May 8 19 64

THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Peter Benji Mason

ENTITLED Effect of Solvent Type and Composition on the Chemiluminescence of Luminol

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science

Timothy A Nieman

Instructor in Charge

APPROVED: [signature]

HEAD OF DEPARTMENT OF Chemistry

O-1944

EFFECT OF SOLVENT TYPE AND COMPOSITION UPON THE CHEMILUMINESCENCE OF LUMINOL

By

Peter Brent Mason

THESIS

for the

DEGREE OF BACHELOR OF SCIENCE

IN

CHEMISTRY

College of Liberal Arts and Sciences

University of Illinois

Urbana, Illinois

1990

ACKNOWLEDGEMENTS

I would like to thank everyone responsible for making this past year such a valuable and enjoyable experience.

To Dr. Nieman, I owe a great debt of thanks for allowing me the opportunity to gain invaluable experience in his laboratory. The support and expertise he brought to my efforts is greatly appreciated.

To everyone in the Nieman group (Janet, Terry, Rao, Nancy), thanks for welcoming me to the group and making me feel comfortable here.

To Janet, thanks for letting me pillage your equipment time and again, and thanks for remaining calm when I interrupted with one of my innumberable and ubiquitous questions.

Also, thanks to Rao for helping me out whenever I asked (and for losing gracefully at basketball).

TABLE OF CONTENTS

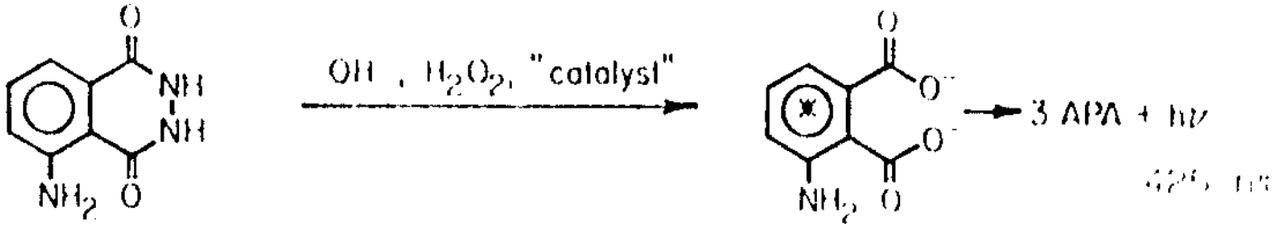
I. Introduction

Chemiluminescence (CL) is the emission of light by electronically excited chemical species which are produced via chemical reaction. This chemical process has been adapted for use in analytical methods and has proven advantageous by providing good detection limits and large linear dynamic ranges. Additionally, the technology for detecting chemiluminescence is well developed and relatively inexpensive, leading to ease of set-up and modification for particular uses (1-3).

A main thrust of the Nieman research group has been the research and development of luminol-based and acridinium ester-based detection schemes in solution-phase assays, in particular high performance liquid chromatographic separations. The luminol reaction [Figure 1] occurs in aqueous solution and utilizes a hydrogen peroxide oxidant and a catalyst, yielding light and the 3-aminophthalate product. Thus the luminol system has been exploited as a means for directly determining hydrogen peroxide concentrations and indirectly determining other analytes which produce hydrogen peroxide. Additionally, the elimination of catalyst and peroxide flow streams by *electrogenerated catalysis and peroxide production has* been studied.

The acridinium ester reaction [Figure 2] also occurs in aqueous solution with hydrogen peroxide as oxidant. However, a catalyst is not needed and this reaction must occur in strongly basic solution due to the fact that it is the dehydrogenated peroxide anion (HO_2^-) which is the oxidant (4). The particular ester of interest here is acridinium phenyl

Figure 1:



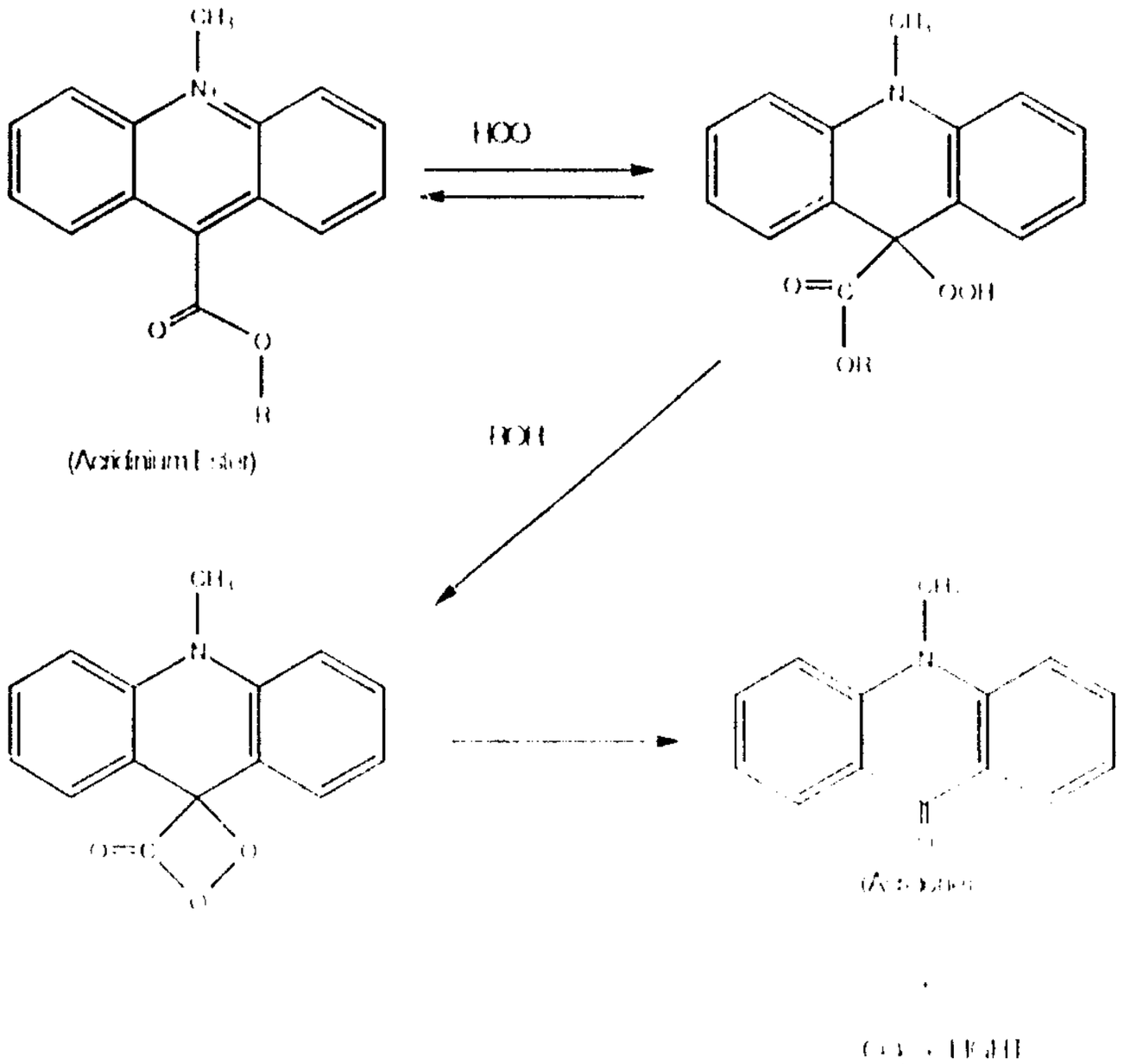
LUMINOL

3 AMINO PHTHALATE

(excited)

The luminol light reaction.

Figure 2:



ester (APE). This system has also been exploited as a means for determining peroxide concentrations in solution. Note that both luminol and APE may be covalently bonded to various compounds and therefore provide a means of directly measuring their concentrations. This derivatization method is hoped to provide excellent detection limits and dynamic range when employed with an HPLC separation.

The main direction of the work presented in this thesis is to examine the aqueous luminol reaction scheme as represented in HPLC detection methods, and to optimize several reaction parameters. The luminol section studied the effect of varying solvent type and composition on luminol chemiluminescence. Additionally, the effect of varying flow cell volume on the chemiluminescence of the APE system was studied.

II. Spacer Thickness Study

A. Background

The use of the single electrode flow cell in the APE chemiluminescence system is illustrated in Figure 3. A flow injection analysis system was employed in this experiment. The flow stream consisted of a solution of 0.05M borate buffer at pH 11. Following injection of the APE solution, the flow stream passed over a glassy carbon electrode held at -0.6V (vs Ag/AgCl) which acted as the hydrogen peroxide source. At pH 11, the peroxide is immediately converted to its dehydrogenated anion which then oxidizes the APE, causing the reaction to proceed to the emission of light. A study of flow cell volume was undertaken to see if a particular volume of solution produced an optimum chemiluminescence.

B. Instrumentation

The experimental set-up is illustrated in Figure 4. The electrochemical flow cell (Figure 3) was designed by the Nieman research group and built by the School of Chemical Sciences machine shop. The cell consisted of an opaque Kel-F backing in which the electrode (supplied by Bioanalytical Systems) was embedded. A transparent plexiglas face was then fitted over the backing, with teflon spacers between to vary the cell volume. The flow cell was placed directly in front of an RCA model 1P28 photomultiplier tube biased to -900V. The PMT signal was amplified by a Pacific Instruments model 126 photometer and read by a Curken linear

Figure 3: single electrode flow cell

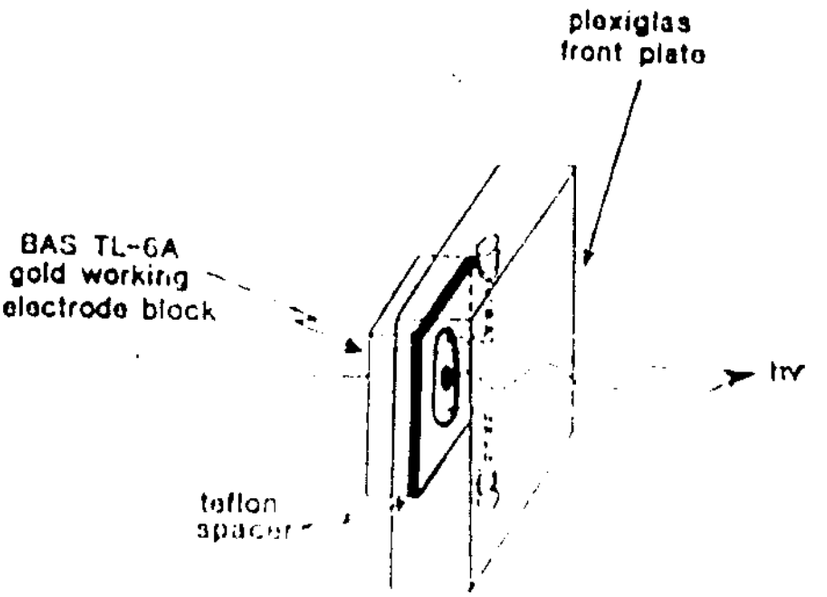
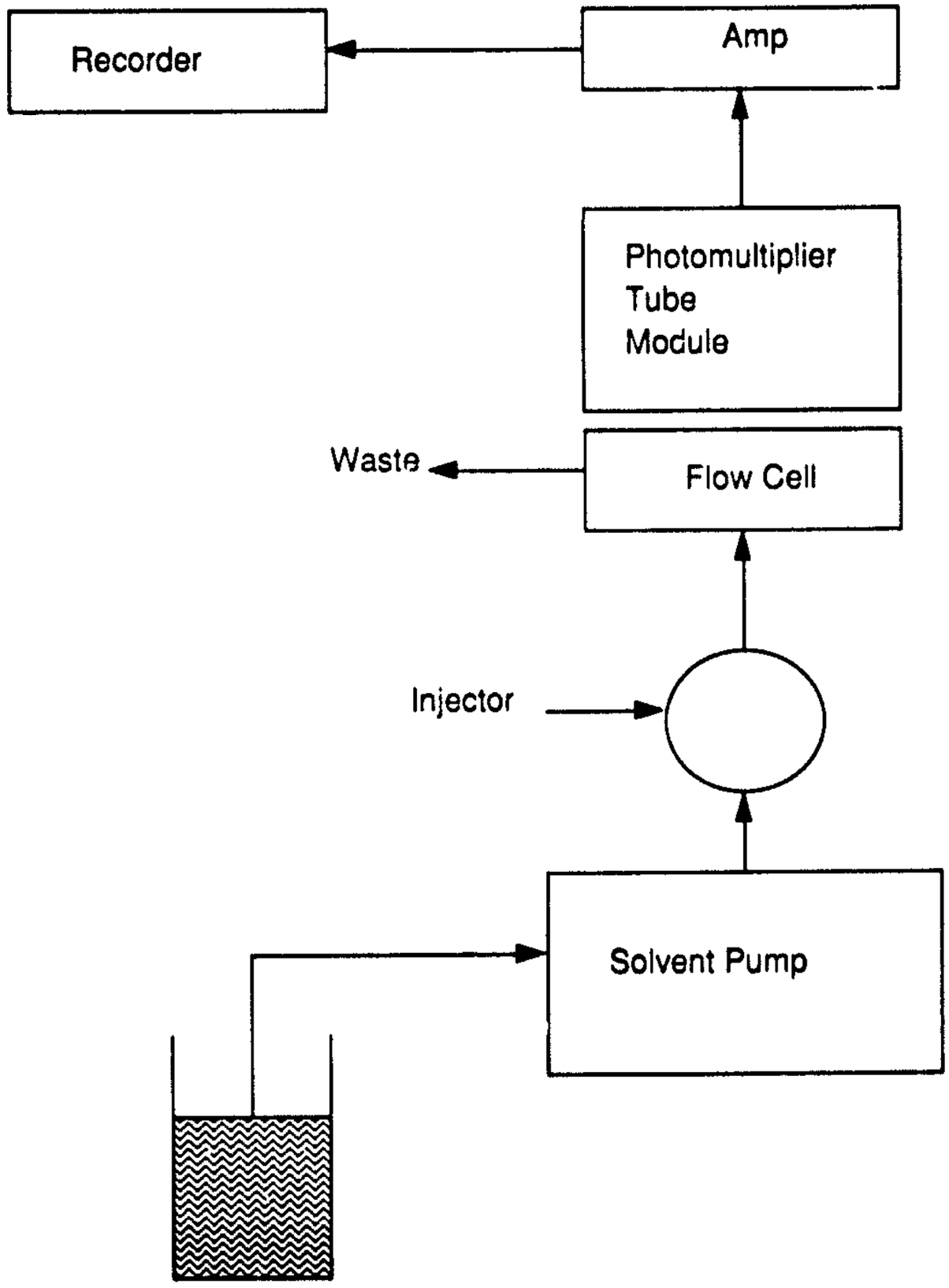


Figure 4: Schematic of Spacer Set-Up



recorder. A Rainin peristaltic pump model HP1 was employed using manifold tubing with a 3.16 mm internal diameter. Pump speed was set at 300 resulting in a flow rate of approximately 1.2 mL/min per channel. A Valco Instruments auto injector with 10 µL loop was placed in the Millipore water flow stream. The glassy carbon electrode was held to -0.6V using a Pine model AFRDEF bi-potentiostat. A silver/silver chloride reference electrode was screwed into the flow cell and a stainless steel counter electrode was placed downstream.

C. Reagents

All injections were made using a 1µM APE solution. The APE used was obtained from the Aldrich Chemical Company. The buffer was made using sodium borate obtained from the J.T. Baker Company. All chemicals were reagent grade quality, and water obtained was purified by a Millipore Continental purification system.

D. Procedure

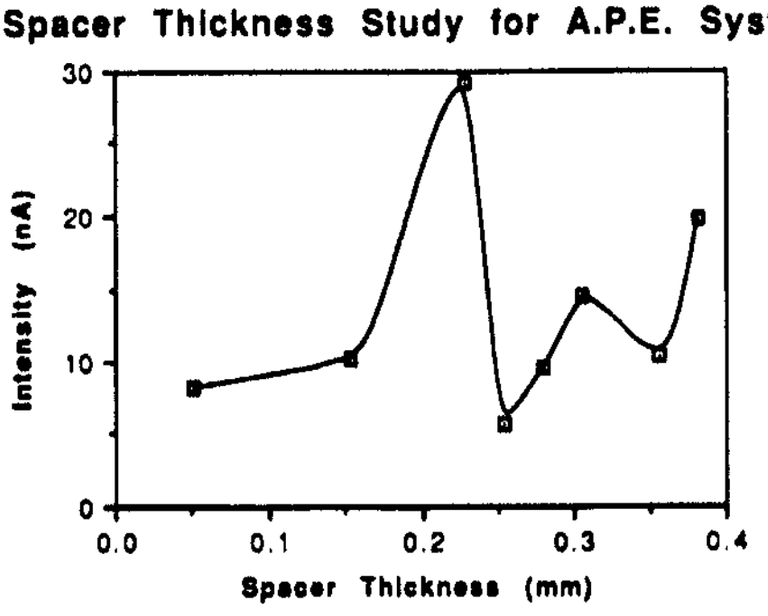
The experiment employed the flow injection system as illustrated. All instruments were powered up at least twenty minutes before the injections began. The solutions were pumped through the flow cell for several minutes and the cell was checked periodically for air bubbles. Due to the small cell volume, the presence of air bubbles would have greatly affected the experimental results. Once the absence of air bubbles was established, the cell was placed in a "black box" and the electrode leads were attached. All injections were made in triplicate. Following each set

of injections, the cell was removed, opened, and a different spacer thickness was placed between the opposing faces of the cell. Spacer thicknesses ranged from 0.051mm to 0.381mm.

E. Results and Discussion

Figure 5 illustrates the results of the experiment. The variations in chemiluminescence intensity with spacer thickness are large, in particular at a thickness of 0.229 mm. This spacer thickness gave a resulting light intensity nearly three times that of its immediate neighbors, and was one and a half times more intense than the 0.381 mm

Figure 5:



thickness. Increasing spacer thickness has the effect of decreasing the linear flow rate of solution through the cell. This may cause a change in the chemiluminescence due to kinetic factors. Also, the increase in flow

cell volume would decrease the concentration of APE since a fixed injection volume of APE was passed through a larger and larger flow cell volume. Finally, all else being equal, the larger the solution volume exposed to the detector, the larger should be the resulting CL intensity, an effect not observed here. An additional, more intensive study is called for, conducted with varying spacer thicknesses around 0.229 mm to examine the behavior of the APE at linear flow rates corresponding to these flow cell volumes.

III. Effect of Solvent on Luminol Chemiluminescence

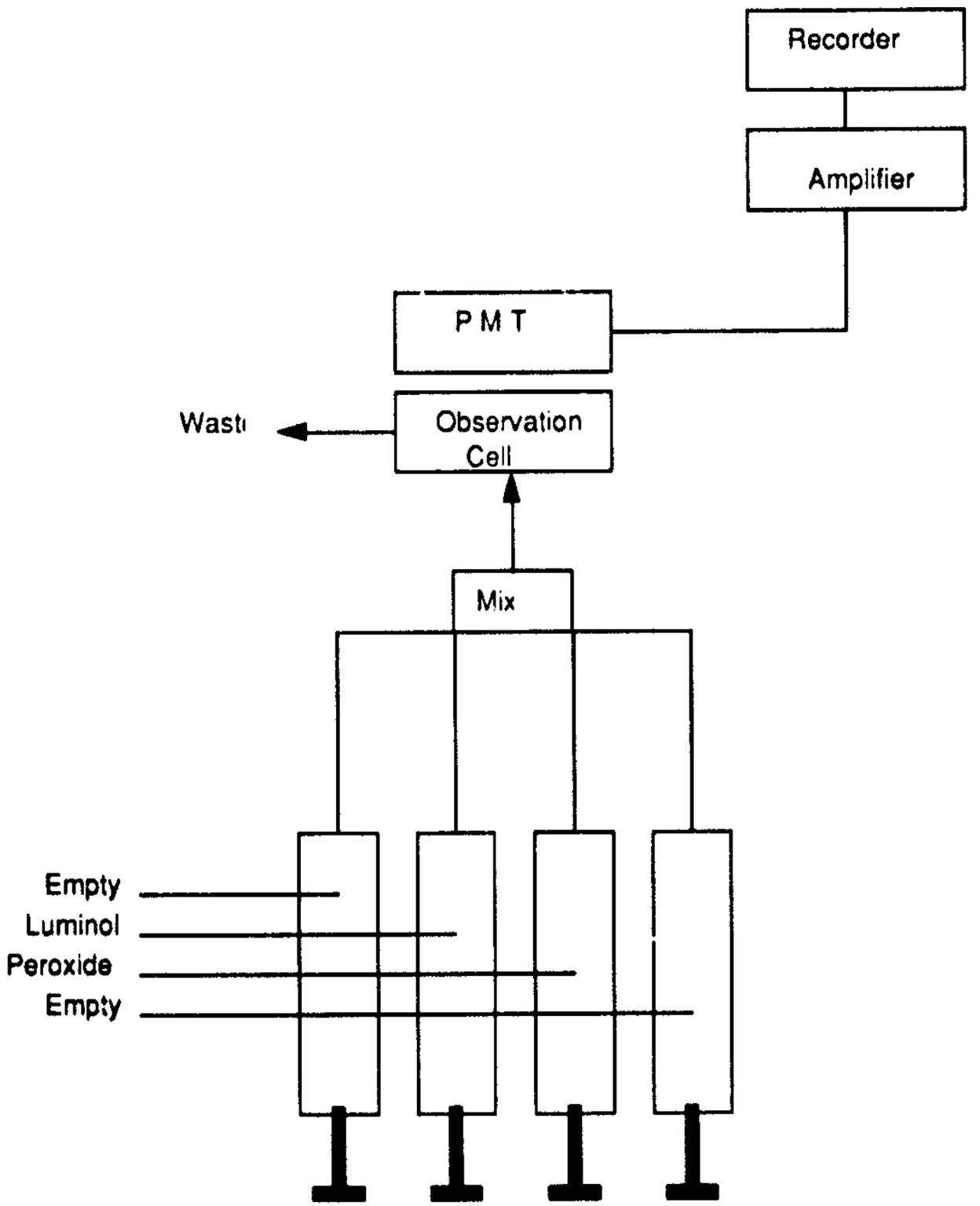
A. Background

As mentioned earlier, much of the work done in the Nieman research group revolves around the use of chemiluminescence-based detection of analytes in an HPLC separation. Most if not all HPLC separations require the use of an organic component of the mobile phase, usually methanol or acetonitrile. Therefore, the effect of these two solvents upon the chemiluminescence of the luminol system was studied. In this set of experiments, a stopped-flow injection apparatus designed by the Nieman research group was employed (Figure 6). In stopped-flow analysis, several solutions are mixed and then immediately injected into a cell. The flow of the solutions is then halted and various reaction parameters can be studied versus time.

B. Instrumentation and Set-Up

The experimental set-up is illustrated in Figure 6. The stopped-flow injection apparatus and cell were designed by the Nieman group and built

FIGURE 6: Stopped-flow set-up



by the School of Chemical Sciences machine shop. The cell consists of a teflon backing in which inlet and outlet holes are bored. The middle section consists also of teflon, with an elongated oval bored through the teflon to act as the entire cell volume. Finally, a transparent plexiglass plate is placed over the two teflon pieces. Due to the high pressures involved during injection (>500 psi), the cell is placed in a brass pipe assembly, with a counter pipe screwed in to very tightly compress the three layers of the flow cell. The cell was then placed directly in front of a Hamamatsu model R372 photomultiplier tube biased to -900V. This was then connected to a Pacific Instruments model 124 photometer followed by a Curken linear recorder. A compressed air switching system was employed to load the syringes and then inject the two solutions into the cell. Check valves were placed prior to and after the syringes to prevent leakage or backflow of solution into and out of the flow cell during an experiment.

Two solvents were examined: methanol and acetonitrile. For each solvent, there were two series of solutions involved. For methanol, one series consisted of varying amounts of 100 μM hydrogen peroxide diluted in methanol to form solutions of 0, 5, 10, 20, 30, and 40% peroxide in methanol. The second series consisted of a solution of 50 mM Phosphate buffer, 2 μM bovine hemin, and 100 μM luminol, at pH 11, mixed with varying amounts of methanol to produce solutions of 0, 5, 10, 20, 30, and 40% methanol. For the acetonitrile study, the plexiglas plate made it impossible to exceed a solution of more than 20% acetonitrile: beyond that concentration the plexiglas was degraded. Solutions of 0, 5, 10, and 20% acetonitrile were utilized. Therefore, with increasing amounts of the organic solvent, the reactant concentrations were correspondingly

reduced. In order to take into account the effect of the reagent dilutions on chemiluminescence, two additional series of solutions were made with the peroxide and luminol stock solutions and then diluted with water (to 0, 5, 10, 20, 30, and 40%) instead of an organic solvent. These latter solutions acted as blanks and were used to normalize the data obtained.

Upon initial completion of the methanol and acetonitrile studies, it was decided to use a method of internal blanking to increase the accuracy and precision of the acetonitrile study. In this experiment the percent acetonitrile was varied from 0 to 50% while the concentrations of the peroxide and luminol solutions were kept constant. Thus no consideration had to be taken for changing reagent concentrations. Additionaliy, in this experiment, a thin sheet of Saran was placed between the teflon flow cell and the plexiglass face, thereby allowing acetonitrile concentrations up to 50%.

C. Reagents

Chemicals used were as follows: dibasic sodium phosphate (Fisher), hydrogen peroxide as 30% (Fisher), bovine hemin (Sigma), and luminol (Aldrich). All chemicals were reagent grade quality, and water obtained was purified by a Millipore Continental purification system.

D. Procedure

The experiment employed the stopped-flow injection system as follows. All instruments were powered up at least twenty minutes prior to the first injection. In between each injection, the syringes and flow cell were flushed with Millipore water to ensure no cross contamination. Because the flow cell volume was much smaller than that of the syringes

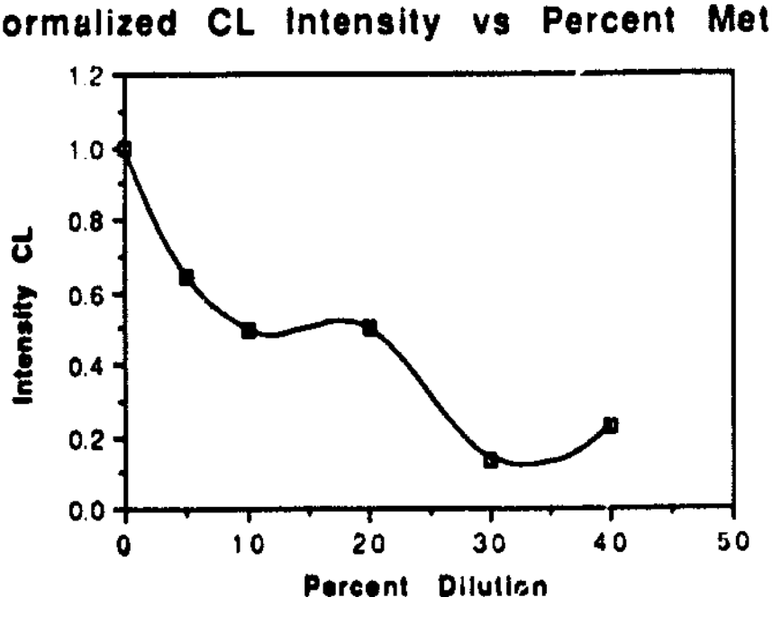
and various tubing, the cell was flushed with each reagent solution three times to ensure that all excess water was removed from the cell and tubing. Three parameters of the chemiluminescence intensity versus time plots were examined: peak intensity, intensity at one-half plot time, and total peak area (determined by weight).

E. Results and Discussion

1. Methanol

Figures 7, 8, and 9 illustrate the results of the methanol study. The so-called "normalized" values are the measurement for the solution containing methanol divided by the measurement for the same concentrations in a completely aqueous solution. Figure 7 indicates in a plot of chemiluminescence intensity versus percent methanol that there is an immediate decrease in luminol CL upon the addition of 5% methanol.

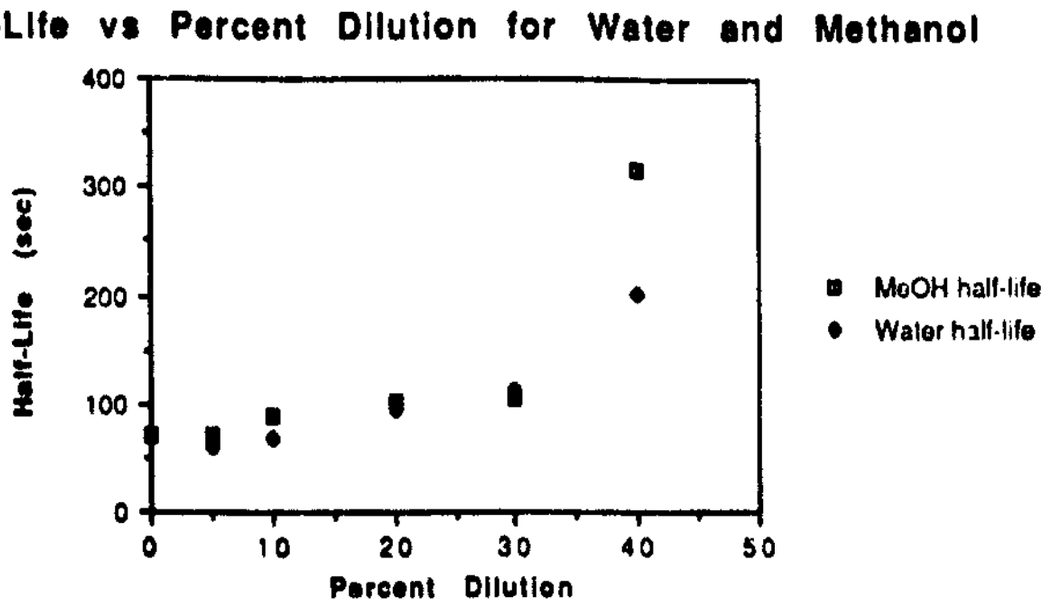
Figure 7:



This is followed by a lessening degree of decrease from 5% to 10%, a plateau at 10% to 20%, then another large drop to 30% where the chemiluminescence intensity increases slightly.

Figure 8 is a graph of intensity half-life versus percent water and methanol. There is a general slow increase in the half-lives of both

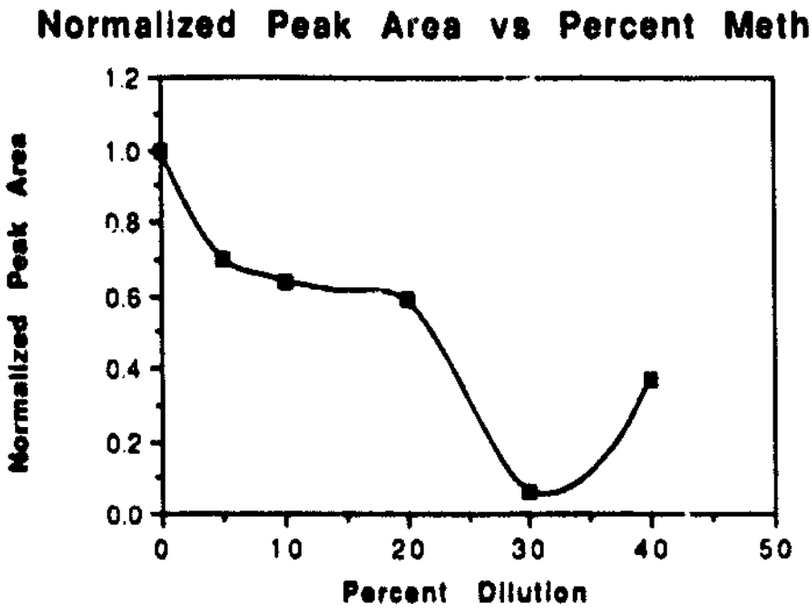
Figure 8:



water and methanol dilutions. Note that both are roughly identical with the exception of the 40% data points. The slow increase in half-life seems to be independent of solvent. It is worth noting that from 0% to 30% dilution the half-life increased by 35 seconds (or about 45%), a fact that may have some practical significance in HPLC separations.

Finally, Figure 9 plots the peak areas versus percent methanol.

Figure 9:



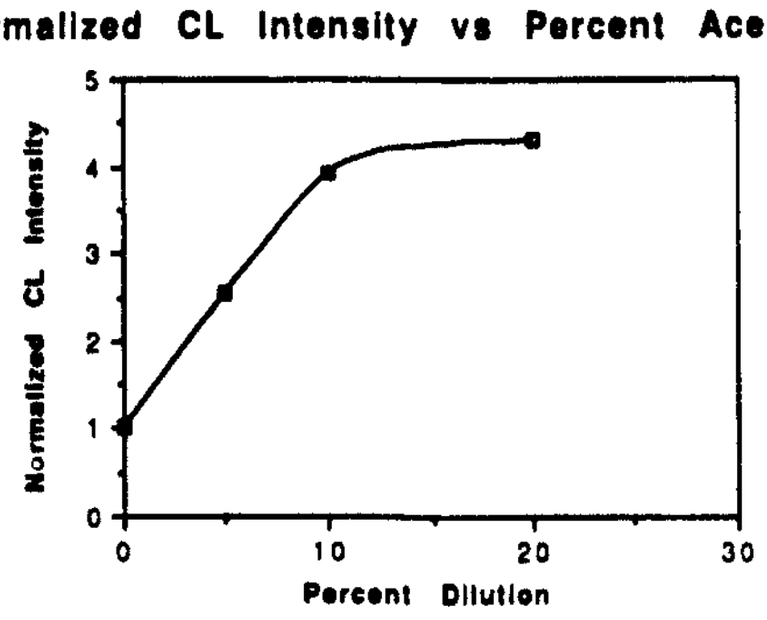
Thus, upon addition of 5% methanol, a 40% decrease in initial intensity and a 40% decrease in the total number of photons emitted is seen. The decrease in peak area may indicate a decrease in the quantum efficiency of the system or a decrease in the rate and/or extent of the CL reaction. In any case, when employing methanol in chemiluminescence-based detection schemes, these factors should be taken into account.

2. Acetonitrile

The initial acetonitrile study was run using 0, 5, 10, and 20% acetonitrile solutions as mentioned previously. The data are summarized in Figures 10, 11, and 12. The so-called "normalized" values are the measurement for the solution containing methanol divided by the

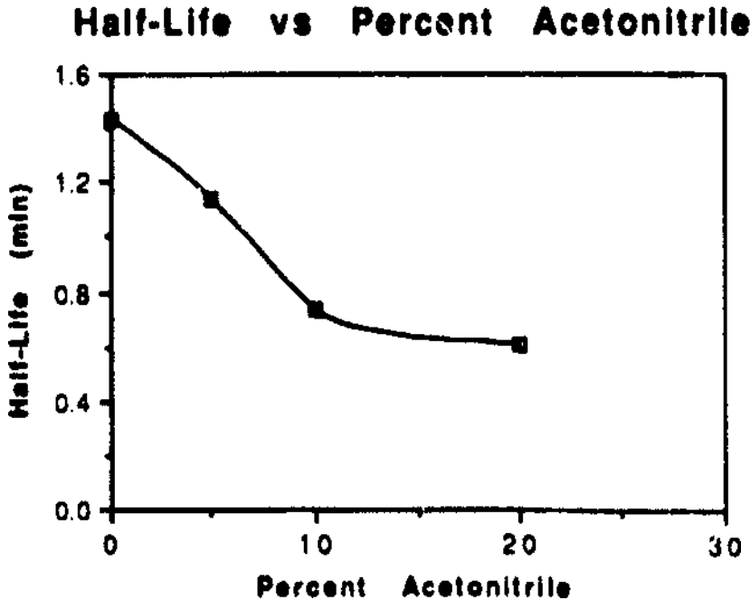
measurement for the same reagent concentrations in a completely aqueous system. This normalization method therefore accounts for any change in the CL data due to dilution of the reagents.

Figure 10:



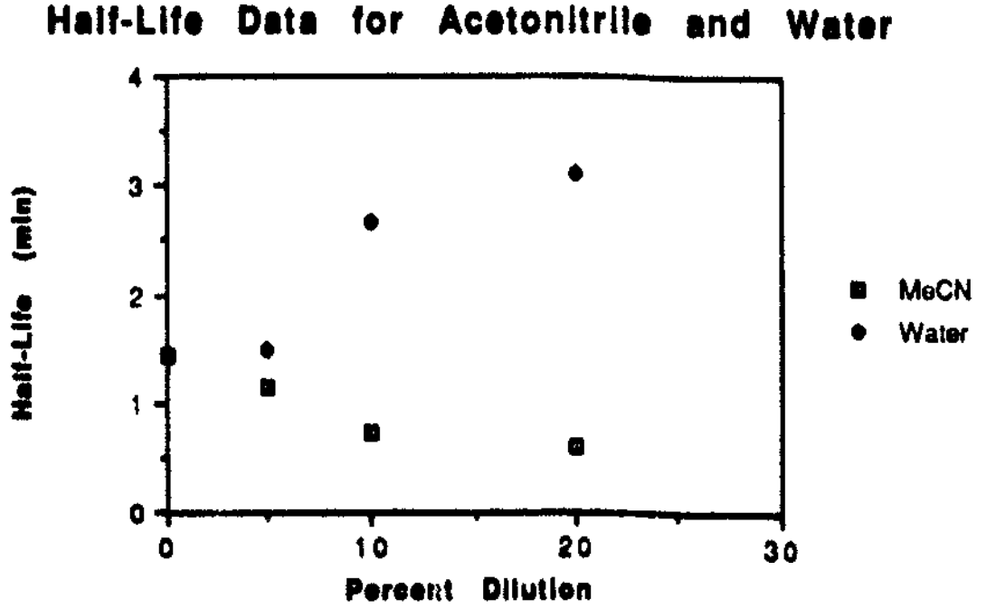
As indicated in Figure 10, the addition of acetonitrile to the reagent solutions increased the initial light intensity greatly. At 5% acetonitrile, the chemiluminescence intensity was 1.5 times greater. With increasing amounts of acetonitrile, the intensity increased at a lesser pace, leveling off at 20%. This concentration of acetonitrile yielded light intensities slightly greater than 4 times that of the 0% solution. However, Figure 11 indicates that with this increase in initial intensity went a marked decrease in the total time of light emission.

Figure 11:



The half-life data decrease sharply from 0% to 10% and then levels off slightly from 10% to 20%. Note that the data indicate half-lives for the acetonitrile system that are significantly shorter than those of the water blanks. A comparison of the raw data is given in Figure 12.

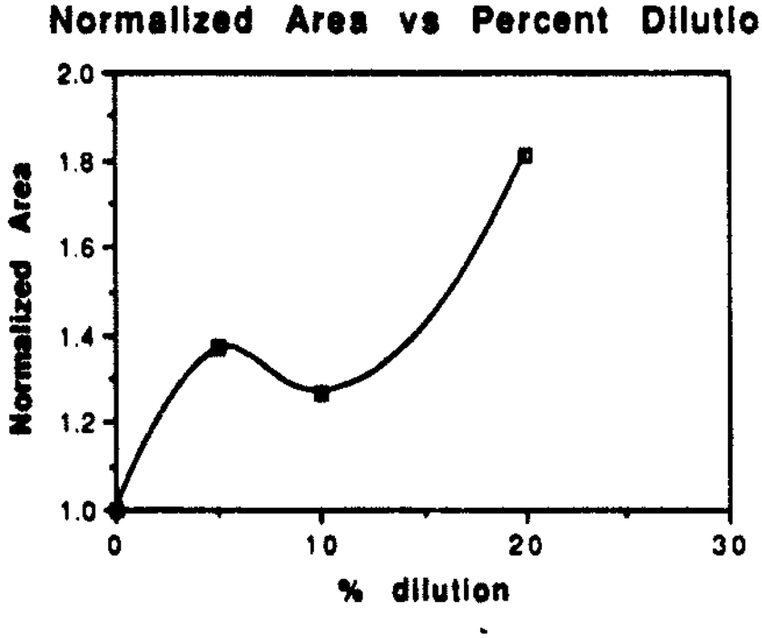
Figure 12:



Clearly, with an increase in dilution by water, the half-life of the chemiluminescence was greatly extended. Conversely, a significant decrease in half-life is observed for the acetonitrile system.

Figure 13 is a plot of peak area versus concentration of acetonitrile.

Figure 13:



This plot exhibits a general increase in the peak areas with increasing percent acetonitrile. Thus, the number of photons emitted with increasing acetonitrile increases when compared to the equivalent amount of water. Apparently, the acetonitrile promotes the emission of photons by the luminol system. This benefit must be weighed with the fact that the emission half-life is greatly decreased. Therefore, the light detector must be placed in close or immediate proximity of the reaction, or perhaps faster flow rates must be employed.

Finally, a comparison of the effects of acetonitrile and methanol on the luminol system may be more readily seen by combining the data for the

two experiments. Figure 14 is a graph of normalized CL intensity versus percent dilution for both methanol and acetonitrile.

Figure 14:

Comparison of Acetonitrile and Water CL Intensities

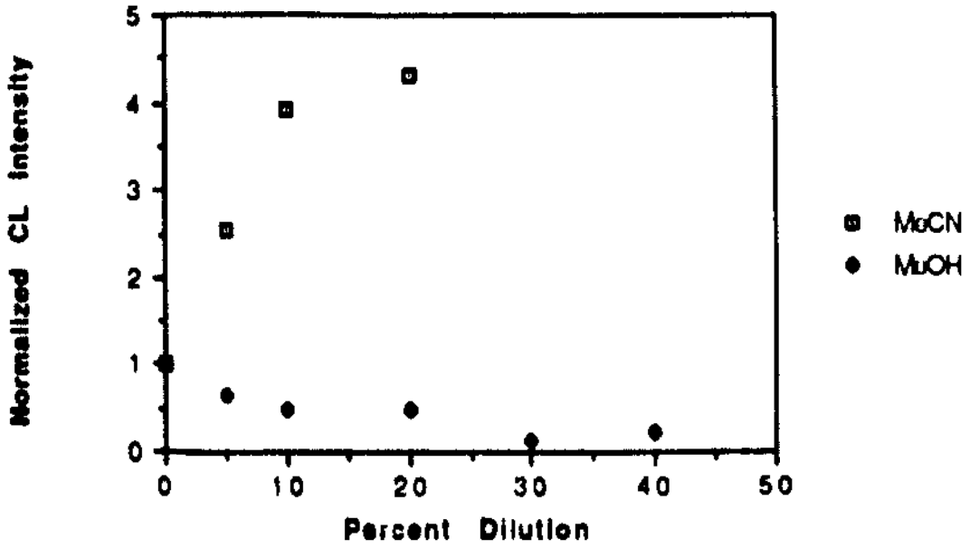
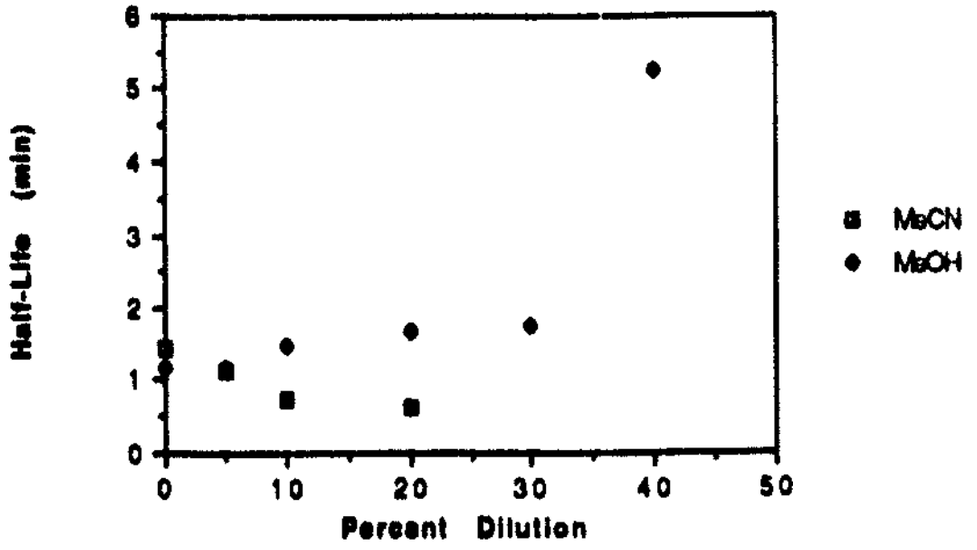


Figure 15 compares half-lives for the acetonitrile and methanol systems.

Figure 15:

Comparison of Half-Lives for Methanol and Acetonitrile



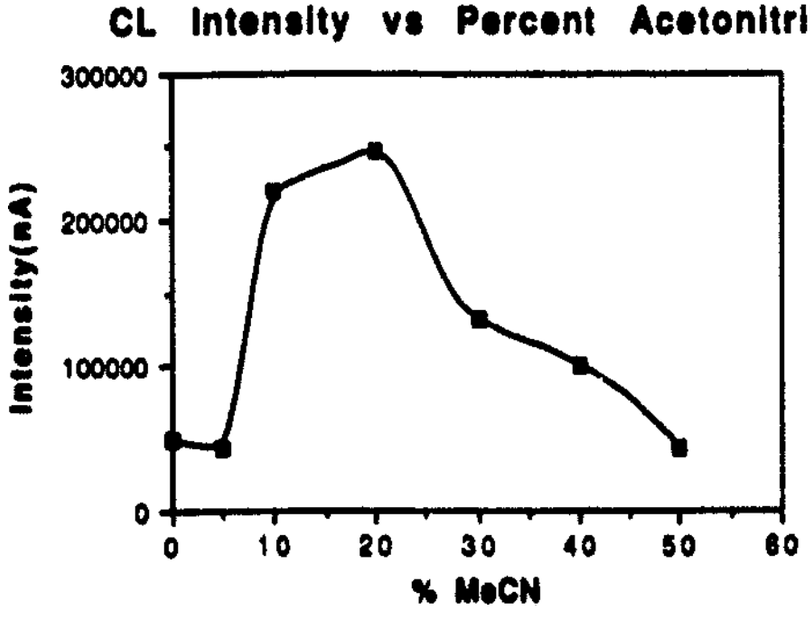
Note that the 0% dilution points are not identical for the acetonitrile and methanol studies, apparently due to experimental error. However, they are in relatively close proximity.

The above data show that for chemiluminescence detection in HPLC, methanol and acetonitrile have generally opposite effects on signal. Because acetonitrile resulted in enhanced light emission intensities, further study of the acetonitrile system was desired, in particular a more accurate and comprehensive examination of the effect of the solvent at higher concentrations.

In this experiment, the luminol concentration was kept constant in all solutions while the acetonitrile concentration was varied. Thus in this experiment no normalization of the data was necessary. Also, an acetonitrile-impervious Saran lining was used to protect the plexiglas from degradation. The results are as follows.

Figure 16 illustrates the effect of increased acetonitrile on the initial chemiluminescence of luminol.

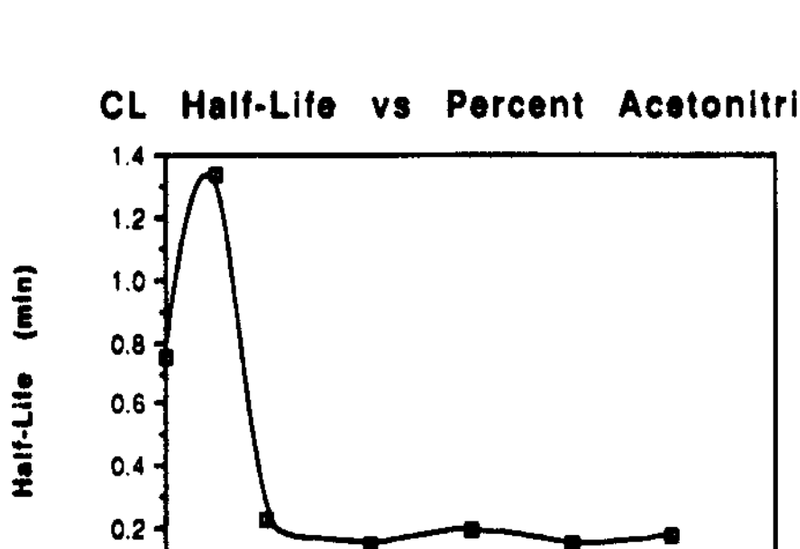
Figure 16:



The increase in intensity upon application of 5% acetonitrile is absent in this experiment. In fact there was a slight decrease in emission. However, the general curve shape and magnitudes seen here for 0-20% acetonitrile agree with that seen in Figure 10 over this same solvent composition range. At 20% there is a slight increase, followed by a steady decline in chemiluminescence from 20% to 50%.

Figure 17 is a plot of half-life versus percent acetonitrile.

Figure 17:



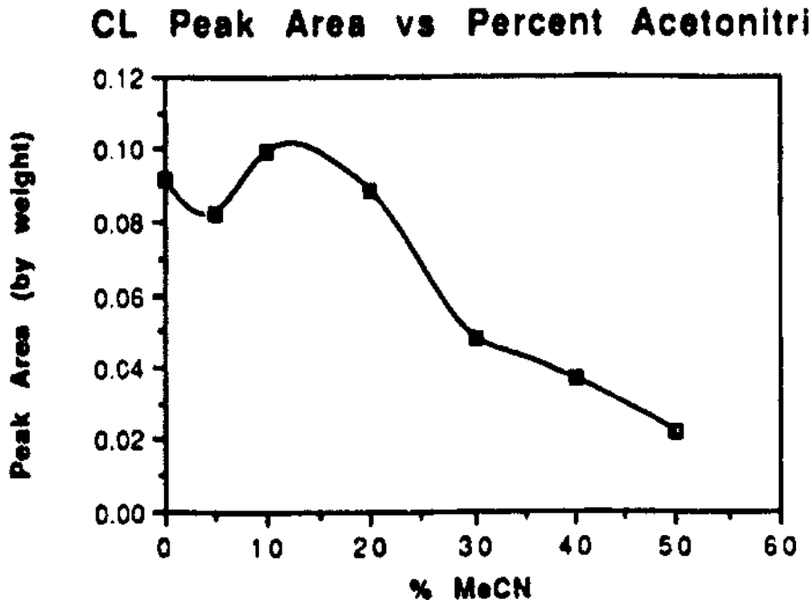
Note the discrepancy for the 0% data points between Figure 17 above and Figures 11 and 12 previously. Although the three data points were taken for a 0% concentration of acetonitrile, the luminol, hemin, and peroxide concentrations differed between Figure 17 and Figures 11 and 12. In order to eliminate the need for normalization of the data, the concentration of luminol in the 0-50% experiment was constant at 50 μM in all solutions,

while in the 0-20% acetonitrile experiment the concentration of the CL reagents varied depending upon the amount of solvent added. Thus the discrepancy.

At 0% and 5% the half-lifes are 0.73 min and 1.3 min respectively. However, from 10% to 50% the half-life is quite constant, remaining at approximately 0.2 min across the entire range of concentrations. There can be little practical importance placed upon the greatly extended half-life at 5% since there is no beneficial increase in chemiluminescence intensity here. It is quite important to note, however, that the half-lives of the 10% and 20% solutions were less than 20 seconds. Therefore, the light detector must be placed in immediate proximity of the reaction cell.

Finally, Figure 18 illustrates the plot of peak area versus percent acetonitrile.

Figure 18:



This graph exhibits an immediate decrease in area at 5% followed by an increase at 10%. From 10% to 50% there is a steady decrease in peak area and therefore a decrease in the total number of emitted photons. This agrees with the previous acetonitrile study and indicates a loss of reaction efficiency. It is impossible to determine the cause of this loss from this data. It would be interesting to examine the effect of increasing acetonitrile concentration on the electrogenerated peroxide/catalyst system to see if this effect is still present. This would at least exclude the possibility that the acetonitrile is in someway enhancing the catalytic effect of the hemin.

As was mentioned earlier, in the 0-20% acetonitrile experiment the CL data was normalized using data from 0-20% dilutions of water to account for the dilution of the luminol/peroxide solutions. Thus in Figure 13, normalized data was computed by dividing the acetonitrile data points by their respective water data points.

Figure 19 is a graph of the unnormalized water and acetonitrile data used to construct Figure 13. It is noted that the areas of the acetonitrile peaks and the areas of the water blank peaks both decrease with an increase in percent dilution, the major difference being that the water peaks decrease faster and further (Figure 19).

Figure 19:

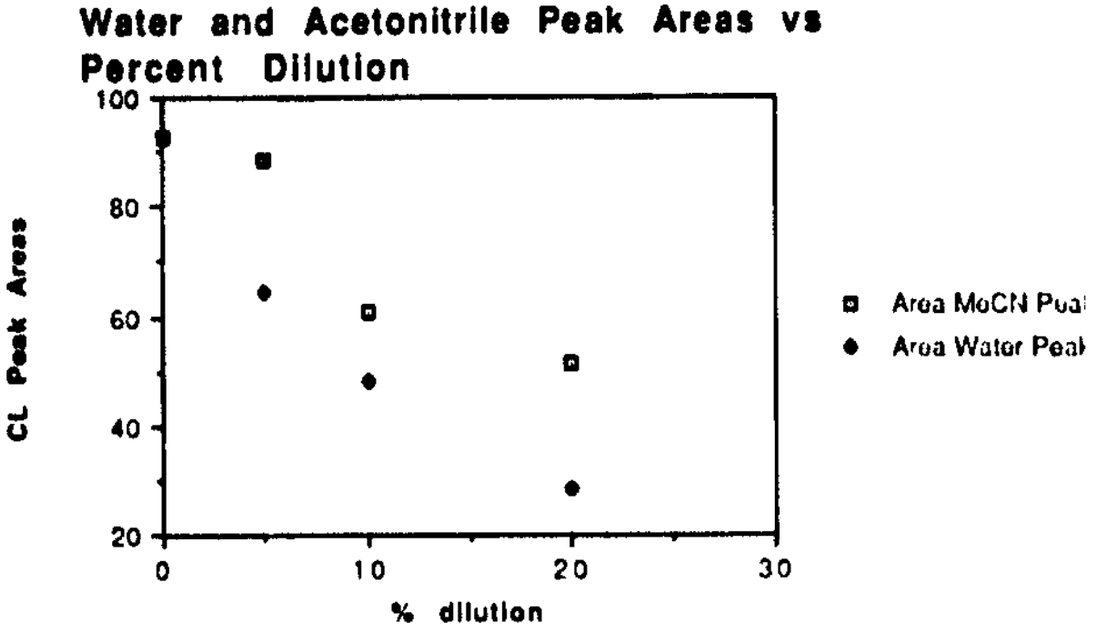
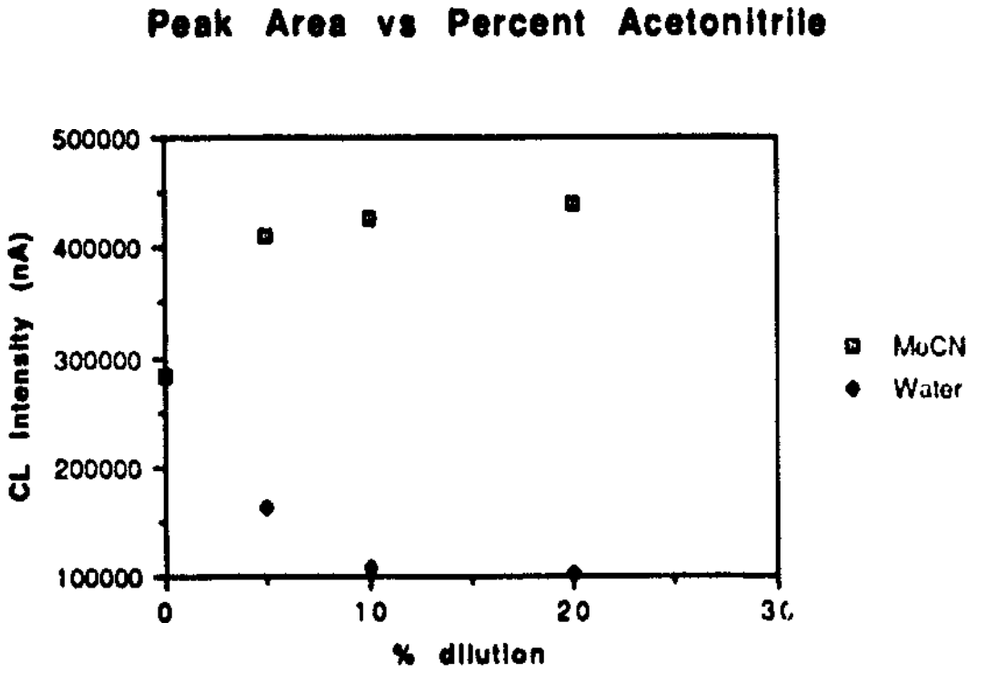


Figure 20 illustrates the acetonitrile and water intensity versus percent dilution data. As mentioned above, the data plotted here was used to produce Figure 10 via the normalization procedure.

Figure 20:



REFERENCES

1. A. Campbell, Chemiluminescence, Ellis Horwood Ltd., Chichester, England, 1988, pp. 18-58.

2. Clinical and Biochemical Luminescence, Vol. 12, ed. L. Kricka and T. Carter, Marcel Dekker Inc., New York, 1982, pp. 2-8.

3. M. Tuttle, B.S. Thesis, University of Illinois, Urbana, IL (1987)

4. A. Campbell, Chemiluminescence, Ellis Horwood Ltd., Chichester, England, 1988, pp. 447-448.