C is
    a carrier fluid, P is a pump, V is a valve for inter-
    posing a volume of sample in the flowing reagent stream,
    T is a coil of tubing and D is a detector.

A liquid sample is injected into a flowing nonsegmented con-
tinuous carrier stream of a suitable liquid. The injected
sample is transported through a coil of tubing to a detector
which records an appropriate analytical parameter of the
sample as a function of time, As it flows down the tubing,
the sample mixes with the carrier stream which may contain
reagent that reacts with the sample or may only serve to
transport the sample to a detector,

The mixing process occurs under conditions of laminar
flow by natural hydrodynamic forces, The process is illus-
trated in Figure 4. Instead of flowing down the tube as a
compact plug, the sample develops a parabolic profile because
the solution at the walls is retarded by friction while the
solution in the center of the tube moves twice as fast as the
average flow rate. The result is that the sample is diluted
and spread out.

FIA deals with sample spreading by controlling the flow
conditions so that the sample mixes with the carrier stream
by radial mass transfer. One mechanism of radial mass trans-
fer is diffusion. At the front part of the parabolic profile,
sample diffuses from the center of the tube toward the walls,
while at the back part, sample diffuses from the wall toward
the center. The net result is mixing of the sample with the
carrier and minimal sample spreading.

Radial mass transfer can be promoted by appropriate
choice of operating parameters: (1) slow flow rate to allow
time for diffusion to occur, (2) small tubing diameters
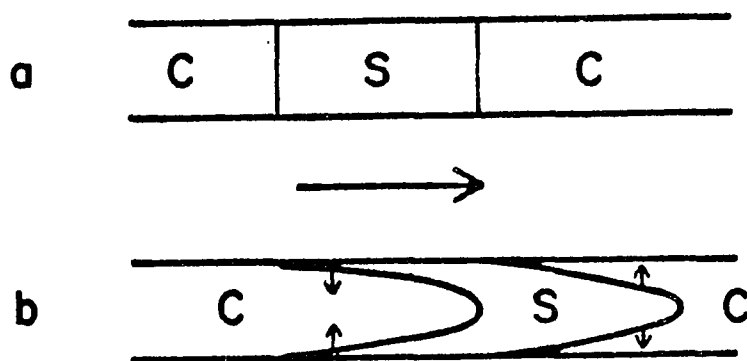
Figure 4. Representation of flow of sample, S, in an unseg-
    mented stream of carrier, C. a shows the distribution
    of sample at the moment it is introduced to the carrier
    before any flow has taken place. b shows the distri-
    bution of sample after some flow has occurred. The
    small arrows in b indicate the direction of sample dif-
    fusion in response to radial concentration gradients
    that develop in the flowing stream.

(typically .5mm) so that diffusion distances are small, and (3) coiling the tube to induce radial mass transfer.

Sample dilution and spreading is a critical parameter in FIA. The parameter used to express the extent of dilution is the dispersion, D, which is defined

$$D = C_O/C_{max} \tag{4}$$

where C_O = initial concentration of sample before dilution and C_{max} = maximum concentration of sample that flows past the detector. Thus a dispersion of 2 means a 1:1 sample dilution. Dispersion is related to flow rate and time as shown in the following approximate equation (Ruzicka and Hansen, 1978):

$$D = \left(2/W\right)FT^{\frac{1}{2}} t^{\frac{1}{2}} \tag{5}$$

where W is the peak width, F is the flow rate, T is the mean residence time (i.e., the time from injection of the sample to the time when it is detected) and t is a system constant which describes the mixing intensity in terms of a characteristic tube length, viscosity, surface tension between the carrier stream and the wall material, temperature, diffusion coefficient of the sample, etc.

The amount of dispersion changes as sample flows down the tube as shown in Figure 5. Therefore to control dispersion tube length can be varied.

In designing a FIA system, two points must be noted: (1) control of dispersion is the basis of FIA and (2) complete
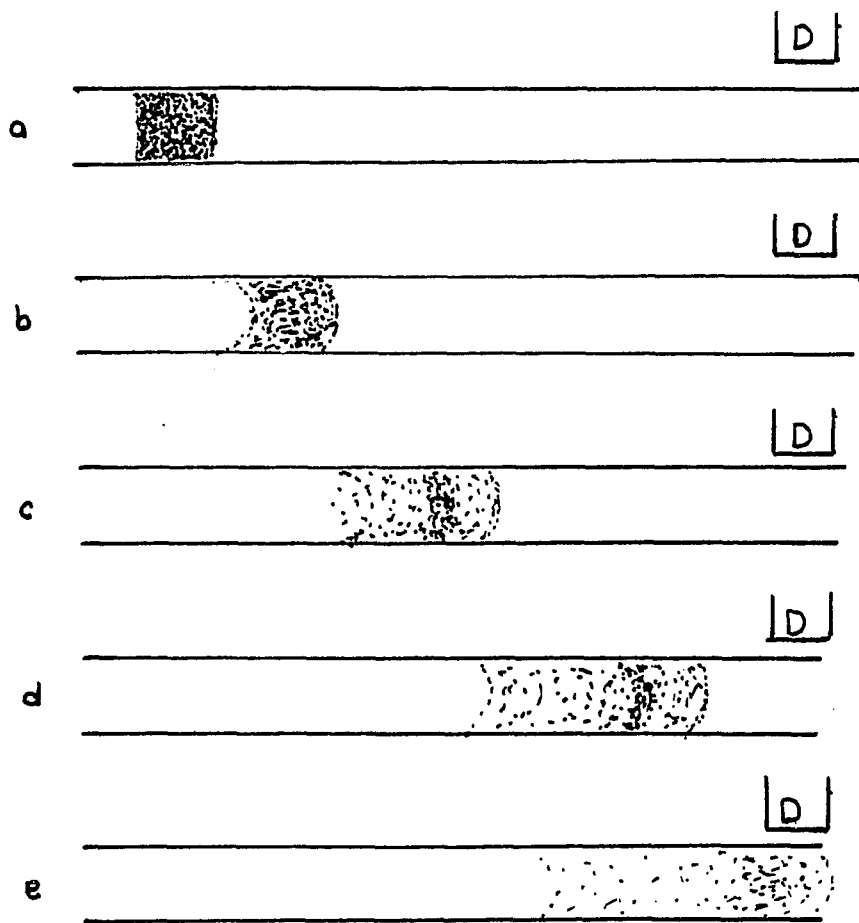
Figure 5. a through e are a representation of the distri-
bution of sample from the moment of introduction into
the carrier (a) as it moves down the coil of tubing
(b-d) until it passes in front of the detector (e).
D represents the detector.

mixing does not occur. Therefore, there are concentration gradients in the system as the sample flows past the detector.

Analytical Characteristics

Several parameters may be varied to achieve rapid, precise analysis with adequate sensitivity. These include (1) sample volume, (2) tubing diameter, (3) tube length, (4) flow rate, (5) kinetics of the analytical reaction, and (6) detector sensitivity. However there is a tradeoff in adjusting each of these. For example, high flow rates and small sample volumes lead to high sampling rates but relatively low sensitivity, by increasing dispersion.

The time allowed for the reaction to occur is the time from sample injection to detection of products. Although the reaction does not have to go to completion in this time, enough product must be formed for adequate sensitivity. This time can be increased by reducing flow rate or lengthening the tube but these measures also reduce sampling rate. As a consequence, FIA works best for reactions with fast kinetics. Continuous flow analysis is often preferred for slower kinetics. This is one of the major limitations of FIA.

Instrumentation

The instrumentation for FIA is very simple as shown in Figure 3. Requirements for constructing a flow injection instrument are: (1) a pulseless, easily controlled flow,

(2) reproducible injection of sample, and (3) some method of detecting and recording the data.

The equipment needed to achieve this is similar to that used in High Performance Liquid Chromatography (HPLC): pumps, flow lines and connectors, sample injection valves, and a detector. The configuration depends on the particular analysis. Two configurations for small volume consumption will be discussed in Chapter II.

Applications

A number of clinically important species have been determined by FIA. These include electrolytes such as calcium (Hansen et al., 1978), sodium (Ruzicka et al., 1977), and potassium (Ramsing et al., 1980), and proteins such as albumin (Braithwaite and Miller, 1980). Sugars such as glucose, lactose, and maltose have also been determined using enzymatic reactions (Nicholelis and Mottola, 1978; Hansen et al., 1977). A variety of detectors have been used for these including spectrophotometry, fluorescence, and electrochemical methods such as potentiometry and voltammetry. Chemiluminescence has been successfully used for detecting in FIA. Rule and Seitz (1979) evaluated a system using the luminol reaction for the detection of hydrogen peroxide. Precisions of 1-2% relative standard deviation were achieved with a sample throughput of six samples per minute. The system was evaluated with both fast and slow kinetics using a Cu(II) catalyst in the presence and absence

of ammonia ligand. Burguera and Townshend used the luminol reaction to detect 1pg - 100ng of Co(II) and 1 - 1000ng of S(-2) in 10 and 1000 μl samples, respectively.

In general sampling rates are often several hundred per hour and precisions range from less than 1% to no more than 5% relative standard deviation.

Chemiluminescence Coupled with Flow Injection Analysis

The coupling of FIA with CL detection offers more than the combined advantages of both methods. As emphasized earlier, CL and BL methods require that sample and reagents be mixed in a reproducible manner and the interval between mixing and observation of CL be carefully controlled. FIA is designed to meet both these requirements. In addition, it achieves these with characteristics desirable to clinical laboratories such as high sampling rates and low sample volume consumption. If CL and BL methods are to be widely used, they must be automated with good precision and high sample throughput. Additional advantages are the introduction of sample into the flow system remote from the mixing chamber, thus avoiding problems that can occur with stray light when a syringe is introduced directly into the mixing chamber with continuous use of the same mixing cell. Problems with replacement of cells after each measurement and of discrete dosing of reagents are eliminated.

CHAPTER II

FLOW INJECTION CONFIGURATIONS FOR MEASURING ATP

Introduction

Design Considerations

The design requirements of a CL detection system for FIA differ from required designs using other detection methods. The intensity of CL is proportional to the *rate* of the reaction rather than to the concentration of the products. Because emission is usually transient, it is desirable to observe the reaction as mixing occurs, since intensity usually peaks at the beginning of the reaction before reagent consumption becomes significant. This is particularly important for reactions with fast kinetics where the intensity is very high initially but decreases rapidly. Thus, with CL detection, it is preferable to observe CL directly from the mixing coil rather than measuring downstream from the coil. To minimize the amount of unobserved light the mixing coil should be placed close to the injection point particularly for reactions with fast kinetics (Rule and Seitz, 1979).

Small Volume Configurations

Flow injection methods normally minimize sample consumption. In some cases, reagent consumption should be minimized because of cost. This is the case when using the

firefly reaction for ATP determination because purified luciferase is quite expensive.

The goal of this work was to evaluate configurations which can be used to minimize sample and reagent consumption while retaining high sample throughputs by using CL detection to examine the effect of the analytical parameters on the mixing. As described above, CL is measured while mixing occurs so that the nature of the CL response gives information about the dispersion of the sample.

Two configurations were studied. The first (Figure 6) is called the "valve within a valve" configuration. In this system, a small volume of reagent is injected into a loop and is introduced into the carrier stream by "sandwiching" it between two plugs of sample. Mixing between sample and reagent occurs as they are transported to the detector by the carrier stream.

The second configuration (Figure 7) is called "merging zones" and has been evaluated as a means of conserving sample and reagent (Bergamin et al., 1978; Ruzicka and Hansen, 1979). In this system, small volumes of reagent and sample are injected into separate streams which then intersect and mix.

Both systems were evaluated with the firefly reaction to determine the nature of the response to ATP (i.e., signal shape and duration), precision, and sample throughput.
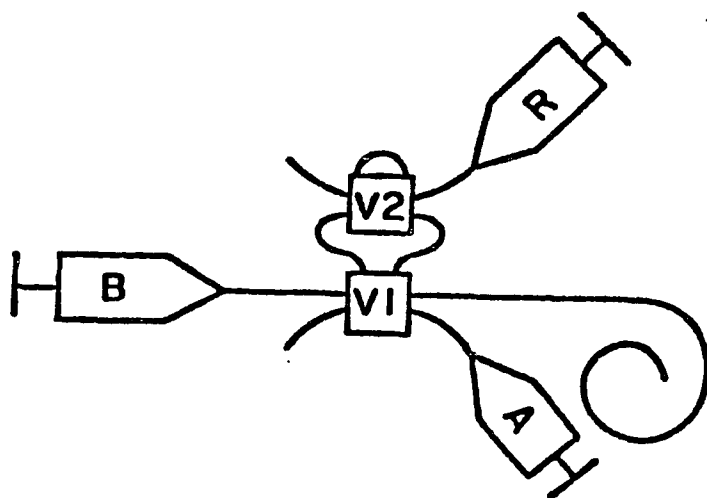
Figure 6. Valve within a valve configuration for flow injection analysis of ATP. Background solution is pumped through flow system, V2 is used to inject a slug of luciferase reagent, R, inside a larger slug of analyte, A, injected through V1. They mix in a coil positioned in front of detector.
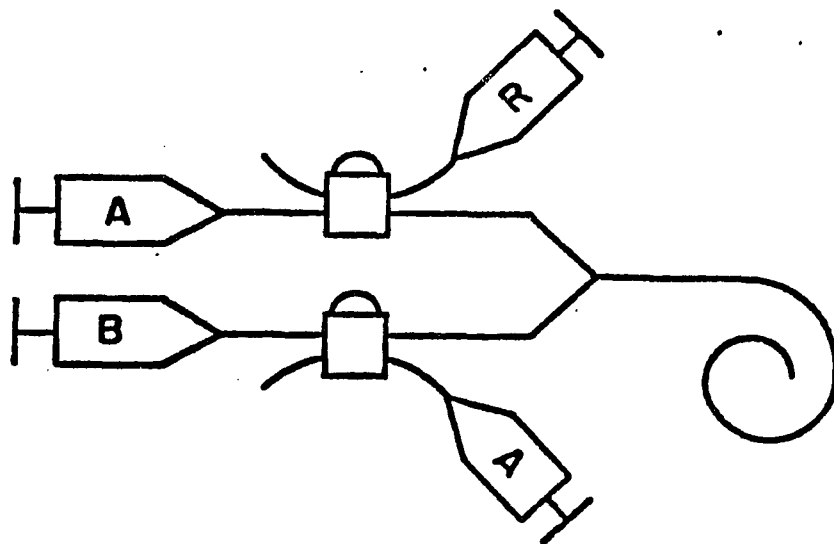
Figure 7. Merging zones configuration for flow injection analysis of ATP. This involves simultaneously injecting slugs of R and A into background flow streams, A and B.

Experimental

Instrumentation

For the valve within a valve configuration, flow was driven by a Cole Parmer 754510 solid state Masterflex Controller Peristaltic Pump. Teflon tubing, .79 mm id, was used throughout. The detection system was a spiral of tubing pressed against the face of an RCA 8852 53CT photomultiplier powered by a PAR 280 High Voltage Power supply operated at 1500 V. The length of the tubing in the spiral was 40 cm with 10 cm of tubing between the sample injection valve and the detector spiral. Tubes were cleaned by rinsing with dilute HCl, then water and, if necessary, an air segment to remove bubbles. Signals were recorded and displayed on a Digital Equipment MINC 11 minicomputer or on a Heath Schlumberger SR 204 strip chart recorder. Peak height, peak area, and relative standard deviation for each set of replicates were recorded on the computer using a program written for this purpose (Appendix). During the initial work, sample and reagent were injected using pneumatically actuated Altex 201-06 sample injection valves. The volume of the reagent loop was 25 µl, while the volume in each sample wing loop was varied from 25 to 65 µl. Samples were injected into the loops using Becton Dickinson disposable syringes.

To improve precision, samples were later introduced using Rheodyne Type 50 sample injection valves. Each sample volume wing size was 22.5 µl. Reagent was injected into the

valve using a Harvard Apparatus Model 794 Compact Infusion pump operated for 5.0 seconds at a flow rate of 1.35 ml/min. The sample was injected using Sage Instruments Model 255.1 Syringe Pump operated for 5.0 seconds at a flow rate of 0.80 ml/min.

The same equipment was used for the merging zones configurations. The Rheodyne valves were used for these measurements. Sample and reagent volumes were each 25 μl.

One set of data was also taken using a commercial flow injection instrument, the BIFOK FIA-OF Flow Injection Analyzer.

Reagents

The carrier stream was .05 M Trizma buffer from Sigma adjusted to pH 7.2 with HCl. The reagent was LUMIT PM luciferin-luciferase reagent for PMT instruments provided by LUMAC. Each vial was diluted to 20 ml using Hepes buffer from Baker adjusted to pH 7.75 containing 10 mM Magnesium acetate. The sample was Adenosine 5' Triphosphate, Sigma grade diluted with water. ATP standards from LUMAC were also used. LUMIT and ATP solutions were prepared fresh daily.

Results and Discussion

The response of the FIA system was characterized by observing the effect of variables on the CL signal. Several variables can be adjusted to minimize time of measurement so that throughput is increased while maximizing signal. Because

the valve within a valve configuration had never been studied
before, tne variables flow rate and sample volume were eval-
uated for this system. Both the valve within a valve and the
merging zones configurations were evaluated for their response
to ATP, precision, and sample throughput.

Typical Data

Figure 8 shows typical response for ATP samples using
the valve within a valve configuration, illustrating both the
precision and the time required for measurement. Figure 9
shows representative data for the merging zones configura-
tion. For both systems, a relative standard deviation of
about 3% was typical for five sequential replicate measure-
ments of peak area for the same sample. For the example
shown, sample throughputs are 5 and 10 samples per minute
for the valve within a valve and the merging zones configu-
ration respectively. These throughputs are based on peak
width which depends on such variables as flow rate and sample
volume.

Effect of Flow Rate and Sample Volume for Valve Within a Valve Configuration

As explained in Chapter I, the effects of FIA variables
are inter-related. When using CL, these effects are con-
voluted with the mixing and kinetic properties of the CL
measurement. With FIA, one looks at a section of the CL
intensity-time curve. Since CL intensity is proportional to
the area under the curve, the peak area and duration were

12 sec
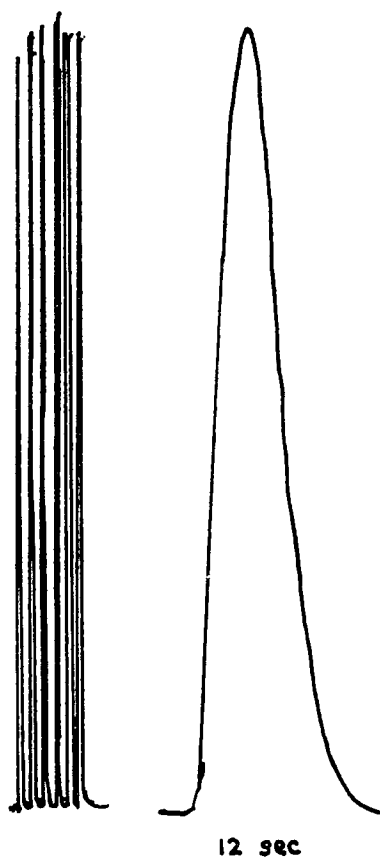
Figure 8. Typical data from FIA valve within a valve con-
figuration for ATP with chemiluminescence detection.
(10^{-8} M ATP, a sample volume of 70.0 µL, and a luci-
ferase reagent volume of 25.0 µL.)

6 sec
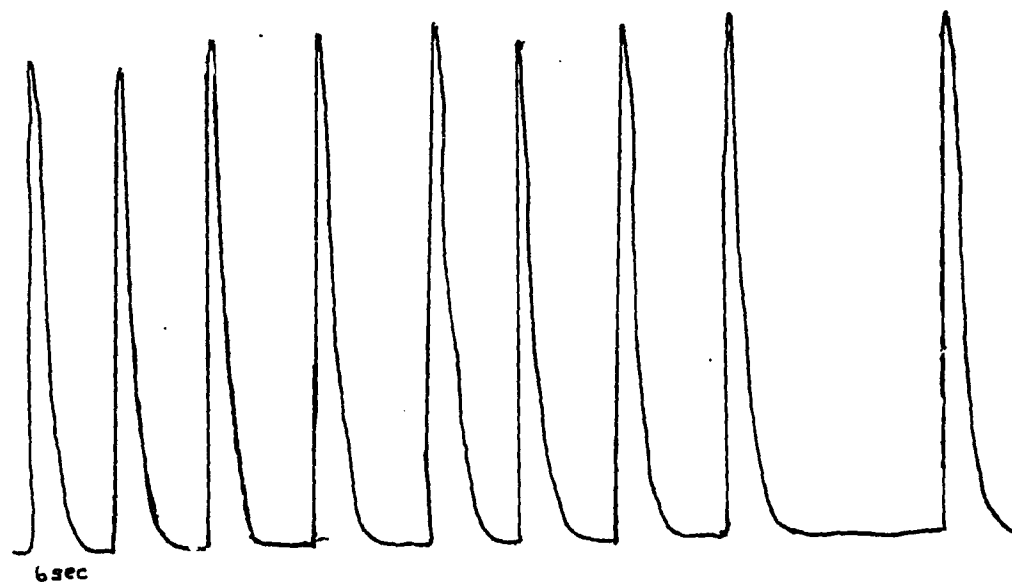
Figure 9. Typical data for FIA merging zones configuration for ATP with chemiluminescence detection. (10^{-8} M ATP with sample and reagent volumes of 50 µL each.)

determined as a measure of mixing and dispersion in the system.

Effect of Flow Rate. The effect of increasing flow rate on the signal is shown in Figure 10. Area is inversely related to flow rate because the sample spends less time in front of the detector at higher flow rates. As expected Figure 10 shows a decrease in both area and duration of the peak. For a kinetically slow process such as the firefly reaction one would expect the area to be halved when the flow rate is doubled but Figure 10 shows slightly less decrease than expected.

These results can be explained by increased dispersion at higher flow rates, as shown in equation (5). The sample spreads out more because there is less time for radial mass transfer to occur. The extent of spreading can be determined by calculating the distance occupied in the tube at different flow rates (Table 1). Based on length of sample in the tube and dead volume (distance between injection point and detector), the time of the sample in front of the detector was calculated assuming no spreading. This was then compared with the actual time in front of the detector and the extent of spreading measured in length of the tube occupied. At the lowest flow rate the 14 cm segment of sample and reagent has spread to occupy approximately 60 cm while at the highest flow rate measured, the segment occupied 81 cm.

Effect of Sample Volume. The results of varying the sample volume by changing the length of the tubes used for
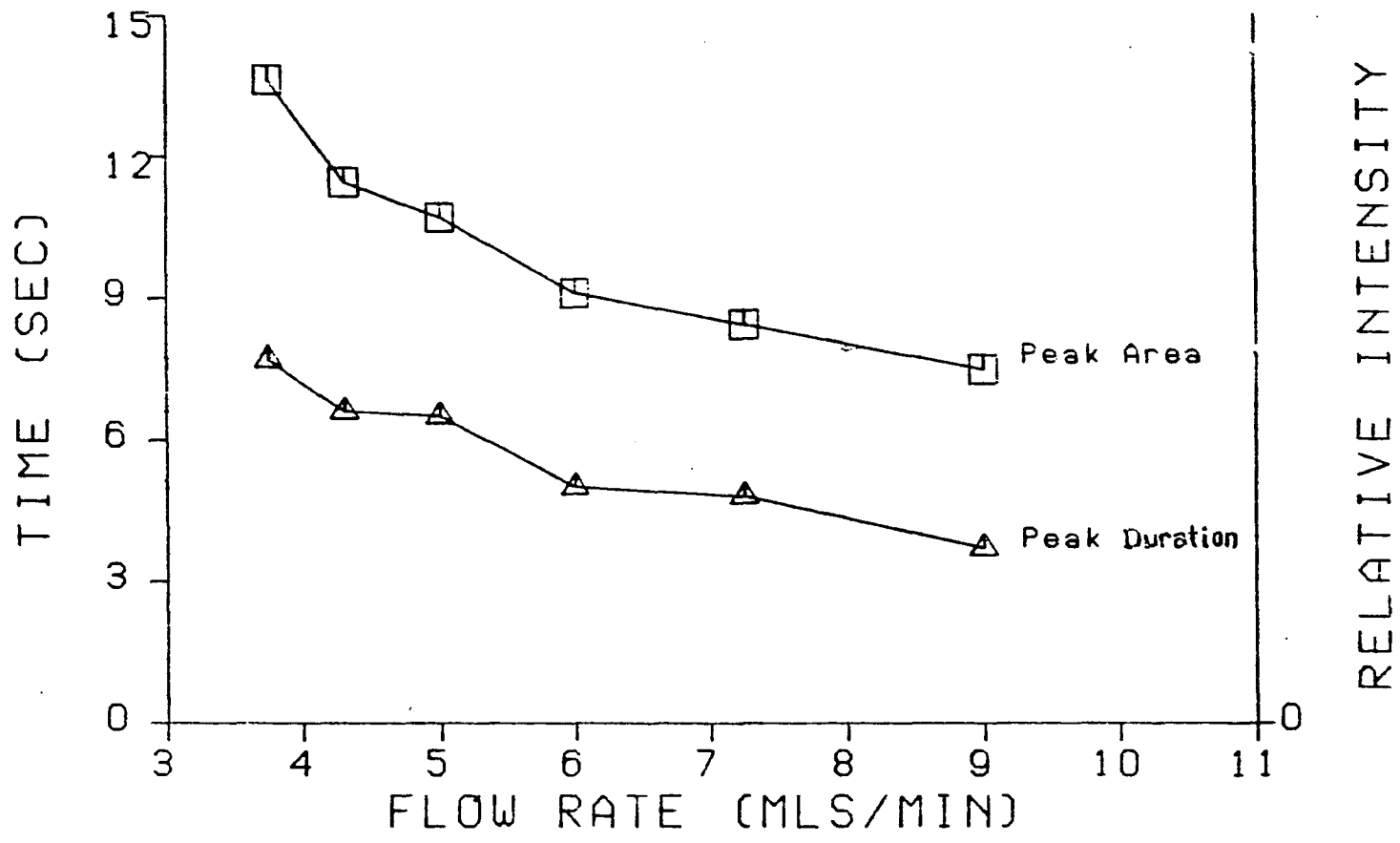
Figure 10. Effect of flow rate on signal intensity and
duration for valve within a valve configuration.
(10^{-8} M ATP, a sample volume of 70.0 µL and a reagent
volume of 25.0 µL.)

TABLE 1

Effect of Flow Rate on Dispersion

Flow Rate (mL/min)	Linear Velocity (cm/sec)	Time in front of detector (assuming no dispersion) (sec)	Actual time in front of detector (sec)	Distance occupied by dispersed sample* (cm)
3.80	12.7	4.22	7.75	59.8
5.03	16.8	3.23	5.00	70.5
8.70	29.0	1.86	4.20	80.9

*Distance occupied by undispersed sample is 14.0 cm.

the wings in the valve within a valve configuration are shown in Figure 11. An increase in volume increases both the area and the duration of the signal. This is as expected since with smaller volumes the detector sees less CL. A large area is desirable for sensitivity but the sample throughput is decreased.

Sample Throughput and Precision

The effect of flow rate and sample volume on throughput and precision are shown in Table 2 for the valve within a valve configuration. The inter-relationship between effects can be seen here. As expected, throughput increases with higher flow rates but decreases with larger sample volumes. The effect of sample volume on throughput is more pronounced at higher flow rates because of increased dispersion.

Precision tends to be better at low flow rates and smaller sample volumes probably because there is less variability in hydrodynamics. Also, pump pulsation would have less effect at low flow rates. Precision was measured under a variety of experimental conditions. By using the conditions described in the Experimental section, i.e. automated injection and appropriate choice of flow rate and sample volumes, precisions of 2-3% relative standard deviation are possible. The type of injection valve also affects precision. The signal from the Altex valves could be changed simply by touching the sample loop or by removing and replacing the same loop because hydrodynamic properties are changed with
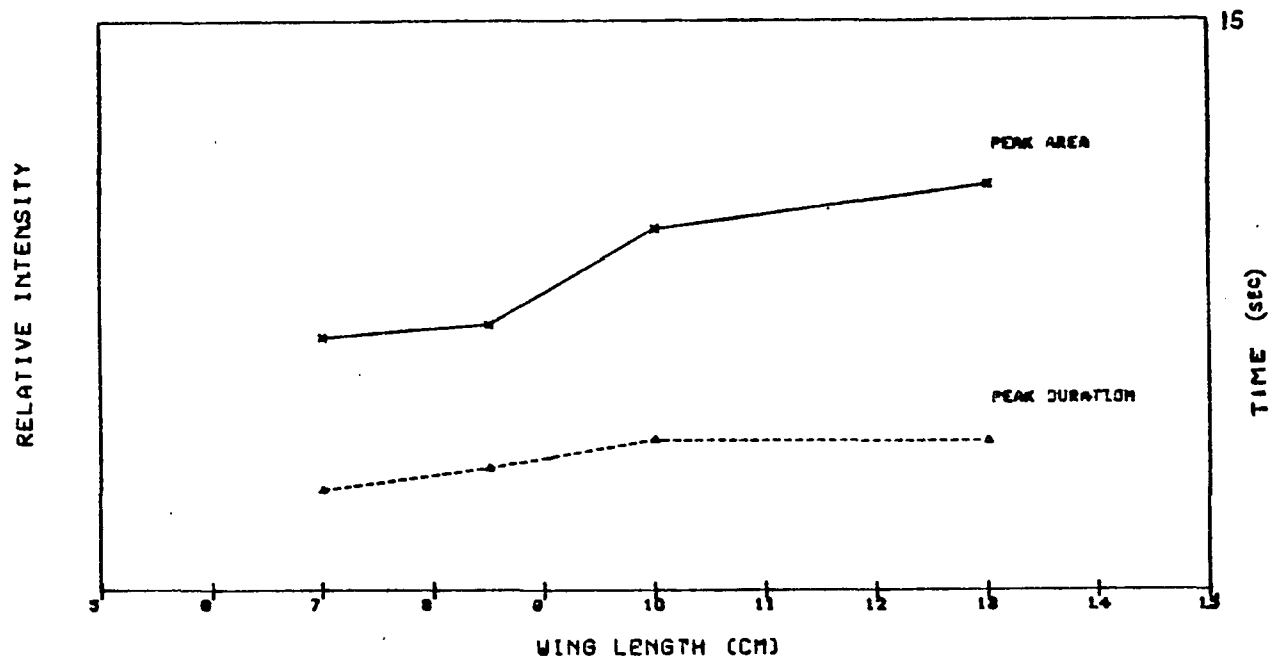
Figure 11. Effect of sample volume (in terms of wing length of tubing .75 mm i.d.) on signal intensity and duration. (10^{-8} M ATP, flow rate of 6.30 mL/min.)

TABLE 2

Effect of Flow Rate and Sample Volume
on Throughput and Precision

Throughput (measurements/min)

Flow Rate mL/min Wing L (cm)	3.8	5.0	6.3	7.5	8.7
7.0	13.3	18.0	20.8	28.8	37.7
8.5	13.5	18.4	21.5	24.7	31.0
10.0	11.5	13.9	19.6	24.7	25.2
11.5	12.5	18.5	19.4	27.1	31.2
13.0	11.0	14.7	16.2	22.4	25.4

Precision (% Relative Standard Deviation)

Flow Rate mL/min Wing L (cm)	3.8	5.0	6.3	7.5	8.7
7.0	3.2	4.6	4.3	2.5	3.4
8.5	3.5	5.8	6.9	8.1	6.0
10.0	3.2	7.1	4.3	4.3	9.2
11.5	3.7	6.8	4.7	10.9	5.7
13.0	2.4	3.2	3.6	8.0	3.6

repositioning of the loop. Therefore Rheodyne valves were preferred.

Precision is also limited by memory effects associated with luciferin-luciferase reagent. When several peaks are measured in sequence, the first peak is usually lower than the others indicating that some reagent remains in the loop. The viscosity of the reagent also affects precision by altering the mixing efficiency. These problems could be reduced by using a surfactant.

Area is not usually measured in FIA. However, since CL is proportional to area under the curve, peaks were integrated by a computer program to improve precision. Precision measuring peak heights was typically 4-5% R.S.D. and precision integrating peak areas was typically 2-3% R.S.D. The precision achieved with the commercial FIA instrument was comparable to those of the one described above suggesting that the imprecision was not because of equipment but is related to effects of the CL reagent.

Response

Linear response to ATP was observed for the valve within the valve configuration using a reagent volume of 25 µl, a sample volume of 22.5 µl in each wing and a flow rate of 6 ml/min. Linear response to ATP was also observed with the merging zones system using reagent and sample volumes of 25 µl each and a flow rate of 4.3 ml/min. Under these conditions the signal for each lasted about seven seconds

indicating that as many as 8 measurements per minute are possible.

The two methods are comparable in throughput and precision, but the merging zones configuration consumes less sample.

Kinetics. These results are related to the kinetics of the reaction. The firefly reaction has slow kinetics with a rise to maximum intensity within a few milliseconds but with a slow decay. Thus a linear response is observed because the measurement is made somewhere on the maximum intensity part of the curve. Nonlinear responses are observed for other systems such as the luminol reaction (Rule and Seitz, 1979). The effect of the parameters described above also relate only to slow kinetics systems and would have to be reevaluated for reactions with fast kinetics.

Detection Limits. Detectability is limited by luminescence observed when no sample is injected. The lowest detectable amount was determined to be about 10-11 ATP.

Conclusions

FIA is a convenient method for handling CL and BL mixing and detection. FIA with CL detection is capable of achieving the desired throughput and precision necessary for clinical laboratories. Sample and reagent volume consumption can be minimized with the appropriate configurations.

The effects of several variables on these configurations have been evaluated for the slow kinetics firefly reaction

and these results can be used in designing a FIA system.
FIA can be used for a variety of applications. Results from
this work would provide the basis for selecting an appro-
priate system depending on needs.

PART II

CHEMILUMINESCENCE IMMUNOASSAY

CHAPTER III

BACKGROUND OF IMMUNOASSAY AND PEROXYOXALATE CHEMILUMINESCENCE

Immunoassay

In 1959 Berson and Yalow developed a technique called immunoassay which has revolutionized the analysis of many biologically important compounds (Berson and Yalow, 1959). In 1977 their work was recognized by the Nobel Prize in Physiology and Medicine. The impact of this technique is based on its specificity, sensitivity, and batch sampling capabilities. Detection limits are generally on the order of 10-12 to 10-15 M (O'Donnell, 1981).

A variety of compounds including peptide hormones, steroids, and drugs have been measured by immunoassay or competitive protein binding assays. The competitive principle of immunoassay may be extended to nonimmunological systems. Any substance that can be bound specifically to a macromolecule can be measured by methods based on the principle of competitive binding. This macromolecule may be a serum protein or tissue receptor as well as an antibody (Markowitz and Jiang, 1976).

Principles

Immunoassay is based on the fact that antibody molecules have binding sites that react specifically with particular

structural features of antigens.

$$Ag + Ab \rightleftharpoons Ag - Ab \qquad (6)$$

The resulting complex is favored thermodynamically with very large equilibrium constants.

$$K = \frac{(Ag-Ab)}{(Ag)\,(Ab)} \qquad (7)$$

where (Ag) is the concentration of the antigen, (Ab) is the concentration of the antibody and (Ag-Ab) is the concentration of the antigen-antibody complex.

Normally, an immunoassay involves labeling an antigen or antibody and allowing labelled and unlabelled species to compete for binding sites. Two assumptions are made in the mathematical treatment of these assays: (1) the reactants obey the law of mass action and (2) the antibody is unable to distinguish between the labelled antigen and the unlabelled antigen. In a typical assay, the analyte (Ag) is allowed to compete with labelled antigen for binding sites on the antibody. The reaction is

$$\begin{matrix} Ag \\ + \ Ab \\ Ag^* \end{matrix} \xrightleftharpoons{K} \begin{matrix} Ag\text{-}Ab \\ \\ Ag^*\text{-}Ab \end{matrix} \qquad (8)$$
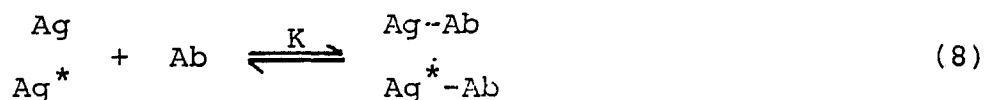
where Ag^* is the labelled antigen and Ag^*-Ab is the labelled antigen-antibody complex.

Data Treatment

The analyte (Ag) is quantitated by relating it to the

amount of free or bound labelled antigen (Ag^*). In a typical immunoassay, a known amount of labelled antigen is allowed to complex with an excess of antibody (Ab). A standard curve to determine Ag may be prepared by adding a set of standards of increasing Ag concentrations to aliquots of the equilibrated mixture and determining the per cent bound by measuring (Ag^*-Ab). As Ag is added, it displaces Ag^* from the antibody and the %Ag^* bound decreases. The standard curve of % bound vs. (Ag) added is then plotted and unknowns estimated from this (Walker, 1977).

The concentration of Ag^*-Ab may be quantitated in one of two ways. The complex may be separated from free antigen and antibody before being measured or it may be measured while in the presence of the free components. The former case is called heterogeneous immunoassay and is shown schematically in Figure 12. The three preferred methods for separating free and bound antigen are (1) adsorption, (2) precipitation and centrifugation, and (3) the use of solid phase antibodies (Parsons, 1981). The equilibrium in equation (6) must be sufficiently irreversible for separation.

Quantitation is sometimes possible without a separation step. If a change in the properties of the labelled antigen occurs on binding, a separation is not necessary. This is called homogeneous immunoassay and is represented in Figure 13. The advantage of this method is the reduced sample handling.
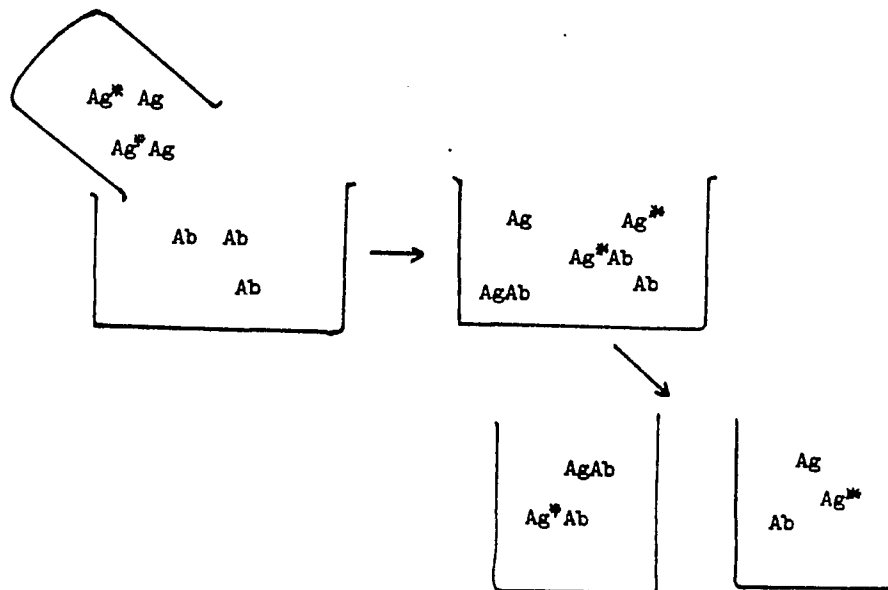
Figure 12. Schematic of heterogeneous immunoassay. Ag =
    antigen, Ab = antibody, Ag - Ab = antigen-antibody
    complex, Ag* = labelled antigen, Ag* Ab = labelled
    antigen-antibody complex.
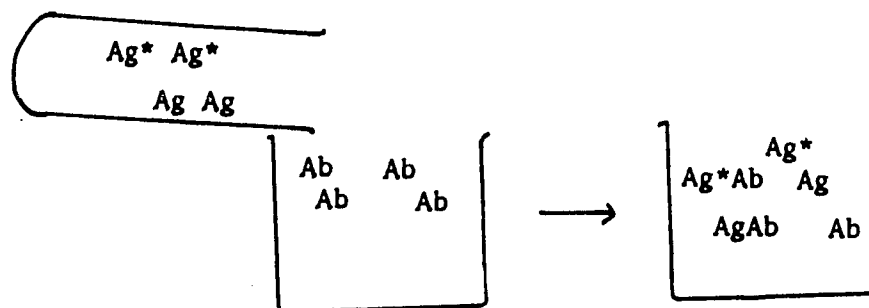
Figure 13. Schematic of homogeneous immunoassay. Ag = anti-
     gen, Ab = antibody, Ag - Ab = antigen-antibody complex,
     Ag* = labelled antigen, Ag* Ab = labelled antigen-anti-
     body complex.

Labels

Most assays use radioactive labels and are called Radio-
immunoassays (RIA). However, this places several restric-
tions on immunoassay because production, shipping, handling,
and disposal of radioactive materials is regulated. The
cost and maintenance of the counting equipment must also be
taken into consideration. In addition, long term standardi-
zation is not possible with radioactive labels because
activity changes with time as isotopes decay (Chait and
Ebersole, 1981).

Consequently, a new generation of immunological tech-
niques has been developed using a variety of non-radioactive
labels including enzymes, fluorophors, particles, and cells.
These assays are preferable because of user safety, extended
reagent shelf life, and ready adaptability to conventional
instruments (Chait and Ebersole, 1981).

Heterogeneous immunoassays have been developed using
fluorophor lebelled antibodies (O'Donnell and Suffin, 1979),
solid phase fluorescent labelled antibodies (Burgett et al.,
1977), and chemiluminescent labels (Simpson et al., 1979).
Metal atom tags have also recently been used. Once the
immune reaction is complete, the antigen-antibody complex
is pyrolyzed in a vacuum and the metal atoms volatilized for
analysis (Chait and Ebersole, 1981).

Homogeneous methods include immunoassay by differential
pulse polarography (Heineman et al., 1979), voltammetric
immunoassay (Weber and Purdy, 1979), fluorescence polarization

(Tengerdy, 1967; McGregor et al., 1978), fluorescence excitation transfer immunoassay (Ullman et al., 1976), enzyme linked immunoassay (Rubenstein et al., 1972; Engwall and Perlmann, 1971), and chemiluminescence (Kohen et al., 1981).

A major limitation of non-radioactive labels is that detection limits are not as low as for radioactive labels. Even fluorescent methods, usually noted for low detection limits, are not comparable for most applications. However, CL offers adequate detection limits and successful immunoassays have been developed using luminol labels.

Peroxyoxalate Chemiluminescence

The most efficient nonenzymatic chemiluminescent reaction known is the peroxyoxalate reaction discovered by Chandross in 1963. He observed that the reaction of oxalyl chloride and hydrogen peroxide in the presence of a fluorophor yielded a bright flash of light with a color characteristic of the fluorophor. He concluded that the emission was due to the first excited singlet state of the fluorophor. Since then, CL has been observed from the reaction of a number of different oxalic esters and hydrogen peroxide. The general reaction scheme is represented in Figure 14. This mechanism is tentative and the key intermediate, 1,2-dioxetanedione has not been detected (Rauhut, 1979). However, a great deal of work has been done which supports the proposed mechanism (Rauhut, 1969; Rauhut et al., 1967; Bollyky et al., 1971; Schuster, 1979). The energy transfer step is the key

Figure 14. Proposed mechanism of peroxyoxalate reaction.

to CL and excitation of the fluorophor. Under optimum conditions, it generates the singlet excited state of the fluorophor with an excitation yield of 60% (Rauhut, 1979).

Several groups have suggested mechanisms for the decomposition of the dioxetanedione. Two bonds of the dioxetane must be broken for decomposition to carbon dioxide. McCapra (1968) and Kearns (1969) suggested a concerted bond cleavage while Richardson (1974) advocated an excitation mechanism that proceeds by rate determining oxygen-oxygen bond cleavage to initially generate a 1,4 biradical.

Parameters

Efficiency of the chemiluminescent reaction is affected by a number of different parameters such as esters, solvents, and fluorophors.

High efficiency peroxyoxalate CL requires an oxalic acid derivative with a good leaving group to facilitate ring closure to the dioxetanedione. Efficient chemiluminescent oxalates include electronegatively substituted aromatic or aliphatic esters (Rauhut, 1969), amides (Bollyky et al., 1969), mixed oxalic-carboxylic anhydrides (Bollyky et al., 1967), and oxalyl chloride (Chandross, 1963). Quantum yields as high as 22-27% have been reported for oxalate esters such as bis(2,4,6-trichlorophenyl) oxalate (Bollyky et al., 1971), bis(2,4-dinitrophenyl) oxalate (Rauhut et al., 1967), and bis(3-trifluoromethyl-4-nitrophenyl) oxalate (Bollyky et al., 1971) with suitable fluorophors.

High yield fluorophors are used for maximum CL intensity.
These fluorophors emit in the near infrared to visible
regions (Rauhut et al., 1975). Excitation yields generally
decrease as the excitation energy of the fluorophor
increases, and chemiluminescence quantum yields for high
energy (blue) fluorophors tend to be relatively low (Turro
and Lechtken, 1974). The fluorophors yielding maximum effi-
ciences are rubrene and 5,12 bis(phenylethynyl) napthacene.
Even at low fluorophor concentrations the yield of excited
fluorophor can be high by careful selection of reaction con-
ditions to minimize non-luminescent side reactions.

Another factor affecting CL efficiency is the use of an
"activator." Most peroxyoxalate reactions are catalyzed by
bases and the reaction rate, chemiluminescence intensity and
CL lifetime can be varied by selection of the base and its
concentration.

One last parameter which must be considered for analyt-
ical applications is the solvent system. Side reactions can
be avoided by appropriate solvent choice. Solubility of the
oxalic ester is another consideration. Because the esters
are only soluble in organic solvents, reaction conditions
usually require a high concentration of an organic component
such as benzene or dimethylphthalate. However, most analytes
of interest are in aqueous media. Therefore the reaction
must be compatible with water. This requirement is met for
the peroxyoxalate reaction, since both the fluorophor and
hydrogen peroxide are water soluble. In fact, a small water

component is required for some peroxyoxalate systems (Rauhut et al., 1966).

Applications

Peroxyoxalate CL is the only chemical light reaction with significant use at present as a light source. American Cyanamid Company's light stick contains the components for peroxyoxalate CL. When the components are mixed, light emission is immediate and can last up to 12 hours (Rauhut in Chemiluminescence and Bioluminescence, p. 451). Peroxyoxalate chemiluminescence has also been used to carry out photochemical reactions (Guston, 1977) but does not appear to produce the triplet excited state (Turro and Lechtken, 1974).

Most analytical applications of peroxyoxalate chemiluminescence involve the determination of hydrogen peroxide. Williams, Huff, and Seitz (1976) measured glucose by producing hydrogen peroxide with glucose oxidase. Williams and Seitz (1976) also measured the reduced form of nicotine amide dinucleotide by using it to reduce oxygen to hydrogen peroxide.

Only four papers have appeared in the literature using peroxyoxalate CL to determine fluorophors by energy transfer. In 1977 Curtis and Seitz used the peroxyoxalate reaction to chemically excite fluorophors on a TLC plate. More recently, Kobayashi and Imai (1980) adapted this idea to HPLC detection. By mixing the peroxyoxalate reactants with the effluent from a HPLC column, they were able to determine 50 fmol of dansylated amino acids. These detection limits are greatly

improved over fluorescence measurements suggesting that CL might improve the detection limits of fluorescence immuno-assay.

The use of peroxyoxalate chemiluminescence to determine fluorophors by energy transfer is the key to the immunoassay application. Fluorophors will be used as labels but will be excited by the chemiluminescent reaction in the measurement step rather than with photoexcitation as is normally done in fluorescence methods. The major advantages of CL detection for immunoassay are simple instrumentation and low detection limits. As discussed in Chapter I, no light source is used for CL so the measurement is not limited by source fluctuations or stray light. The most serious limitation for the peroxyoxalate reaction is the solvent system, particularly for homogeneous immunoassay in which the antigen-antibody complex would be disturbed by organic solvents.

CHAPTER IV

DETERMINATION OF FLUOROPHORS BY PEROXYOXALATE CHEMILUMINESCENCE

Preliminary Experiments

Introduction

As mentioned in the preceding chapter the peroxyoxalate reaction has been used to detect as little as 50 femtomoles of fluorophor-labelled amino acids separated by liquid chromatography. Our goal was to try to achieve comparable detection limits for fluorophors used as labels in a medium suitable for either homogeneous or heterogeneous immunoassay. To achieve this goal four questions must be answered: (1) Can fluorophors other than dansyl chloride be determined by energy transfer? (2) Under what conditions can the reagents be mixed with satisfactory precision for an immunoassay? This must be achieved by manual mixing because automated mixers are not satisfactory for the mixed solvent system required by the peroxyoxalate reaction. (3) Does binding affect the CL intensity such that a homogeneous immunoassay is feasible? and (4) Can a system be developed with a high water to organic solvent ratio for homogeneous immunoassay? In addition, detection limits must be comparable or superior to present immunoassay methods, if this approach is to be useful.

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Constraints. Certain restrictions are imposed on the measurement system because of the immunoassay application. In addition to solvent constraints, a major restriction is the volume of analyte. Because of the cost and difficulty of purifying immunochemicals, immunoassays generally use sample and reagent volumes in the microliter range which complicates the problem with the peroxyoxalate measurements.

Parameters. To develop the measurement system, effects of various parameters on the CL intensity-time curve must be determined. The most desirable measurement systems will have a high intensity and a relatively slow decay to maximize sensitivity while still retaining acceptable precision.

The following variables were evaluated for their effect on the intensity-time curve:

(1) Fluorophors were compared to determine which should be used as a label.

(2) Buffers for the analyte were evaluated to determine their effect as activators.

(3) Solvents for the ester 2,4,5 trichloro-6-carbopentoxy phenyl oxalate were evaluated with respect to their effect on CL.

(4) Concentrations of both the ester and hydrogen peroxide were also considered. To analyze for low concentrations of fluorophors, the concentrations of these other components should be as high as possible to increase the CL intensity.

Experimental

Reagents. Buffers were prepared with THAM (Tris(hydrox-ymethyl)amino methane) (Fisher, primary standard), potassium phosphate (Fisher, reagent grade), and sodium hydrogen carbonate (Fisher, reagent grade). All buffers were .05 M and adjusted to pH 8.0.

Stock solutions of 10^{-3} M rhodamine B (Eastman Kodak, Practical Grade), 2',7' dichlorofluorescein (Eastman Kodak), and hydrolyzed dansyl chloride (Pierce) were made in each buffer and diluted as needed.

Bis(2,4,5-trichloro-6-carbopentoxyphenyl) oxalate (CPPO) was donated by American Cyanamid. The ester was dissolved in each of the following: acetonitrile (Baker, reagent grade), acetone (Fisher, reagent grade), and tetrahydrofuran (Baker).

Hydrogen peroxide (30%, Baker, reagent grade) solutions were diluted with distilled water.

Instrumentation. All measurements were read from the meter of an Aminco Chem-Glo Photometer.

Procedure. Reagents were added to a test tube in the following order: 9.0 mL of fluorophor solution in buffer and 1.0 mL of ester solution which were shaken until homogeneous. 0.30 mL of hydrogen peroxide solution was added to this solution. This mixture was shaken for 3 seconds and aliquots pipetted into borosilicate tubes for the Aminco Photometer. Recording of CL signal began 15 seconds after mixing and intensity was measured for up to 10 minutes.

Results and Discussion

Effect of Fluorophors. Structures of the three fluoro-
phors studied are shown in Figure 15. The isothiocyanate
forms of rhodamine and fluorescein are commonly used to label
proteins, and immunochemicals are commercially available with
these labels. 2'7'-dichlorofluorescein has fluorescence
characteristics similar to fluorescein but it is less effi-
cient. Dansyl chloride is often used to derivatize compounds
for fluorescence detection, particularly for HPLC. Dansyl
derivatives were also used in all the earlier studies involv-
ing excitation of fluorophors by energy transfer (Curtis and
Seitz, 1977; Kobayashi and Imai, 1980).

Figures 16a, b, and c show the effect of fluorophor on
the intensity-time curves in three buffers. In all cases,
the reaction yields the highest intensity with Rhodamine.
Of the three fluorophors, Rhodamine is the longest wavelength
emitter at 570 nm. 2'7'-dichlorofluorescein emits at 520 nm
and dansyl chloride at 470 nm. Intensities in THAM and
phosphate buffers with dansyl chloride were lower than the
other two fluorophors. These results are consistant with
those of Turro and Lechtken (1974) which show that the lowest
energy emitters are the best energy acceptors. An advantage
to using a long wavelength emitter is that fluorescent con-
tamination is usually more of a problem at shorter wave-
lengths so background in serum measurements could be opti-
cally resolved.

COOH

Cl⁻

$(C_2H_5)_2N$

$N^+(C_2H_5)_2$

RHODAMINE B

COOH

Cl

Cl

HO

O

2, 7 DICHLOROFLUOROSCEIN

$N(CH_3)_2$

SO_2Cl

DANSYL CHLORIDE
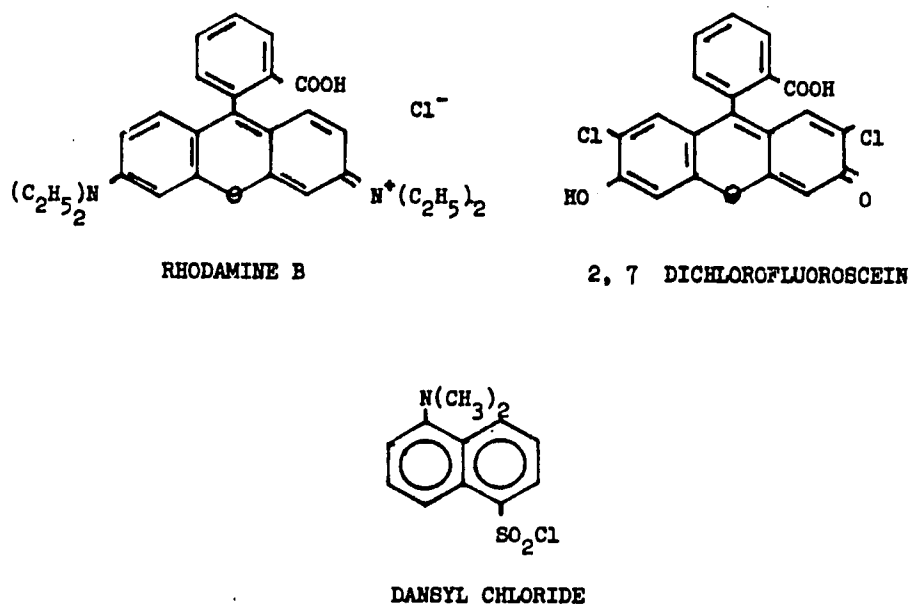
Figure 15.   Structures of fluorophors.
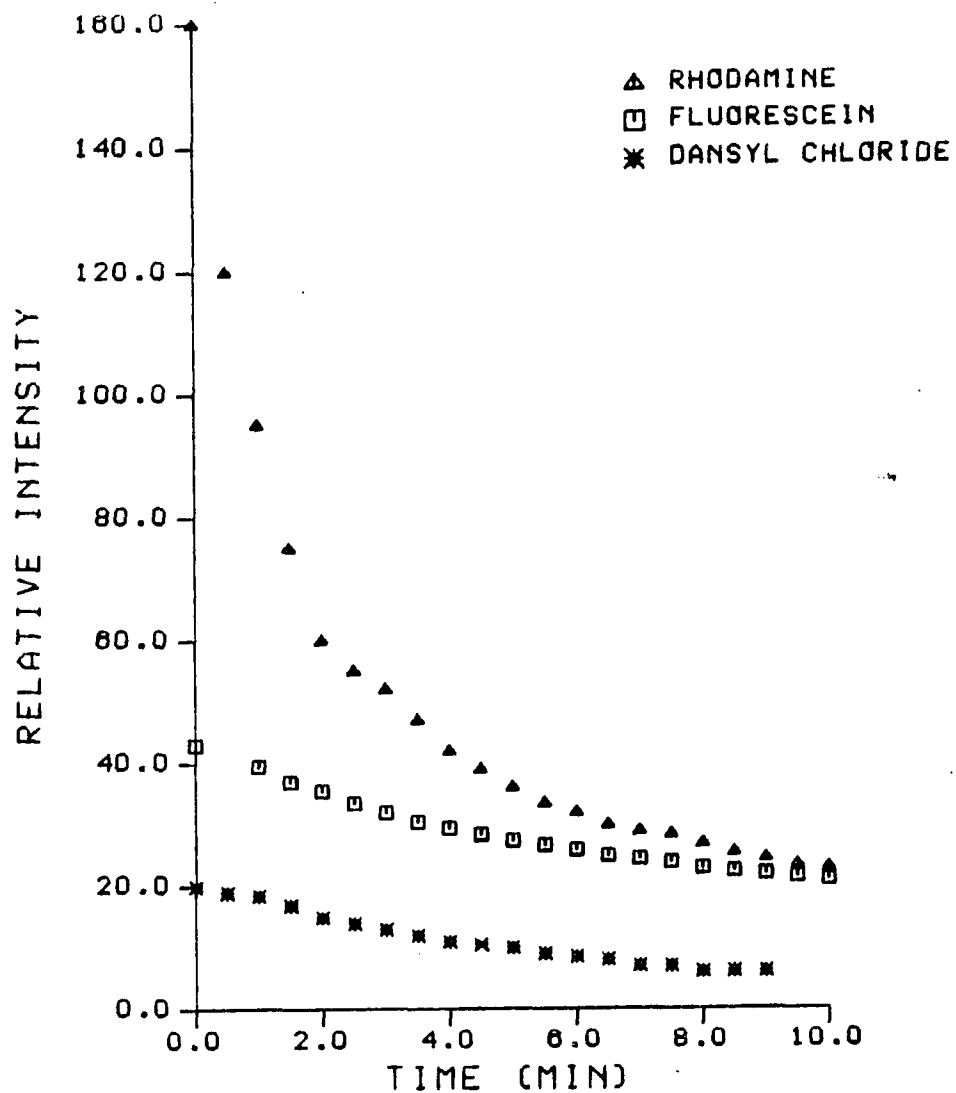
Figure 16a. Chemiluminescence intensity-time curves for
    three fluorophors in .05 M THAM buffer, pH 8.00.
    (10^{-7} M fluorophor, 10^{-3} M CPPO in acetonitrile, 10^{-3} M
    hydrogen peroxide in water.)
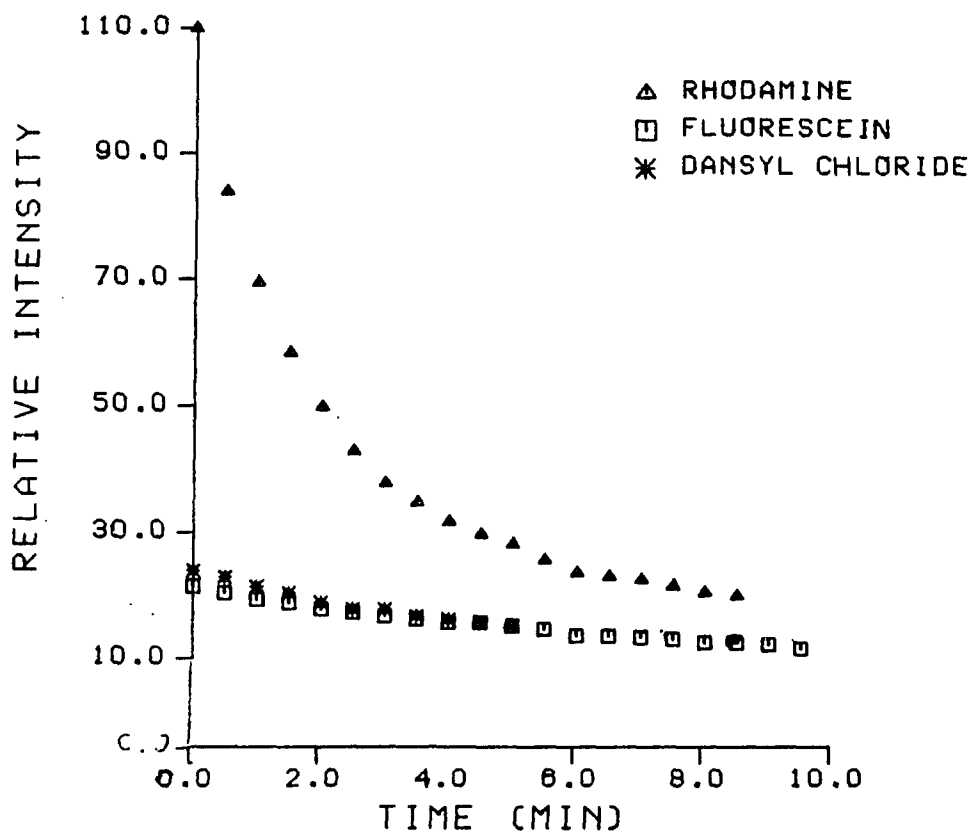
Figure 16b. Chemiluminescence intensity-time curves for three fluorophors in .05 M phosphate buffer, pH 8.00. (10^{-7} M fluorophor, 10^{-3} M CPPO in acetonitrile, 10^{-3} M hydrogen peroxide in water.)
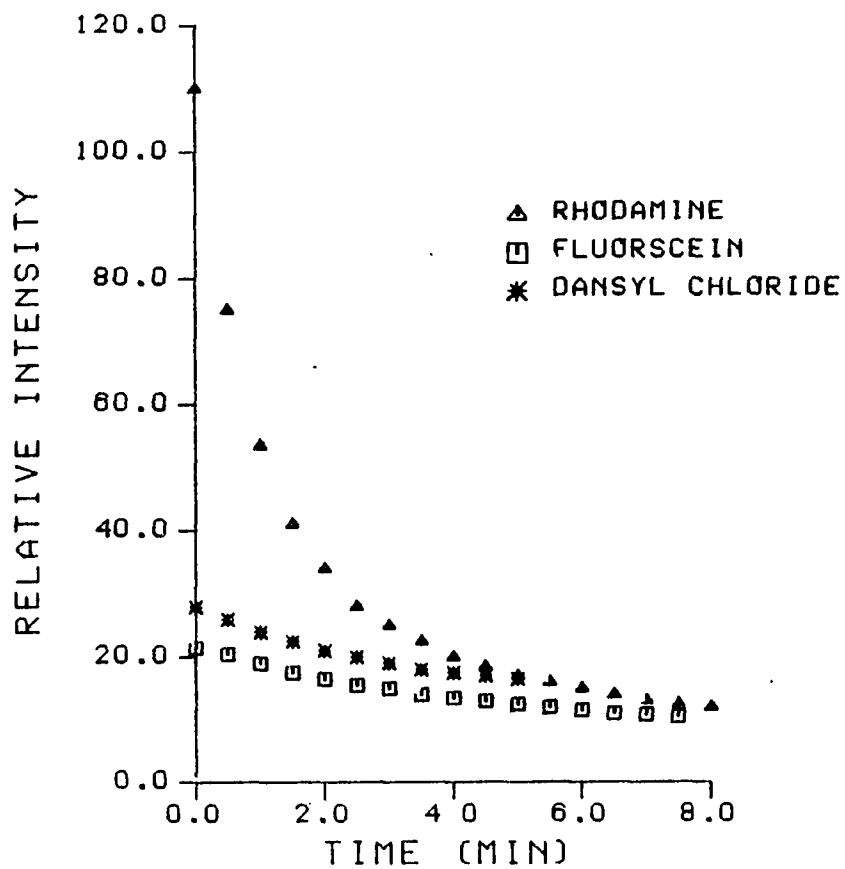
Figure 16c. Chemiluminescence intensity-time curves for three fluorophors in .05 M carbonate buffer, pH 8.00. (10^{-7} M fluorophor, 10^{-3} M CPPO in acetonitrile, 10^{-3} M hydrogen peroxide in water.)

<u>Effect of Buffers</u>. Both pH and concentration of buffer affect the intensity-time curve. Generally, high pH and concentration increase CL intensity. For immunoassay, physiological conditions were chosen so that buffers of concentration 0.05 M were evaluated at pH 8. Figures 17a, b, and c show intensity-time curves for fluorophors in three buffers. Highest intensity is observed in THAM. Often an "activator" such as triethylamine is used in addition to the buffer. It is possible that THAM behaves similarly to this activator which explains why intensity is higher in THAM.

<u>Effect of Solvent</u>. Intensity-time curves for the ester CPPO in various solvents are shown in Figures 18a, b, and c. Intensities were higher in acetone and acetonitrile than in THF. Since THF is less miscible with water than the other two solvents, miscibility may be related to the lower intensity. Generally esters and ethers are used for the most efficient CL reactions (Rauhut, 1979). These results show that solvents more miscible with water are also an acceptable medium.

The shapes of the intensity-time curves of acetone and acetonitrile should be noted. Intensity in acetonitrile is much higher initially but has a more rapid decay than acetone. Kinetic characteristics affect precision which will be considered in the next section.

<u>Effect of Reagent Concentration</u>. Ester concentration is limited by solubility and the highest concentration possible in acetone is shown in Figure 19. As shown, there is no
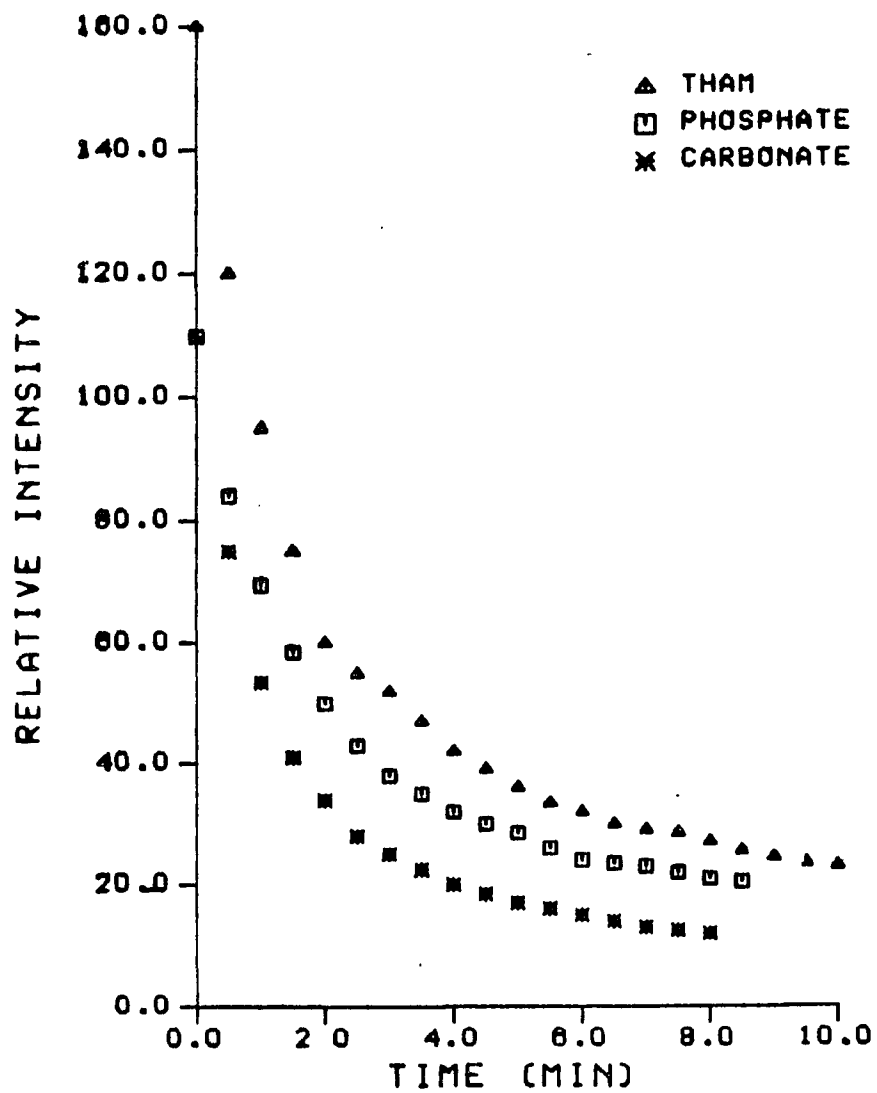
Figure 17a. Chemiluminescence intensity-time curves for three buffers with 10^{-7} M rhodamine B. (.05 M buffer, pH 8.00, 10^{-3} M CPPO in acetonitrile, 10^{-1} M hydrogen peroxide in water.)
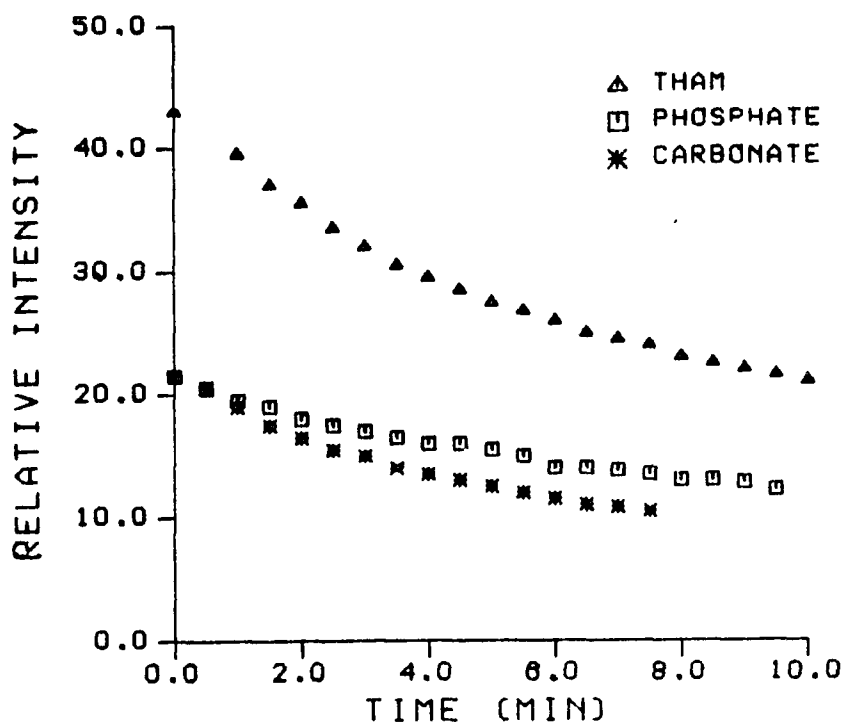
Figure 17b. Chemiluminescence intensity-time curves for three buffers with 10^{-7} M 2'7' dichlorofluorescein. (.05 M buffer, pH 8.00, 10^{-3} M CPPO in acetonitrile, 10^{-1} M hydrogen peroxide.)
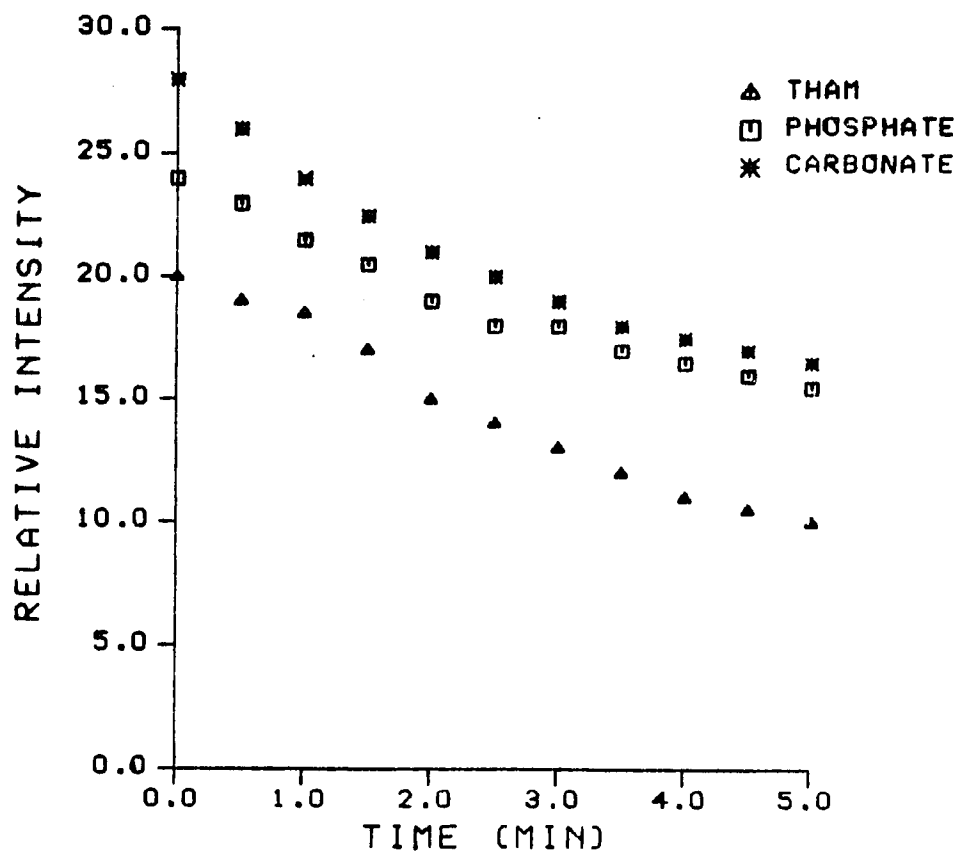
Figure 17c. Chemiluminescence intensity-time curves for three buffers with 10^{-7} M dansyl chloride. (.05 M buffer, pH 8.00, 10^{-3} M CPPO in acetonitrile, 10^{-1} M hydrogen peroxide.)
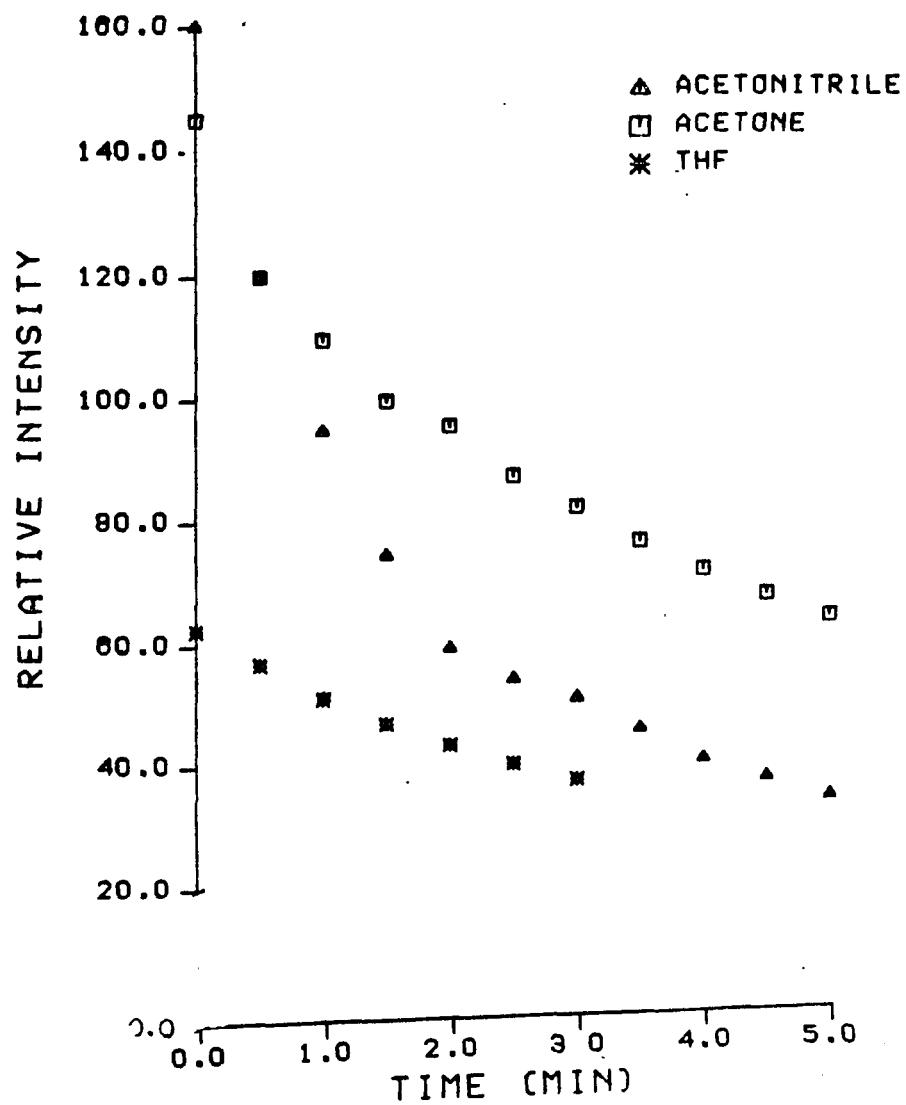
Figure 18a. Chemiluminescence intensity-time curves for three solvents for CPPO. (10^{-3} M ester, 10^{-7} M rhodamine in .05 M THAM buffer, pH 8.00.)
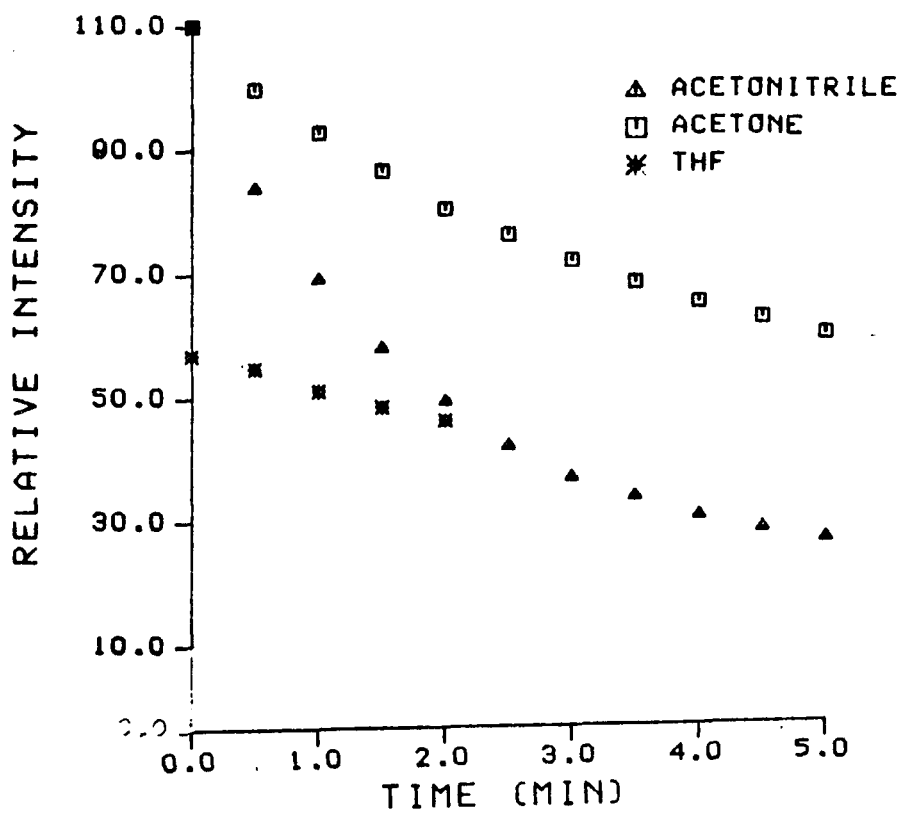
Figure 18b. Chemiluminescence intensity-time curves for three solvents for CPPO. (10^{-3} M ester, 10^{-7} M rhodamine in .05 M phosphate buffer, pH 8.00.)
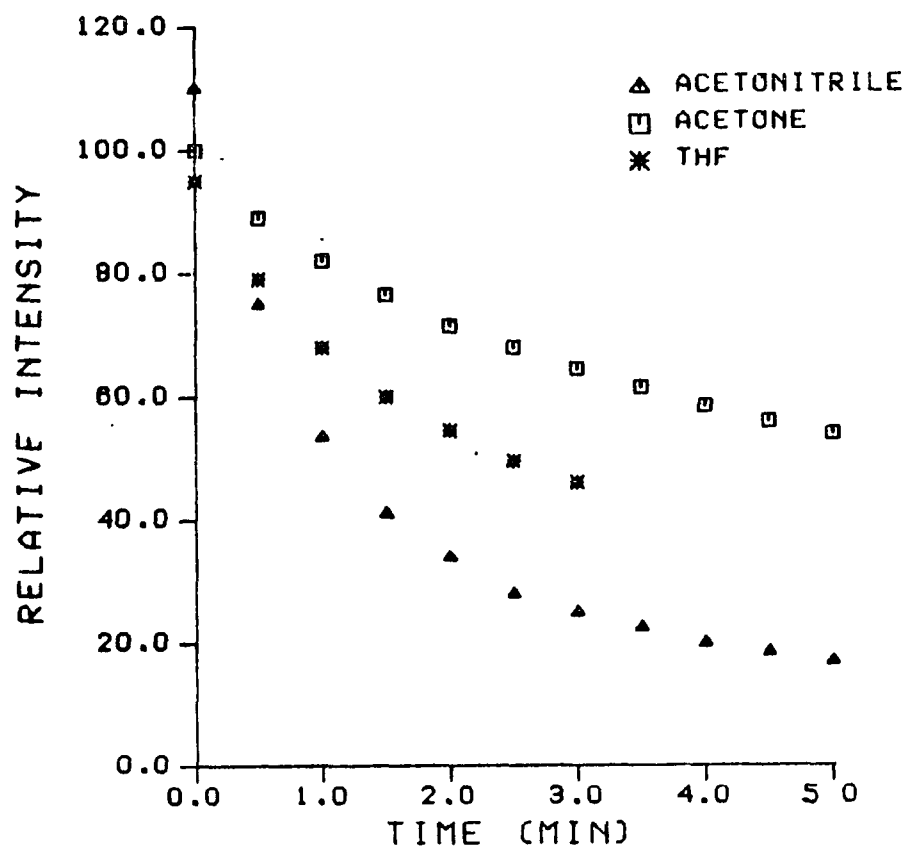
Figure 18c. Chemiluminescence intensity-time curves for three solvents for CPPO. (10^{-3} M ester, 10^{-7} M rhodamine in .05 M carbonate buffer, pH 8.00.)
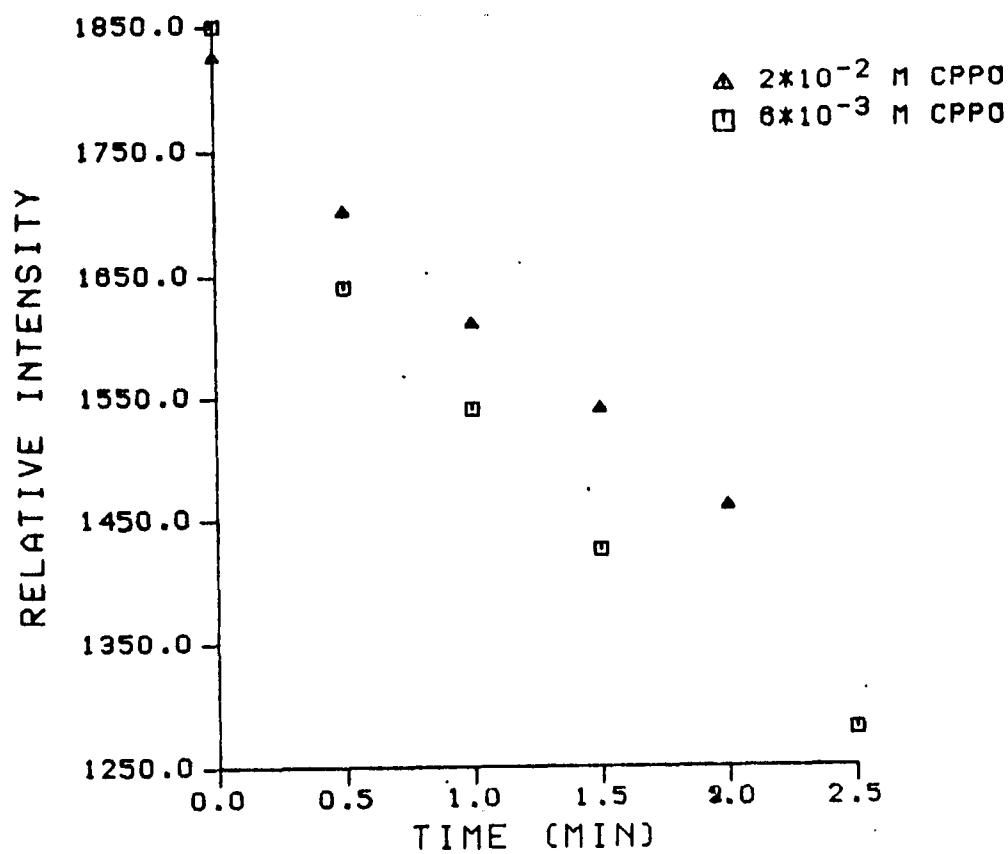
Figure 19. Chemiluminescence intensity-time curves for two concentrations of CPPO in acetone. (10^{-5} M rhodamine B in .05 M THAM buffer, pH 8.00, 10^{-1} M hydrogen peroxide in water.)

advantage to using a lower concentration since the intensity is lower and the intensity-time curves are similar in decay rate.

Of the variables studied, concentration of hydrogen peroxide has the most significant effect on the intensity-time curve. Kinetics range from very fast in 0.88 M hydrogen peroxide to much slower in 0.05 or 0.025 M. This indicates that the concentration of hydrogen peroxide is a suitable variable for controlling reaction kinetics (Figures 20a and b).

Preliminary results demonstrate that a CL measurement can be made with a high water concentration and that fluorophors other than dansyl chloride can be determined by energy transfer. Based on these results several measures were taken in future experiments: (1) Rhodamine was used in preference to the other fluorophors because it yields higher intensity. Since fluorescence contamination is more likely to be a problem at shorter wavelengths, detection limits should be better using rhodamine because it is the longest wavelength emitter of the three. (2) THAM buffer was used as the fluorophor solvent. (3) THP was no longer used as an ester solvent. Since reactions in acetone and acetonitrile yield high intensities, these were evaluated as solvents for other esters in future experiments. Precision in these solvents is considered in the next section based on differences in intensity-time curves. (4) Hydrogen peroxide concentration was used as a parameter to control kinetics of the reaction. (5) The concentration of esters used was determined by solubility
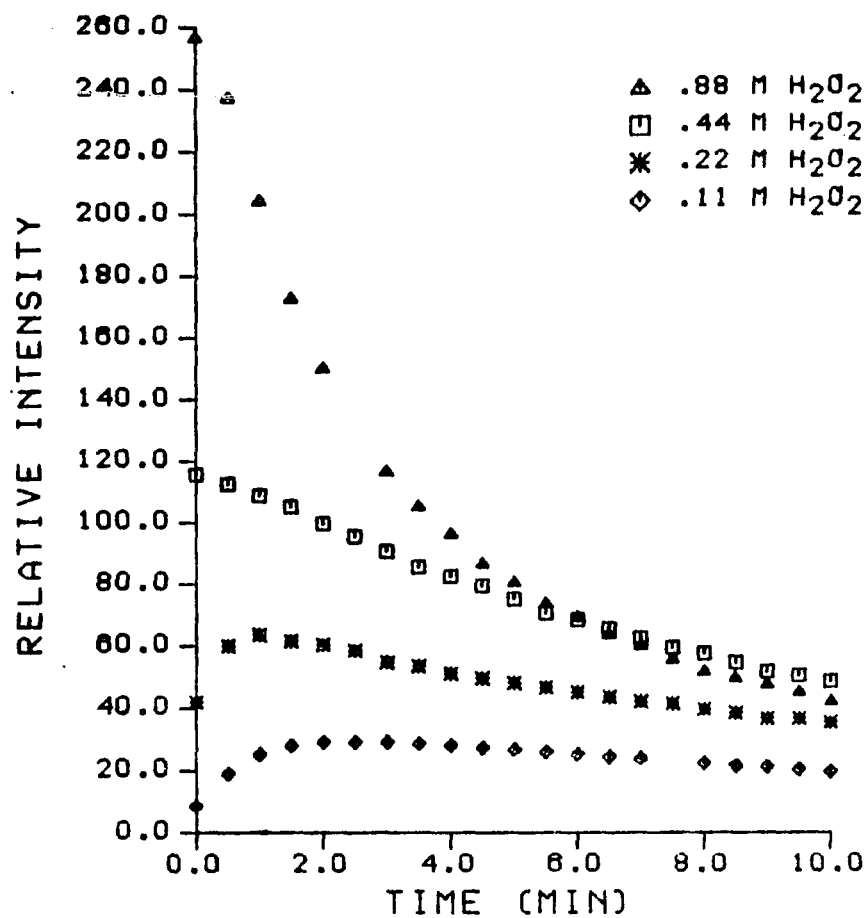
Figure 20a. Chemiluminescence intensity-time curves for various hydrogen peroxide concentrations in water. (10^{-7} M rhodamine B in .05 M THAM buffer, pH 8.00, 10^{-3} M CPPO in acetonitrile.)
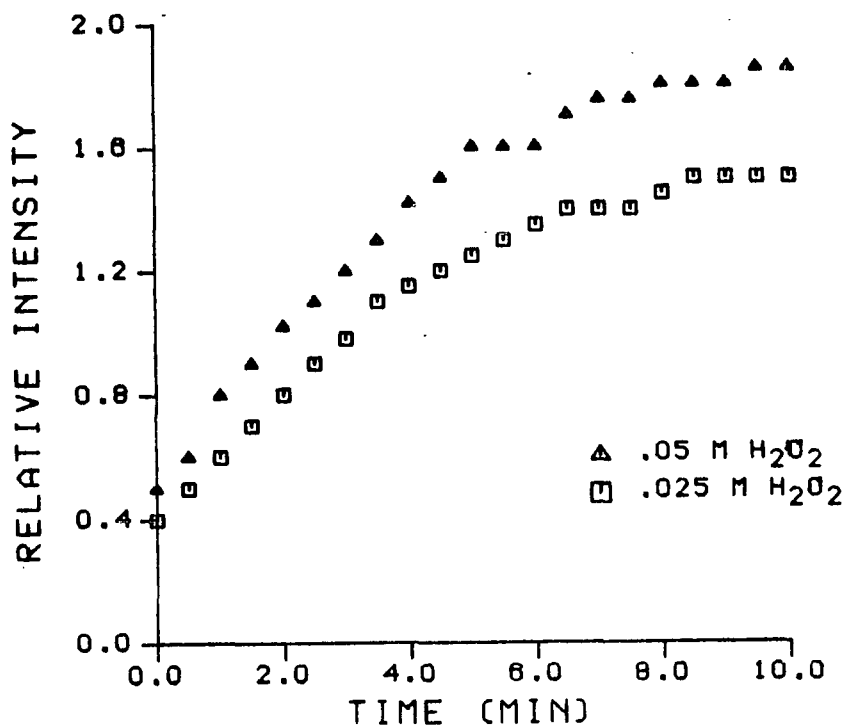
Figure 20b. Chemiluminescence intensity-time curves for various hydrogen peroxide concentrations in water. (10^{-7} M rhodamine B in .05 M THAM buffer, pH 8.00, 10^{-3} M CPPO in acetonitrile.)

limitations.

Limitations. Three problems were noted as a result of these experiments:

(1) A very high background emission was observed when no fluorophor was present. The kinetics of the background were faster than the kinetics of the fluorophors. Because of this background, the lowest concentrations that could be detected were on the order of 10^{-8} M rhodamine B and 10^{-7} M 2',7'-dichlorofluorescein or dansyl chloride.

(2) Precision is unacceptable for initial intensity measurements. Imprecision is a problem even with slower kinetics. Systems with acceptable precision use higher organic concentrations (Kobayashi and Imai, 1981). One reason for the problem could be the solvent ratio so a higher organic concentration (20%) was used for the next set of experiments.

(3) Measurements were made using 9.0 mL of analyte. For immunoassay applications, much smaller volumes must be used (in the μL range at most). Therefore, future measurements were made in this range and mixing conditions were changed so that all three reagents were mixed simultaneously instead of analyte and ester being mixed first.

The system was reevaluated to deal with these limitations.

Improving the System

Introduction

Because the blank may be due to contamination, solvents were purified to reduce fluorophor contamination and esters were recrystallized to remove impurities. In an attempt to obtain purer esters, two other esters were prepared and recrystallized: bis(2,4,6-trichlorophenyl) oxalate (TPCO) and bis(2,4-dinitrophenyl) oxalate (DNPO) whose structures are compared with CPPO in Figure 21.

New solvent systems were also examined for these esters. Acetone and acetonitrile were chosen initially because of their miscibility with water. Later, ethyl acetate was also examined because TCPO is stable in it (Kobayashi and Imai, 1980). However, ethyl acetate is immiscible with water. To promote mixing of the aqueous and organic phases, several organic solvents for hydrogen peroxide were also examined. In addition to water, acetone and acetonitrile were evaluated as solvents for hydrogen peroxide.

Initially these systems were evaluated with an increased organic concentration of 20%. To improve precision, several systems with varying organic concentrations were compared.

All intensity-time curves were evaluated using a measurement volume no larger than 500 µL. A new instrument, LUMAC Biocounter M 2000 was used instead of the Aminco Photometer for several reasons. The cells are reproducible optically and have larger volumes (3 mL vs. .5 mL), making it
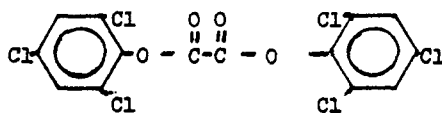
BIS (2,4,6 TRICHLOROPHENYL) OXALATE
TCPO
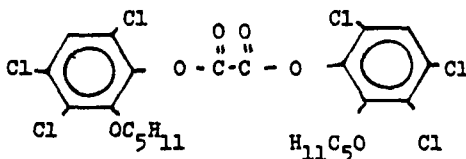
BIS (2,4,5 TRICHLORO-CARBOPENTOXYPHENYL) OXALATE
CPPO
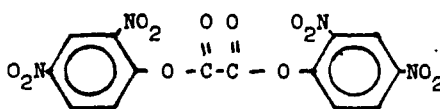
BIS (2,4 DINITROPHENYL) OXALATE
DNPO

Figure 21. Structures of esters.

easier to mix reagents. The LUMAC instrument also has integrating capabilities.

Experimental

Reagents. Bis(2,4,5-trichloro-6-carbopentoxyphenyl) oxalate (CPPO) (American Cyanamid) was recrystallized twice from acetonitrile. Bis(2,4,6-trichlorophenyl) oxalate (TCPO) was prepared from 2,4,6-trichlorophenol (Aldrich) according to the method of Mohan and Turro and recrystallized twice from petroleum ether. Bis(2,4-dinitrophenyl) oxalate (DNPO) was prepared from 2,4-dinitrophenol (Sigma) according to Mohan and Turro (1974).

Solutions of rhodamine B were prepared in .05 M THAM buffer adjusted to pH 7.5.

Acetone (Fisher, reagent grade), acetonitrile (Baker), and ethyl acetate (Eastman Kodak) were used to prepare solutions of the esters and hydrogen peroxide (30%, Baker, Reagent Grade). Distilled water was also used for hydrogen peroxide. For the detection limit data, HPLC Grade acetonitrile, ethyl acetate, and water (Fisher) were used.

Instrumentation. Data was taken with a LUMAC Biocounter M 2000 and recorded on a Heath Schlumberger Strip Chart Recorder, Model SR 255B. Reagents were dispensed with a Gilson Pipetman adjustable pipet.

Procedure. Aliquots of the solutions were added in the order of 400.0 µL fluorophor, 50.0 µL ester, and 50.0 µL hydrogen peroxide, then mixed by inverting 5 times for 3.0

seconds. Recording of intensity began 5.0 seconds after mixing. Since most of the information is obtained from the initial part of the intensity-time curve, intensities were integrated for up to 60 seconds.

Glassware was cleaned by soaking at least one hour in concentrated nitric acid (Baker) followed by several rinses with deionized water.

Results and Discussion

Effect of Solvents for Hydrogen Peroxide. Intensity-time curve for hydrogen peroxide in different solvents are shown in Figure 22. Both acetone and acetonitrile gave higher intensities than water so they were both employed with the ester solvents. As expected, a higher organic component in the reaction mixture results in increased intensity. Because acetonitrile gave the highest intensity, these results are shown in the next section.

Effects of Esters. Intensity-time curves for the esters are shown in Figures 23a, and b. Intensities in acetonitrile were significantly lower and are shown in Figure 23c, but it should be noted for comparison that these results are an order of magnitude lower. TCPO and CPPO behaved similarly in that they both have slow kinetics. On the other hand, reaction kinetics with DNPO are much faster and a sharp decay was observed. This response is unacceptable because of the difficulty in reproducing the initial intensity. Most of the signal for DNPO has decayed before it is recorded.
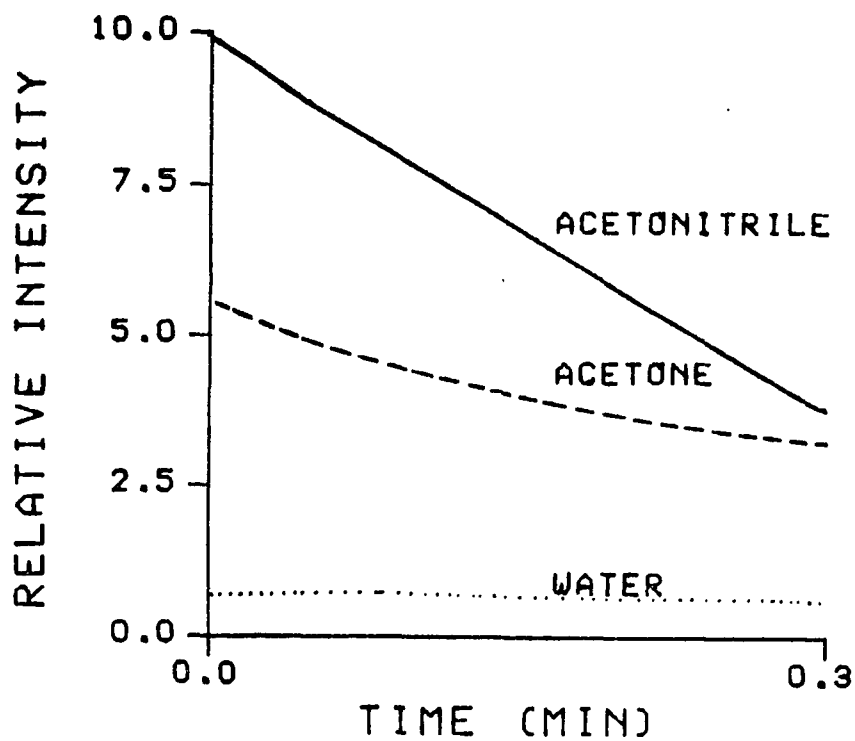
Figure 22. Chemiluminescence intensity-time curves for solvents for hydrogen peroxide. (10^{-1} M hydrogen peroxide, 3×10^{-2} M TCPO in acetone, 10^{-6} M rhodamine B in .05 M THAM, pH 8.00.)
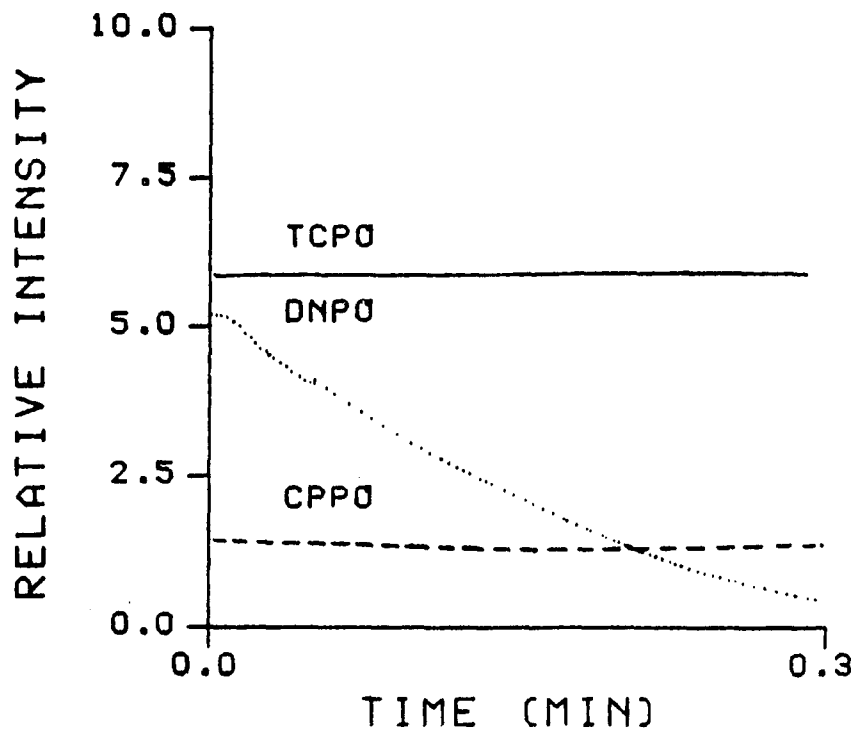
Figure 23a. Chemiluminescence intensity-time curves for three esters in ethyl acetate. (10^{-2} M ester, 10^{-1} M hydrogen peroxide in acetonitrile, 10^{-6} M rhodamine B in .05 M THAM buffer, pH 8.00.)
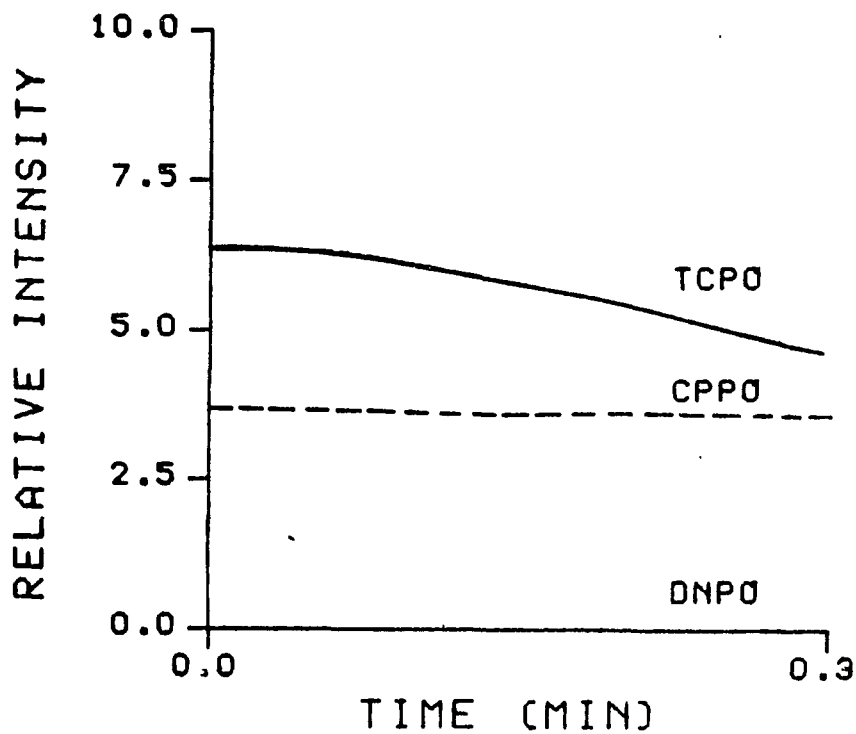
Figure 23b. Chemiluminescence intensity-time curves for
three esters in acetone. (10^{-2} M ester, 10^{-1} M
hydrogen peroxide in acetonitrile, 10^{-6} M rhodamine B
in .05 M THAM buffer, pH 8.00.)
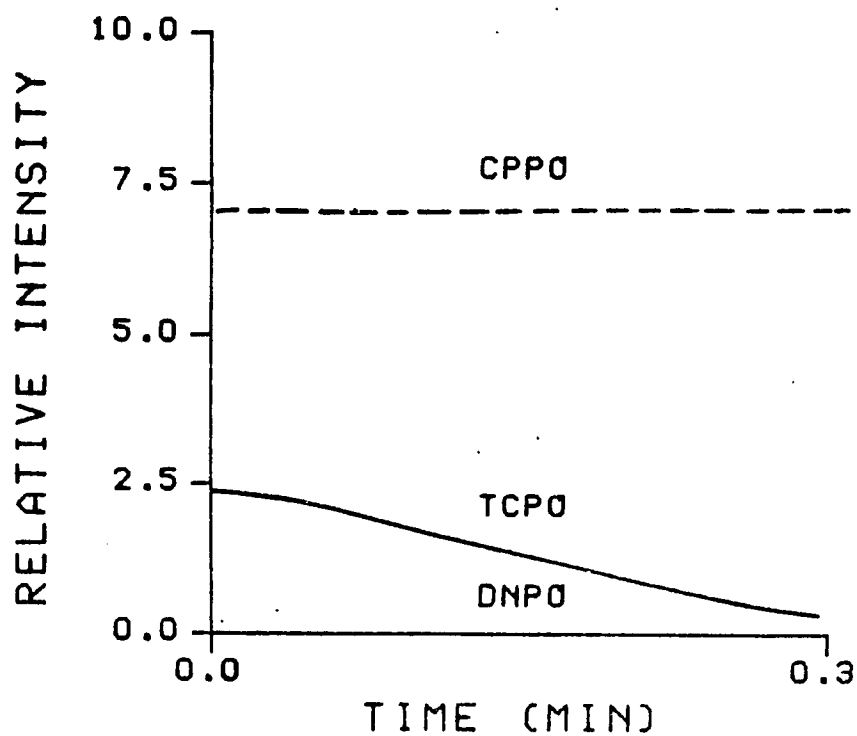
Figure 23c.   Chemiluminescence intensity-time curves for
three esters in acetonitrile.   Intensities are an order
of magnitude lower than in Figures 20a and b.   (10^{-2} M
ester, 10^{-1} M hydrogen peroxide in acetonitrile, 10^{-6} M
rhodamine B in .05 M THAM buffer, pH 8.00.)

Recrystallization of the esters did not improve the signal/background, but an advantage is gained in that the CL efficiencies of TCPO and DNPO are higher. Both TCPO and DNPO are reported to have been used for the most efficient CL reactions (Rauhut, 1979). Although the kinetics for the DNPO reaction are unacceptable using the hydrogen peroxide concentrations in these experiments, TCPO is suitable for use in this measurement system.

Effects of Solvents. Results with solvents ethyl acetate, acetone and acetonitrile are shown in Figures 24a and b. Of all the intensity-time curves studied, the most suitable kinetics for measurement are shown here. Intensity for TCPO in ethyl acetate or acetone is high initially with a very slow decay rate. Because TCPO is most stable in ethyl acetate and the decay rate is even slower than in acetone, ethyl acetate was used for further experiments.

System for Measurement and Limitations. Based on the above parameters, the chosen measurement system consisted of rhodamine B in THAM, TCPO in ethyl acetate, and hydrogen peroxide in acetonitrile. All solvents must be purified to reduce the background so HPLC grade solvents or purer should be used. A significant improvement in signal/background was observed by purification and the improvement in detectability is reported in the section on detection limit. The limitation of this system is the precision. The most suitable reaction kinetics for acceptable precision were chosen based on the previous experiments. However, mixing imprecision
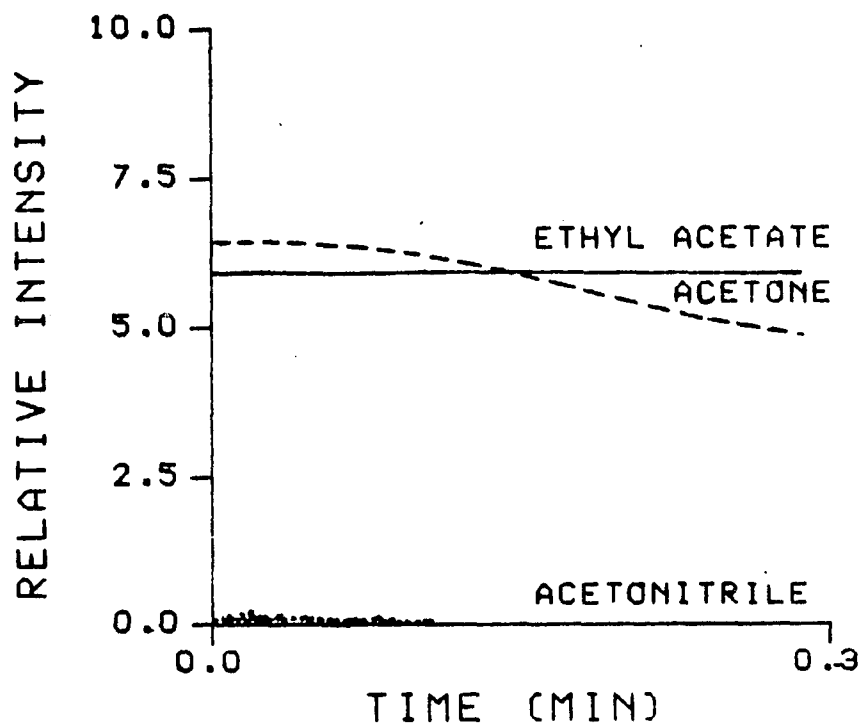
Figure 24a. Chemiluminescence intensity-time curves for
     TCPO in three solvents. (10^{-2} M TCPO, 10^{-1} M hydrogen
     peroxide in acetonitrile, 10^{-6} M rhodamine B in .05 M
     THAM buffer, pH 8.00.)
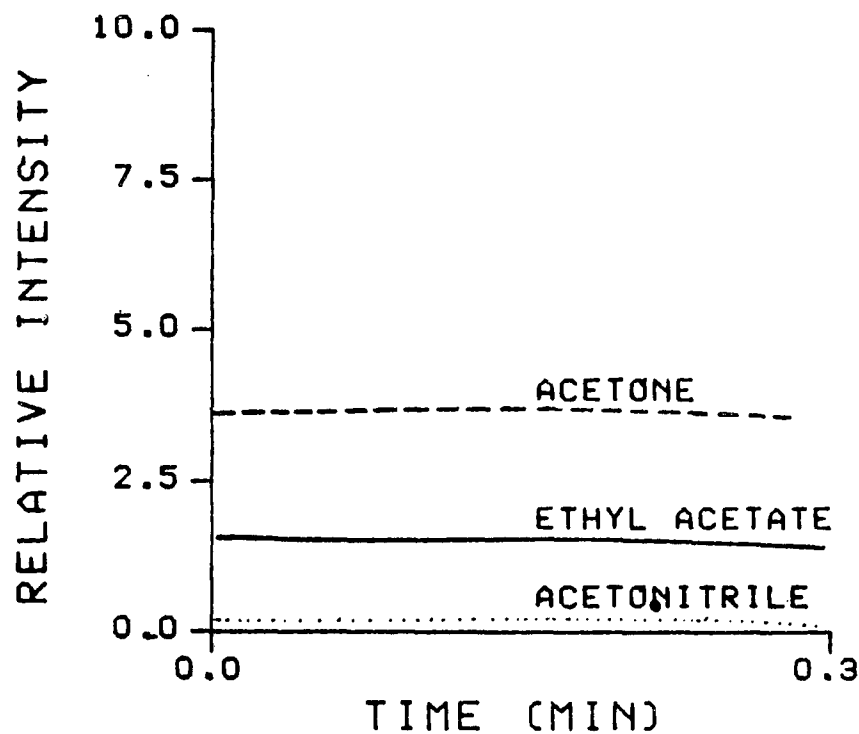
Figure 24b. Chemiluminescence intensity-time curves for CPPO in three solvents. (10^{-2} M CPPO, 10^{-1} M hydrogen peroxide in acetonitrile, 10^{-6} M rhodamine B in .05 M THAM buffer, pH 8.00.)

was still a problem. To improve precision, the effect of
solvent proportions was evaluated.

Precision. The ratio of organic solvent to water was
varied and the effect on the intensity-time curve is shown
in Figure 25. Each measurement was made in triplicate. The
area of these curves was integrated and the data were evalu-
ated using Bartlett's test to compare precisions. Bartlett's
test is used to determine whether standard deviation for sets
of measurements are homogeneous: i.e., is precision for one
different from (better than) another? A chi-square value is
calculated and the hypothesis that the precisions are homo-
geneous is tested at an appropriate confidence level. Results
of Bartlett's test (Table 3) show that the standard devia-
tions are not from a homogeneous group at the 99% confidence
level. Further inspection shows that the variances increase
as the aqueous component of the mixture increases suggesting
that better precision can be achieved with a higher organic
concentration.

To achieve the highest ratio of water/organic and still
have precision of 2-5% R.S.D., a mixture which contains no
more than 50% water should be used.

Detection Limits. Detection limits for immunoassay are
usually reported in absolute amounts rather than concentra-
tion. Using the system developed from these results, the
peroxyoxalate reaction can be used to determine fluorophors
in the fmol range. The conditions which achieve this is
rhodamine B in THAM buffer adjusted to pH 8.00, 10^{-3} M TCPO
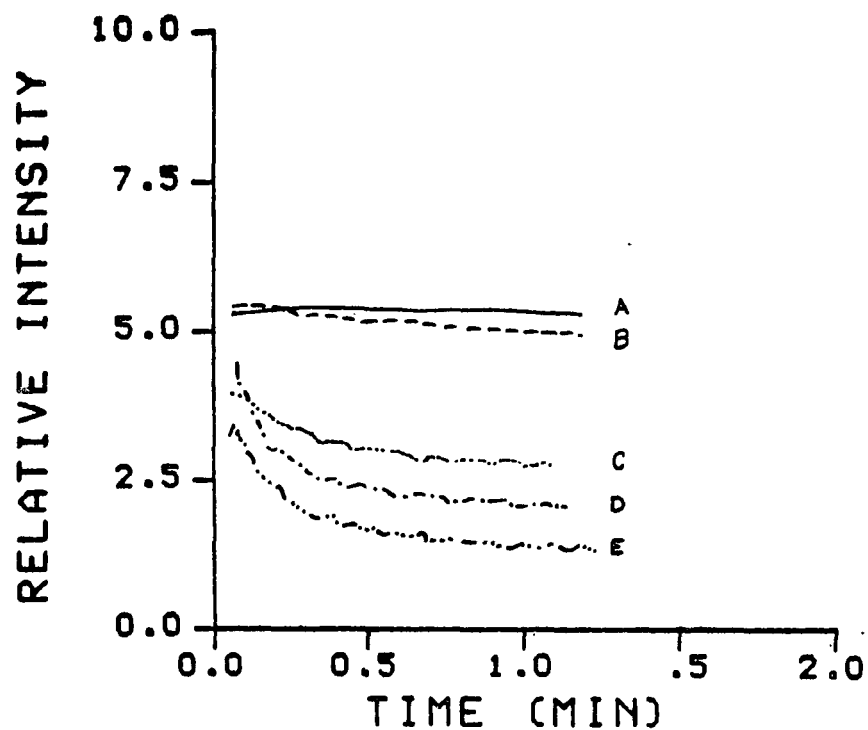
Figure 25. Chemiluminescence intensity-time curves for various organic/water solvent ratios. The ratios for (10^{-5} M rhodamine B in .05 M THAM buffer, pH 8.00, 10^{-3} MTCPO in ethyl acetate, 10^{-3} M hydrogen peroxide in acetonitrile) are A (1,2,2), B (2,1.5,1.5), C (3,1,1), D (3.5,.75,.75), E (4,.5,.5). Total volume of each measurement is 500 µL.

TABLE 3

Results from Bartlett's Test for Precision of
Various Water/Organic Solvent Systems

Solvent System (µL)					Relative
Buffer	Ethyl Acetate	Aceton- itrile	Relative Intensity	Variances	Standard Deviation
20	100	380	1.68 1.55 1.52	.0072	5%
100	50	350	4.45 4.30 4.45	.0075	1.9%
100	100	300	4.90 5.15 4.90	.0208	2.8%
200	150	150	5.70 5.80 5.30	.1033	4.7%
300	100	100	8.90 6.70 6.30	2.3433	2.1%

$$\chi^2 = 23.2198$$

$$\chi^2_{(10,.01)} = 23.2$$

$$\chi^2_{(10,.025)} = 20.5$$

in ethyl acetate, and 10^{-3} M hydrogen peroxide in acetonitrile in the ratio 5:2:3 for the highest acceptable water concentration. Under these conditions, the detection limit for rhodamine B is 10^{-9} M or 250 fmol when measuring 250 μL of sample. Using a higher organic concentration, i.e., the same reagents in the ratio 1:10:14, 25 fmol of rhodamine B are detectable. Detectability is limited by the background luminescence when no fluorophor is present. The above values are based on the imprecision of the background and are detectable at 2 times the standard deviation of the background luminescence. Purified solvents (HPLC grade) reduced but did not eliminate this background. Optical resolution with filters is possible but the trade off is sensitivity. As shown in Table 5, a cut-off filter of 554 nm improves signal/background approximately 1.8 times. An interference filter (577 nm) improves signal/background to 4.6, but reduces both background and analyte. Attempts to identify the source of the background with fluorescence measurements on solvents, etc. were unsuccessful.

Because background and analyte signal are proportional to the volume measured, absolute detection limits could be improved by measuring smaller volumes. In this case, mechanical mixing would be necessary.

TABLE 5

Optical Resolution of Chemiluminescence Signal

	I_{CL} of Sample	I_{CL} of Background	$\dfrac{\text{Sample}}{\text{Background}}$
No filter	60987 67060	59041 55451	$\dfrac{64023.5}{57246} = 1.1$
Long Pass filter 554 nm	20193 34234 28605	12272 19705 12591	$\dfrac{27213.5}{14856} = 1.8$
Interference filter 577 nm	731 832 864	126 152 208	$\dfrac{804}{162} = 4.9$

CHAPTER V

HOMOGENEOUS IMMUNOASSAY

Effect of Labelling on Chemiluminescence Efficiency

Introduction

A label is required for the most sensitive forms of
immunoassay. Some non-radioisotopic labels have the attrac-
tion of permitting measurement of bound label without sepa-
rating it from free label following equilibration. Fluores-
cence polarization (McGregor et al., 1978), the enzyme multi-
plied immunoassay technique (EMIT, Syva) (Rubenstein et al.,
1972), electron spin resonance (Leute et al., 1972), and CL
(Kohen et al., 1981) all work without prior separation. In
order for this to be possible, there must be some analytical
characteristic of the label which changes upon binding.

In the case of peroxyoxalate CL, this characteristic
would be the light intensity because it is a measure of the
quantum yield (CL efficiency) which changes due to the effect
of binding of the antibody to the fluorophor labelled antigen.
To understand how this is possible, the general mechanism
for CL will be considered.

Theory. As explained in Chapter I, the overall quantum
yield (light emitted per mole of chemiluminescent reactant)
for any chemiluminescent reaction is the product of the
yields of separate steps. This general mechanism is shown

below.

> Chemiluminescent reactant + other reactants
>
> Step 1 $\downarrow$ chemical reaction
>
> Key Intermediate
>
> Step 2 $\downarrow$ excitation reaction
>
> Excited State Product
>
> Step 3 $\downarrow$ fluorescence emission
>
> Light and Ground State Product
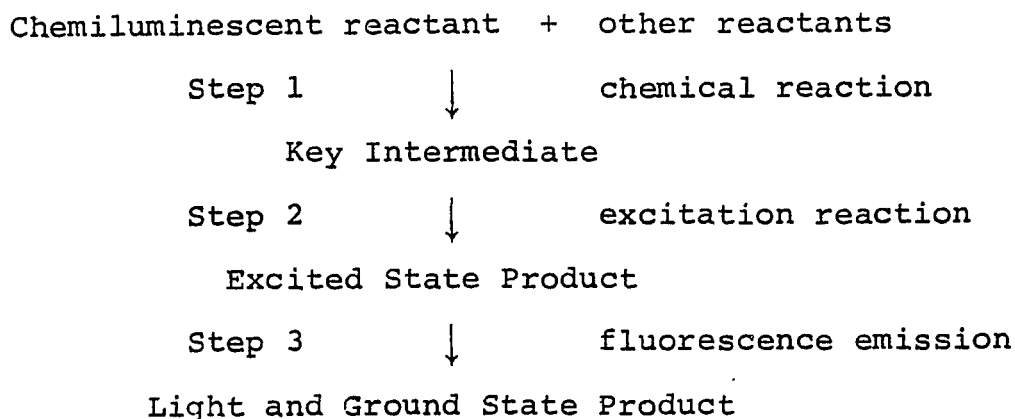
Considering the peroxyoxalate reaction, Step 1 is unrelated to the fluorophor labelled analyte and results in the formation of 1,2-dioxetanedione. Step 3, the fluorescence emission is not always affected by binding (Rauhut, 1979). However, it is conceivable that Step 2, the excitation step will be affected. The excitation of the fluorophor labelled analyte involves energy transfer from dioxetanedione to the fluorophor. When the fluorophor labelled antigen is bound to the antibody, the fluorophor may not be excited in the same way as when it is free which would then affect the quantum yield for the reaction.

To test the feasibility of a homogeneous CL immunoassay, it must first be demonstrated whether binding does indeed affect CL excitation efficiency. One way of doing this is to compare CL intensities with fluorescence intensities of bound and free fluorophors. The basis for this comparison can be shown mathematically.

Combining equations (1) and (3) from Chapter I, the

relationship between CL intensity and excitation efficiency is

$$I_{CL} = \phi_{EX} \; \phi_F \; K(C) \qquad\qquad (9)$$

The expression for fluorescence intensity (I_F) is

$$I_F = \phi_F \; k(C) \qquad\qquad (10)$$

where k is a constant incorporating instrumental factors and other terms are defined previously.

The effect of binding on excitation efficiency can be determined by comparing CL and fluorescence intensities of the fluorophor bound to an antigen $(I_{CL \; bound}, \; I_{F \; bound})$ with the intensities of the free fluorophor $(I_{CL \; free}, \; I_{F \; free})$ as follows

$$\frac{I_{CL \; bound}/I_{F \; bound}}{I_{CL \; free}/I_{F \; free}} = X \qquad\qquad (11)$$

where X = the effect of binding on excitation efficiency. Substituting equations (9) and (10) for I_{CL} and I_F:

$$\frac{K \; \phi_{EX \; bound}\phi_F \; (C)_{bound}/k \; \phi_F \; (C)_{bound}}{K \; \phi_{EX \; free}(C)_{free}/k \; \phi_F \; (C)_{free}} = X \qquad\qquad (12)$$

which simplified to

$$\frac{\phi_{EX \; bound}}{\phi_{EX \; free}} = X \qquad\qquad (13)$$

For X - 1, there is no effect on the excitation effi-
ciency; for X less than 1, the excitation efficiency of the
free is greater than that of the bound fluorophor; for X
greater than 1, the excitation efficiency of the bound is
greater than that of the free fluorophor.

This value was determined by measuring I_{CL} and I_F for
the free fluorophor rhodamine and as a bound fluorophor after
labelling with antigens bovine serum albumin, human albumin,
and folic acid.

Experimental

Reagents. Rhodamine B (Eastman Kodak, Practical Grade)
was used as the free fluorophor. Rhodamine B Isothiocyanate
(RITC) Sigma Grade II was used in the labelling experiments.
Bovine albumin, human albumin and folic acid, all from Sigma,
were each bound to RITC.

Chemiluminescent reagents used were 0.05 M TRIS buffer
(Fisher, primary standard), pH 7.5, 10^{-3} M TCPO in ethyl
acetate (Fisher), and 10^{-3} M H_2O_2 (Baker) in acetonitrile
(Baker).

Instrumentation. Fluorescence measurements were made
on a Perkin Elmer 204 Spectrofluorometer using an excitation
wavelength of 552 nm and an emission wavelength of 571 nm.

Chemiluminescence measurements were made on a LUMAC
Biocounter M 2000 using the 60 second integrate mode.

Labelling. Proteins and folic acid were bound to the
isothiocyanate form of rhodamine B using the methods

described by Udenfriend (1962). The labelled human and bovine albumin were separated from the free fluorophor by ultrafiltration using a membrane with molecular weight cut- off of 10000. The RITC was reacted with a slight excess of folic acid and the reaction was assumed to go to completion so no separation was performed.

Procedure. Labelled antigens were diluted 200 fold with Tris buffer and the fluorescence intensity was measured. The same solutions were used for the chemiluminescence measurements. The reaction mixture for each measurement con- sisted of 250 μL labelled antigen, 100 μL TCPO solution and 150 μL of H_2O_2 solution. These were hand shaken and the intensity integrated for 60 seconds. Duplicates of each solution were measured and the values averaged.

Results and Discussion

To determine the effect of binding on excitation effi- ciency, fluorescence and CL intensities of the free rhodamine and of each of the three labelled rhodamine fractions were substituted into equation (12) (Table 4). Binding either albumin to the fluorophor decreases the excitation efficiency. However the folic acid labelled fluorophor is excited four times more efficiently than free rhodamine.

One might expect that the fluorophor bound to protein would be less efficiently excited. It might be less accessi- ble to the excited state intermediate since the protein occu- pies space near the fluorophor. A possible explanation for

TABLE 4

Effect of Labelling on Relative Excitation Efficiency

Serum Albumin	.85
Bovine Serum Albumin	.22
Folic Acid	4

the increase in excitation efficiency for folic acid is that
the folic acid is excited and transfers its energy to the
rhodamine. The energy transfer step of the peroxyoxalate
reaction is not clearly understood so any explanation of the
effect on the excitation efficiency must be tentative.
Steric effects alone are not sufficient to explain the pro-
cess although it is likely that they play some role.

Binding Curve

Since labelling affects CL efficiency, it would be
expected that binding the antigen to the fluorophor labelled
antibody would also affect intensity. If this is the case,
then bound and free labelled fluorophor can be distinguished
without a separation using an appropriate calibration curve.

One way to study this effect is as follows. Using com-
mercially available fluorophor labelled antibody, a series
of increasing concentrations of antigen are added to aliquots
of the antibody and allowed to bind. The antibody is main-
tained in excess so that all of the antigen will bind. CL
intensity of the antibody with and without antigen is
measured and the results plotted as a function of increasing
antigen concentration. Changes in CL intensity of the
fluorophor labelled antibody could then be attributed to the
effect of binding of the antigen to the antibody.

The success of this experiment would determine whether
or not a competitive binding curve could be prepared. Even
with a high water to organic solvent ratio, there may still

be solvent related problems if the antigen-antibody binding
is affected by the organic components.

Experimental

Reagents. RITC labelled Goat Anti Human Albumin (Bio-
netics) was prepared according to directions provided by
Bionetics in distilled water and diluted as needed. A series
of concentrations of human albumin (Sigma) from 10^{-4} M to
10^{-8} M were prepared. Tris buffer (0.05 M) pH 7.5, with
0.9% NaCl was used for dilutions.

CL reagents used for the first set of experiments were
10^{-3} CPPO (American Cyanamid) in acetone and 5×10^{-3} M H_2O_2
in acetone. For the second set of experiments, 10^{-3} M TCPO
in ethyl acetate and 10^{-3} M H_2O_2 in acetonitrile were
employed.

Instrumentation. CL measurements were made with an
Aminco Chem-Glo Photometer and peak heights were recorded
after 30 seconds from a Heath Schlumberger strip chart
recorder.

Procedure. For the first experiment, duplicates of
200 μL of each of the five albumin concentrations were added
to ten aliquots of 200 μL of RITC antialbumin (of concen-
tration greater than 10^{-8} M). These were incubated at room
temperature for 30 minutes. To make the CL measurement,
50 μL of ester solution and 50 μL of H_2O_2 solution were
added to each tube.

The procedure for the second experiment was the same

except for volume changes. Ten μL each of antibody and antigen solution were incubated in each tube and 100 μL of ester solution plus 350 μL of H_2O_2 solution were added to each tube for the CL measurement.

Results and Discussion

Binding Curve. Results of the first binding study with 80% water component in the measurement system are shown in Figure 26. CL intensity was plotted as a function of concentration of antigen added. The highest intensity was obtained for the free antibody and a decrease in intensity is observed with increasing antigen concentration suggesting that as the albumin binds to the antibody the efficiency of excitation was affected. However the affect is very subtle and measurement imprecision leaves considerable uncertainty in the response function.

Results of the binding study with 4% water concentration in the measurement mixture is shown in Figure 27. A similar response is obtained in that a decrease in intensity was observed with increasing antigen concentration.

Precision. Precision of the CL measurements can be observed from the plots of the binding curves; they range from 2-22% relative standard deviation for duplicates of the same sample. The precision of the measurements for the high organic component solvent system are 2-4% R.S.D. except for two concentrations which exhibited very large scatter. Imprecision in these cases may be due to pipetting errors or

Figure 26. Binding curve of RITC antialbumin with albumin
using a chemiluminescence measurement system with 80%
water. (200 μL each albumin and RITC antialbumin in
.05 M THAM, pH 7.5, 50 L each 10^{-3} M CPPO in acetone,
10^{-3} M hydrogen peroxide in acetonitrile.)

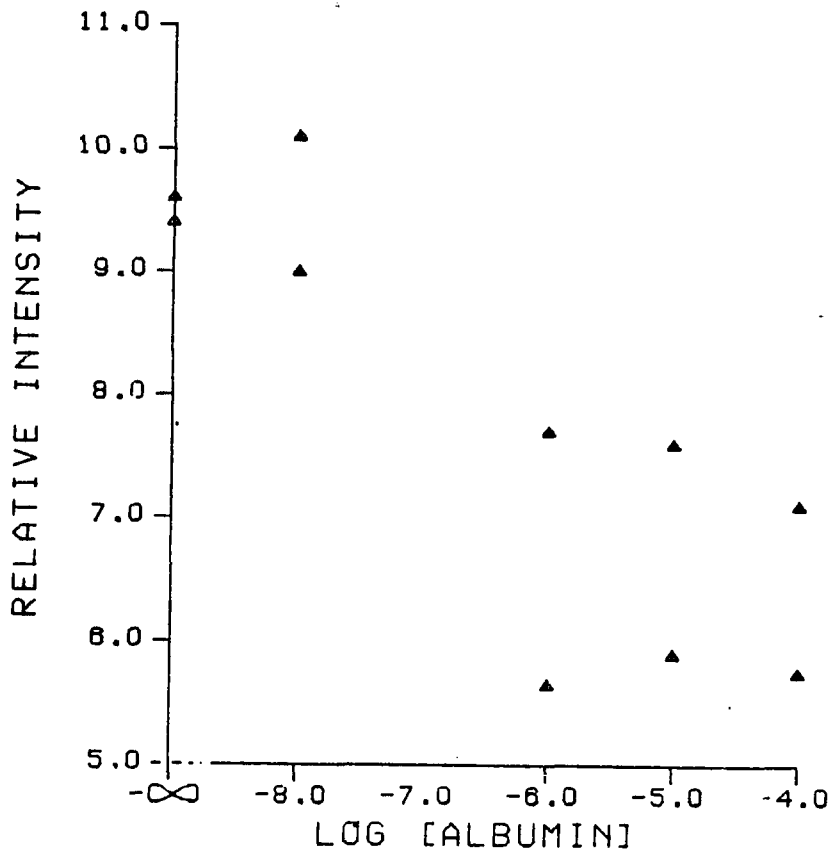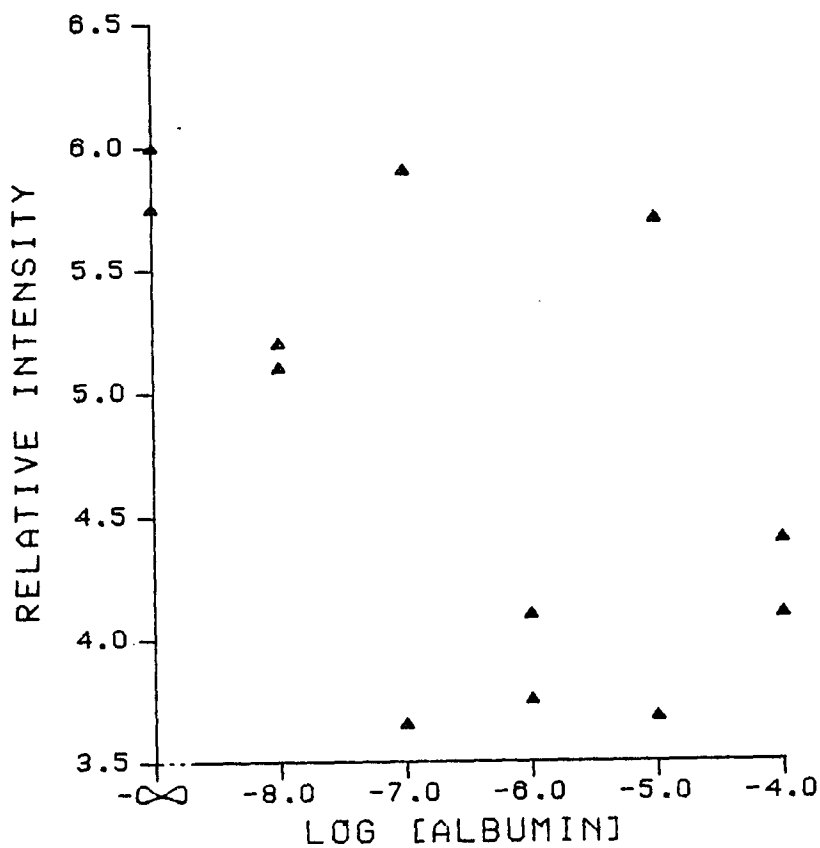Figure 27. Binding curve of RITC antialbumin with albumin
using a chemiluminescence measurement system with 4%
water. (10 μL each albumin and RITC antialbumin in
.05 M THAM, pH 7.5, 100 μL CPPO in acetone, 380 μL
hydrogen peroxide in acetonitrile.)

may reflect the effect of the organ solvent on the antigen-antibody complex. The precision of the measurements of free labelled antibody for both systems is about 2% R.S.D. suggesting antigen related imprecision.

Precision is a major limitation to the CL measurement. Mixing of small volumes of organic and water components is not easily reproducible when mixed manually.

Conclusions. The fact that intensity changes with binding suggests that a homogeneous competitive binding assay is feasible but several problems must be solved to successfully design such an assay. Imprecision is generally a problem with immunoassays and the peroxyoxalate CL method suffers from this imprecision because of difficulties in mixing. Organic solvents are necessary for the measurement, but should be kept to a minimum because of their effect on the antigen-antibody complex. However, as demonstrated in Chapter IV, high water concentration for the measurement causes increased imprecision.

These problems coupled with the fact that the change response is relatively small, suggests that a heterogeneous assay might be preferable. In this situation, a more precise measurement can be designed without concern for the effect on the antigen-antibody complex since it will have been separated before a measurement is made.

CHAPTER VI

HETEROGENEOUS IMMUNOASSAY

Introduction

Heterogeneous immunoassay involves determination of
bound and/or free labelled antigen (or antibody) after they
are separated. This contributes to imprecision in the immu-
noassay because of the additional step in the procedure, but
it imposes fewer restrictions on the method of measurement.
Because solvent constraints are less of a problem once the
bound and free fractions are separated, the peroxyoxalate CL
method should be a suitable way to determine fluorophor
labelled analyte.

To determine if the CL method is suitable for immuno-
assay measurements it can be compared with a fluorescence
immunoassay which uses fluorophor labelled antibody with
fluorescence as the detection method.

There are two commercially available fluorescence immu-
noassays which are comparable in precision and accuracy
(Blanchard and Gardner, 1978). These are the Fiax Immuno-
globin Assay Kit and the Bio-Rad Immuno-Fluor Assay Kit.
Both are used for the determination of immunoglobins and
both use solid phase antibodies for the separation. The
Fiax kit uses a stick with antibodies immobilized on it.
The stick is dipped in the analyte plus reagent solutions

and separation is achieved by removing the stick from solu-
tion, then placing it in a fluorometer for detection. The
Bio-Rad method uses antibodies immobilized on polyacryla-
mide beads. These are equilibrated with the analyte and
fluorescent reagent. The density of the beads is 1.1 g/cm^3
so that after separating they can be resuspended in solution
for the fluorescence measurement. The procedure is shown in
Figure 28.

Because the fluorophor is immobilized on a solid,
neither method is ideally suited for CL energy transfer. The
beads in solution should be more accessible than the stick
so the Bio-Rad method was chosen for comparison. Still, this
method presents several problems for CL measurement. First,
energy transfer involves fluorophor on a solid surface.
Second, the density of the CL measurement solvent system
will be slightly less than that of water so the beads will
have to be resuspended by mixing immediately before intensity
measurements. Third, the conditions of the immunoassay must
be adjusted so that they will be compatible with the CL
measurement. The Bio-Rad kit uses a fluorescein label and
phosphate buffer. From the results described in Chapter IV,
a rhodamine label and Tris buffer are preferable for the CL
measurement.
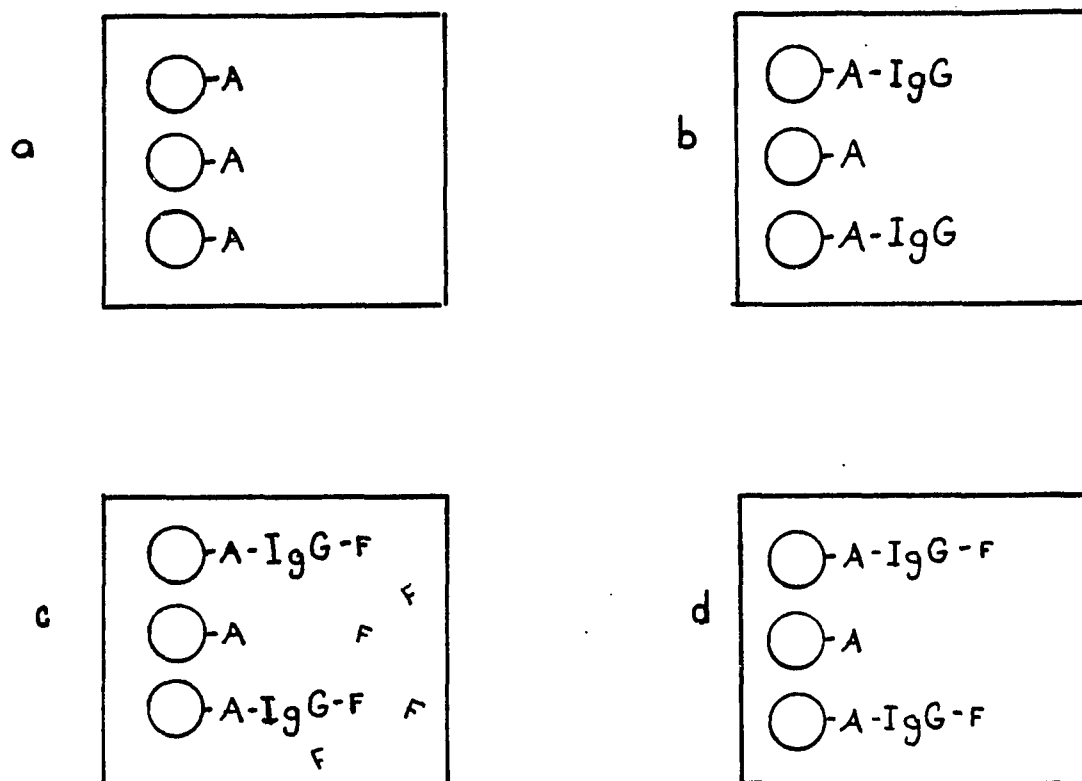
Figure 28. Schematic of Bio-Rad Immunofluor Assay Kit.
(a) Antibody to human immunoglobulins (A) is covalently
bonded to small hydrophilic beads (O) (derivatized
polyacrylamide beads) to form a stable immunoadsorbent.
(b) Sample is added and all of the immunoglobulin in
the sample is bound to the excess solid phase immuno-
adsorbent. (c) Fluorescently labelled monospecific
antiserum (F) is added to the mixture and combines with
the antigen bound to the immunoadsorbent. (d) After
separation from unreacted materials, the stable com-
plexes that are formed are quantitated by standard
fluorometric techniques.

Experimental

Reagents

Biorad ImmunoFluor Ig G Standard and Immunobead Reagent were used from the Biorad Immunofluorescent Assay Kit. Tetramethyl Rhodamine Conjugated Ig G Fraction, Rabbit (Cappel Laboratories, Inc.) was substituted for the Immuno-Fluor Reagent in the kit. Tris buffer (0.01 M, pH 7.5) (Fisher) was prepared from triple distilled water with 0.15 M NaCl and 0.1% NaN_3.

The CL reagents used were 10^{-3} M H_2O_2 (Baker) in aceto-nitrile (Fisher, HPLC Grade) and 10^{-3} M TCPO in ethyl acetate (Fisher, HPLC Grade).

Instrumentation

Fluorescence measurements were made on an SLM Photon Counting Spectrofluorometer with the excitation and emission polarizers crossed with excitation wavelength of 550 nm. An interference filter of 577 nm was used to measure emission. The PMT was operated at 1300 V. The sample holder was modified to receive 12 × 75 mm glass tubes used for the reaction.

The CL measurements were made with a LUMAC Biocounter M 2000 using glass tubes.

Procedure

The Biorad ImmunoFluor Assay Kit instructions were followed with substitution of rhodamine labelled antiserum

and Tris buffer. In this procedure the diluted samples, prepared in triplicate from standards in the kit, were added to an excess of derivatized polyacrylamide beads to which antibody specific to the human immunoglobin in the samples was bound to the solid phase immunoadsorbent during the course of a 1.5 hour incubation at $37^{\circ}C$. Rhodamine labelled monospecific antiserum was then added to the mixture, which was incubated at $37^{\circ}C$ for another hour as the fluorescent antiserum combined with the bound antigen. Two mL of buffer was added to the mixtures and the tubes were centrifuged at 1700 × g for 8 min. Supernates were decanted and the pellets washed twice with 3.0 mL of buffer to remove all unreacted material. Care was taken not to disturb the pellets during decantation and the rims of the tubes were blotted with absorbent paper. After the final centrifugation, 2.0 mL of buffer were added to each tube and each tube followed by vortex mixing.

Fluorescence in the suspension was read as described in Instrumentation.

CL measurements were made by pipetting 200.0 mL of freshly vortexed sample into glass cuvettes and adding 1 mL each of ester solution and water solution. The tube was vortexed for 3.0 seconds and CL integrated for 60 sec.

Results and Discussion

Calibration Curves. A calibration curve for the fluorescence immunoassay is shown in Figure 29. Precision for
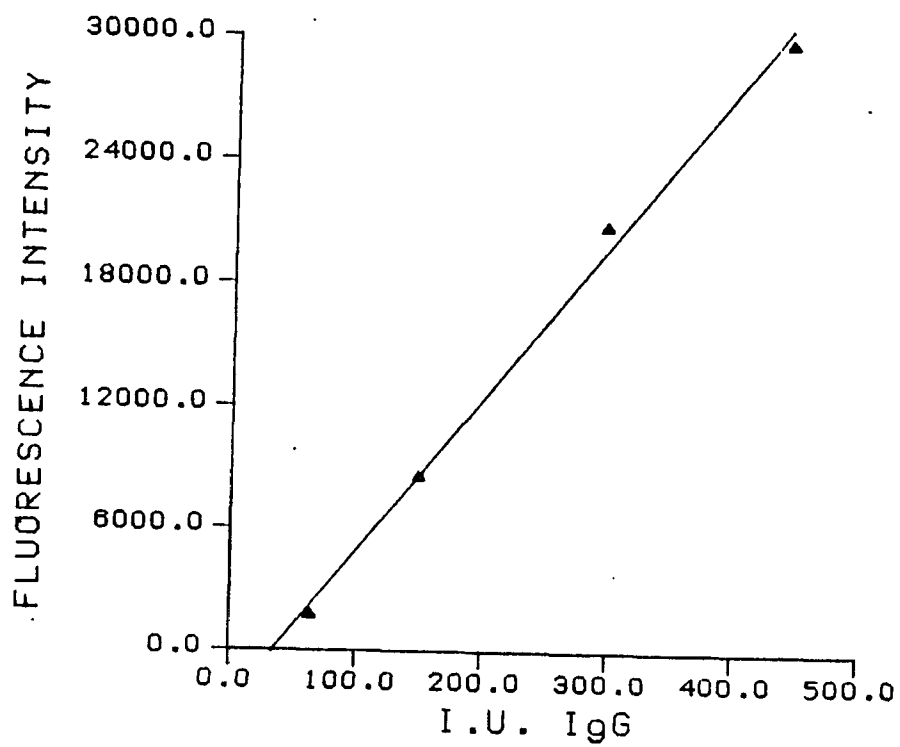
Figure 29. Calibration curve for fluorescence immunoassay. (RITC labelled antiserum, λ_{ex} = 550 mm, λ_{em} = 577 nm.)

random duplicate measurements of the same solution varied from 3-9% relative standard deviation. Part of this may be due to the optical imperfections of the cells and differences in position in front of the detector.

The calibration curve for the CL immunoassay is shown in Figure 30. Precision for the CL measurements range from 4-15% relative standard deviations for random duplicates. Imprecision can be explained to some degree because of the non-homogeneous system containing the beads.

For both calibration curves, the relative standard deviations do not reflect the imprecision of the analytical method because it does not take into account the deviations of the points from the line. These deviations are more evident in the CL method than in the fluorescence. The calibration line for the CL method intersects the intensity axis suggesting a problem with the blank. This is expected in the CL immunoassay.
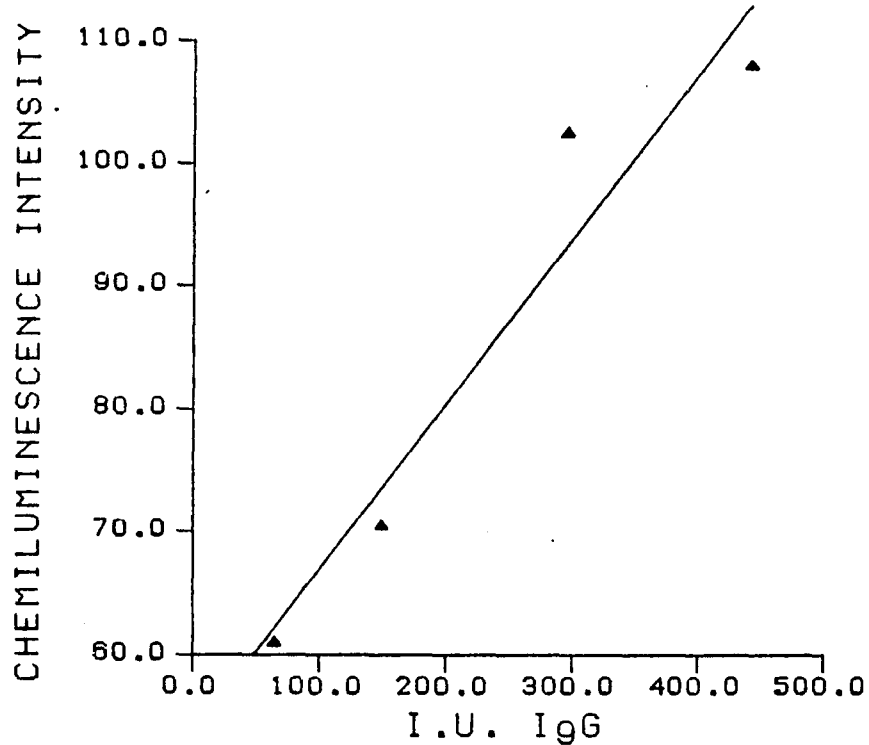
Figure 30. Calibration curve for chemiluminescence immuno-
assay. Chemiluminescent measurement made with 200 µL
reacted mixture RITC labelled antiserum, immunoglobulin
standards and Bio-Rad immunoadsorbent, 1.000 mL 2 ×
10^{-3} M TCPO in ethyl acetate and 10^{-3} M hydrogen
peroxide in acetonitrile.

CHAPTER VII

CONCLUSIONS

Four questions were to be answered in evaluating the peroxyoxalate system as a new approach to CL ummunoassay. (1) Energy transfer to fluorophors other than dansyl chloride has been shown to be possible and in some cases preferable for analytical purposes because of the increase in CL intensity. (2) The feasibility of a homogeneous immunoassay was demonstrated because the CL excitation efficiency of the fluorophor is affected by binding but the imprecision problem must be solved before it can be satisfactorily applied to analysis. (3) The measurement system with a high water component for the homogeneous immunoassay cannot be mixed reproducibly for satisfactory precision. (4) Conditions were evaluated for a measurement system that could be reproducibly mixed manually. The conditions for this include a high organic component and reaction conditions with slow kinetics. Under these conditions, detection limits suitable for immunoassay are attainable.

Future work with this system should approach the problem of mixing since imprecision is the major limitation for this method. Automated methods of dealing with mixed solvent systems could be developed. Once this problem is satisfactorily handled the CL system could be used for immunoassay since it does have the detectability needed for this application.

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APPENDIX

MINC II COMPUTER PROGRAM FOR MEASURING
PEAK HEIGHT, AREA, AND DURATION

```
10   Rem light, bas measures peak ht, and area
20   Rem it calculates re. st. dev. and stores data
30   K=0
40   Print 'beginning set' K+1
50   Print 'how many peaks (up to 15)'; / input L
60   Dim A(15), H(15), D%(511), X(20), S1(20), R1(20),
       Y(20), T1(20)
70   Rem initialize counters
80   C=0
90   A=0 / H=0/ A2=0/ H2=0
100  For I=0 to 511
110  D%(I)=0
120  Next I
130  Print 'ready for data'
135  Set-gain (,1,9)
140  Ain('display, st2', D%(), 512, 1/50, 9)
150  Print "data taken'
160  For I=0 to 19
170  A2=A2+D%(I)
180  If D%(I)<H2 then H2=D%(I)
190  Next I
200  For I=20 to 511
210  A=A+D%(I)
220  If D%(I)<H then H=D%(I)
230  Next I
240  A=A-A2*24.6
250  H=H-H2
260  Rem measure time
270  Z=0
280  For I=0 to 511
290  If D%(I)<H2 then Z=Z+1
300  Next I
310  T=10.24*Z/512
320  Print 'peak height is'H;
330  Print 'peak area is'A
340  Print 'time is'T;' keep peak(Y or N)'; / input Q$
350  If Q$='N' then 90
360  A(C)=A / H(C)=H / T(C)=T
370  C=C+1
380  If C<L then 90
390  X(K)=0 / H1(K)=0 / T1(K)=0
400  For I=0 to L-1
410  X(K)=X(K)+A(I) / H1(K)+H(I) / T1(K)=T1(K)+T(I)
420  Next I
430  X(K)=X(K)/L / H1(K)=H1(K)/L / T1(K)=T1(K)/L
440  X2=0 / Y2=0
450  For I=0 to L-1
```

```
460   X1=(A(I)-X(K))^2 / Y1=(H(I)-H1(K))^2
470   X2=X1+X2 / Y2=Y1+Y2
480   Next I
490   S1(K)=((X2/IL-1))^.5)/X(K)
500   R1(K)=((Y2/(L-1))^.5)/H1(K)
510   Print 'avg. height is'H1(K)'rel. std. dev. is'R1(K)
520   Print 'avg. area is'X(K)'rel. std. dev. is'S1(K)
530   Print 'avg. time is'T1(K)'identify peak'; / input Y(K)
540   Print 'end of set 'K+1' another set(Y or N)'; / input C$
550   K=K+1
560   If C$='Y' then 40
570   Print 'graph area vs. identifier(Y or N)'; / input G$
580   If G$='N' then 600
590   Graph(,K,X(0),Y(0))
600   Print 'store data(Y or N)'; / input S$
610   If S$='N' then 750
620   Print "file name'; / input F$
630   Open F$ as file #1
640   Dim #1,P(20,5)
650   For K=0 to 20
660   P(K,0)=X(K)
670   P(K,1)=H1(K)
680   P(K,2)=S1(K)
690   P(K,3)=R1(K)
700   P(K,4)=Y(K)
710   P(K,5)=T1(K)
720   Next K
730   Close
740   Print 'data stored'
750   Display-clear
760   Go to 830
770   Rem subroutine for peak values
780   Display-clear
790   For I=0 to 511
800   Print D%(I);
810   Next I
820   Return
830   End
```