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

MATTHEW CHAD TUTTLE

ENTITLED OPTIMIZATION OF THE FLOW INJECTION PARAMETERS FOR THE LUMINOLO

CHEMILUMINESCENCE DETERMINATION OF HYDROGEN PEROXIDE

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

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OPTIMIZATION OF THE FLOW INJECTION PARAMETERS
FOR THE LUMINOL CHEMILUMINESCENCE DETERMINATION
OF HYDROGEN PEROXIDE

by

MATTHEW CHAD TUTTLE

THESIS

for the

DEGREE OF BACHELOR OF SCIENCE
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I have many people to thank for helping me through the endeavors of this project. Without their help and support none of this would have been possible.

To everyone in the Nieman group, thank you for putting up with me asking all those questions and taking the time to advise me. A special thanks goes to Phil Koerner and Kevin Hool for helping me set up the flow injection systems and getting them working properly.

To my parents Richard and Sharon, thank you for the endless support you have given me throughout the past four years of my life. Your guidance has proved invaluable and in general without you my education would have been impossible.

To Dr. Timothy Nieman, my research advisor, thank you for your sincere guidance, trust, friendship, and humor throughout my past twelve months in the group. The atmosphere has always been comfortable, warm, and secure.
INTRODUCTION

Chemiluminescence (CL) is observed when light is emitted from a chemical reaction. Electronically excited species are formed, and photon emission from these species completes the process. Assays based on chemiluminescence combine the advantages of speed and sensitivity. In most luminescent assays the peak light emission occurs in less than 1 minute and in some cases in less than 1 second. Usually the peak light emission is proportional to analyte concentration. An added advantage of luminescent assays is the wide range of linear response to analyte concentration; linearity is usually observed over several orders of magnitude. Still another advantage is the simplicity of instrumentation and inexpensive reagents which also are quite safe to use in the laboratory. (1-3)

A number of chemical transformations which produce light are known. Among these the oxidation of the aminophthalic cyclic hydrazide known as luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, see Figure 1) has been studied extensively and is the subject of this work. Luminol undergoes chemiluminescent reactions with a range of oxidants, including oxygen, hypochlorite, iodine, permanganate, perborate, and peroxide. For some oxidants, e.g., hydrogen peroxide, a co-oxidant or catalyst (e.g., peroxidase, hemin, and several transition metals) is required for chemiluminescence. Peroxidase and hemin will be the catalysts used in
Figure 1: The luminol light reaction.
this work in conjunction with hydrogen peroxide as the primary oxidant. Luminol research first began with the famous work by Albrect in 1928. (4-5)

The interest of this work is in optimization of experimental conditions for the determination of hydrogen peroxide via luminol chemiluminescence. The effect of luminol concentration, catalyst identity (hemin, horseradish peroxidase, and mixtures of the two) and catalyst concentration upon the peroxide working and detection limits were investigated. Because hydrogen peroxide is known to decompose in aqueous solution, the stability of hydrogen peroxide standard solutions was a concern. Therefore, an additional matter of study concerned the effect of hydrogen peroxide solution storage conditions (type of container and temperature) upon the rate of decomposition of standard solutions of hydrogen peroxide of various concentrations.
EFFECT of LUMINOL CONCENTRATION

EXPERIMENTAL

A. Instrumentation

The flow injection system used for the optimization of luminol is shown in Figure 2. This system was designed primarily by Mark Nussbaum, Cathy Swindlehurst, and Kevin Hooi. A Rainin peristaltic pump with 4 channel capability was used. For this experiment only 2 channels were used. One channel was used for a 0.1 M solution of phosphate buffer (pH 6.5) and the other channel was used for the luminol-catalyst solutions (pH 11.4). A Rheodyne Model 5020 sample injection loop was used to inject an 80 µL sample of hydrogen peroxide into the flow system. Hydrogen peroxide solutions were prepared in H₂O only. Each channel was set at a flow rate of 1.3 mL/min. The two streams combined into a mixing tee and then went on to the flow cell. A 5.5 cm strip of air permeable tubing (Goretex) was placed between the mixing tee and the flow cell to eliminate occasional air bubbles in the flowing stream.

The flow cell used was built in the University of Illinois Machine Shop (See Figure 3). The flow cell consists of a transparent plexiglas face with a white teflon reflective backing. The flow cell used teflon
Figure 2: Flow injection system 1.
Figure 3: Diagram of the Flow cell used in the flow injection system 1.
spacers which gave it a total volume of 50 ul. Since the volume was small it was quite important that the cell remained free of any air bubbles during operation. The plumbing used with the flow cell and throughout the entire system contained fittings (with 1/4-28 threads) and tubing supplied by Altex. The cell was positioned directly in front of an RCA Model 1P28 photomultiplier tube which was biased at -958 V for all measurements. The anodic current generated by the PMT was amplified by a Pacific Model 126 photometer and output to a Curken stripchart recorder.

B. Reagents

The reagents that were used all were readily available in the laboratory. (Room 37 RAL) They are as listed: luminol (Aldrich); monobasic potassium phosphate (Mallinckrodt); horseradish peroxidase, i.e. HRP, (Sigma Chemical Co.); Hemin (Sigma Chemical Co.) as type I; and hydrogen peroxide (Mallinckrodt) as 30 %, i.e.10 M, solution stored in a plastic container under refrigeration.

All reagents used were of reagent grade quality and were used without further purification or preparation. All solutions were prepared using water purified by a Millipore Continental purification system.

C. Flow injection analysis procedure
The procedure used the flow injection system in Figure 1. The photomultiplier tube, recorder, and photometer was allowed to warm up at least five minutes prior to sample injection. Millipore water was used to purge the system before and after each use. Once the system was purged, the luminol and buffer streams were allowed to flow through the system again for at least five minutes or until a stable baseline was established. Once a stable baseline was established hydrogen peroxide injections were made and the signal recorded. All injections were made in triplicate or until reproducible data was obtained. All glassware was washed with dilute nitric acid and triple rinsed with millipore water before solution preparation. The flow cell was periodically checked for the accumulation of air bubbles. This however was seldom a problem with the installation of air permeable tubing between the mixing tee and the flow cell.
EFFECT of LUMINOL CONCENTRATION

RESULTS AND DISCUSSION

A. Initial

In the optimization studies of luminol concentrations, the luminol concentration was varied while maintaining the pH and catalyst concentrations constant. The pH from the mixing tee through the flow cell was maintained at 11.4; because the concentration of this phosphate buffer was 100 times the concentration of buffered luminol used in the flow stream, the pH of the CL reaction was held at 11.4. The HRP and hemin catalysts were incorporated into the luminol-buffer solution. The HRP concentration was prepared at 8 mg/L and the hemin was prepared at 2 μM for this study. Luminol concentrations were prepared from 40 mM to 4 μM.

B. Luminol Optimization

Figure 4 shows the working curves at various hydrogen peroxide concentrations. Hydrogen peroxide concentrations ranged from 1.0 - 100 μM. All working curves remain log linear over this entire range of hydrogen concentrations. Figure 4 clearly shows that in the 0.2 - 40 mM
Figure 4: Comparison of working curves at various luminol concentrations.
range of luminol concentrations absolute CL intensity varies slightly but the slope remains independent of luminol concentration in this region. The slope of the cluster of working curves was measured at 1.38. A shift in the working curves is seen in Figure 4 at concentrations less than 200 μM luminol. This shift was measured to be approximately 1.5 orders of magnitude. The slope observed at 40 μM luminol has also shifted to 0.820. All working curves showed excellent linearity with correlation coefficients of 0.9990 or higher.

Another way to examine the data from this experiment is to plot log CL intensity vs log luminol concentration. Injections of hydrogen peroxide ranging from 1.0 - 400 μM were made and the CL relationship was observed. It was found that absolute CL intensity varied with hydrogen peroxide concentration but the overall shape of the plot log CL intensity vs log luminol concentration remained constant. A log linear relationship between CL intensity and luminol concentration is observed for the 3 lowest concentration solutions. The slope of this logarithmic plot is approximately 1.54. See Figure 5. This linear relationship exists due to the fact that luminol is still the limiting reagent with respect to the catalyst and peroxide concentrations. Non-linearity is observed at the higher concentrations of luminol and leveling off occurs at approximately 0.4 mM luminol. The maximum CL intensity is observed at 4 mM luminol. This maximum was observed at all concentrations of peroxide. Once the maximum is reached CL intensity is seen to decrease
Figure 5: Plot of log CL intensity vs log luminol concentration.
slightly as the luminol concentration is further increased. This leveling off and subsequent decrease occur because luminol is no longer the limiting reagent with respect to the catalyst and peroxide concentrations in this range of luminol concentrations. Hydrogen peroxide is the limiting reagent in this non-linear region. In addition, self absorbance is thought to be a problem at high concentrations of luminol.

C. Detection limit

As seen in Figure 5 the overall shape of the plot of log CL intensity vs log luminol concentration remains relatively constant with only variations in absolute CL intensity. Now it was necessary to observe how the CL intensity varied at low concentrations of hydrogen peroxide for various concentrations of luminol and see if the same pattern is followed as at the high hydrogen peroxide concentrations. Figures 6-11 show working curves of CL intensity vs hydrogen peroxide concentration at 40 mM, 4.0 mM, 2.0 mM, 0.4 mM, 0.2 mM, and 40 µM respectively. The least squares data is indicated on the Figures 6-11 also. The linearity observed is excellent, i.e. correlation coefficient higher than 0.9. Some of the figures show the water blank data point. The reason all do not show the water blank, i.e. Figures 7 and 9, is due to high instability at the low concentrations of hydrogen peroxide. This instability is especially seen in Figure 6. The detection limit range
Plot of CL intensity vs hydrogen peroxide concentration at 40mM luminol conc. (The least squares data interpretation is as indicated: S=slope, R=correlation coefficient, and Y=y intercept.)
Figure 7: Plot of CL intensity vs hydrogen peroxide conc. at 4.0mM luminol conc..
Figure 8: Plot of CL intensity vs hydrogen peroxide conc. at 2.0 mM luminol conc.
CL INTENSITY (nanoamperes)

CONCENTRATION H2O2 (M)

S = 1.93
R = 0.9863
Y = 9.71

Figure 9: Plot of CL intensity vs hydrogen peroxide conc. at 0.4 mM luminol conc.
Figure 10: Plot of CL intensity vs hydrogen peroxide conc. at 0.2 mM luminol conc.
Figure 11: Plot of CL intensity vs hydrogen peroxide concentration at 40μM luminol concentration.
is from 0.1 μM to 10 nM. The lowest detection limit is seen in Figure 8 using 2.0 mM as the luminol concentration. This plot has an excellent correlation coefficient at 0.9990. The best detection limit reported is 10 nM with high confidence.
EFFECT of CATALYST IDENTITY AND CONCENTRATION

EXPERIMENTAL

A. Instrumentation

The flow injection system used for the study of catalyst effects is the same system as used in luminol study.

D. Reagents

The reagents that were used all were the same as used in the luminol study.

C. Procedure

The procedure used the flow injection system in Figure 2. The procedure followed is the same as the procedure in the luminol study.
EFFECT of CATALYST IDENTITY AND CONCENTRATION

RESULTS AND DISCUSSION

The concentration of catalyst is known to greatly affect CL intensity. The Nieman Group uses two catalysts in research, hemin and horseradish peroxidase (HRP). The purpose of this study was to observe how each catalyst affects absolute CL intensity. HRP concentrations were 0.6, 8.0, and 80 mg/L. Hemin concentrations used were 0.2, 2.0, and 20 μM. The hydrogen peroxide concentrations used were 1.0, 10, and 100 μM. The luminol concentration was held constant at 0.4 mM. Figure 12 shows the plot of log CL intensity vs log hemin concentration. For each of the three concentrations of hemin used there were three more solutions prepared of the same concentration, but spiked with 8 mg/L HRP. Figure 12 shows that CL intensity does not increase significantly with increasing hemin concentration. CL intensity actually appears to level off at higher concentrations. Figure 12 also shows that addition of HRP has a very small effect on CL intensity compared to the hemin catalyst. The hydrogen peroxide concentration affects the absolute CL intensity but not the shape of the plot. Figure 13 shows the plot of log CL intensity vs HRP concentration. In the HRP study as in the hemin study six catalyst solutions were prepared. Three solutions contained HRP only and three solutions were HRP plus 2.0 μM hemin. Figure 13 clearly
Figure 12: Plot of log CL intensity vs log hemin concentration at 1, 10, and 100 μM hydrogen peroxide with and without 8.0 mg/L HRP catalyst.
Figure 13: Plot of log CL intensity vs log HRP concentration at 1, 10, and 100 μM hydrogen peroxide with and without 2.0 μM Hemin catalyst.
shows that hemin greatly enhances CL intensity. The data from Figure 13 shows that hemin enhances the CL intensity by one order of magnitude or 1000%. Figure 13 also shows that increasing HRP concentration affects the CL intensity only slightly; however, it is observed that CL intensity actually decreases slightly at the 80 mg/L concentrations of HRP with the hemin spikes. The hydrogen peroxide concentration as expected only affects absolute CL intensity. Figures 14 and 15 show the excellent linearity in CL measurements. These figures also show that the slope is not dependent on catalyst presence or concentration. The measured slope in both Figures 14 and 15 is approximately 1.11.
Figure 14: Comparison of working curves at 0.2, 2.0, and 20 μM Hemin catalyst with and without 8.0 mg/L of HRP catalyst.
Figure 15: Comparison of working curves at 0.8, 8.0, and 80 mg/L HRP catalyst with and without 2.0 μM Hemin catalyst.
DETERMINATION of HYDROGEN PEROXIDE CONCENTRATION

EXPERIMENTAL

A. Titration method

A.1 Instrumentation/Apparatus

The apparatus in this experiment used for the determination of hydrogen peroxide concentration was a simple titration setup. A 10 mL burette with 0.05 mL division marks was used. A 50 mL beaker was used to hold the analyte and indicator since the aliquots were only 5.0 mL. A white piece of plotter paper was placed under the analyte-indicator aliquot to more easily determine the end-point.

A.2 Reagents

The reagents that were used were all readily available in the laboratory. They are as listed: a stock solution of 0.1 M Ce(IV) diluted in with 1.0 M H₂SO₄; ferroin indicator; and hydrogen peroxide (Mallinckrodt) as 30 %, i.e. 10 M, solution stored in a plastic container under refrigeration.
All reagents used were of reagent grade quality and were used without further purification or preparation. All solutions were prepared using water purified by a Millipore Continental Purification System.

A.3 Titration procedure

The procedure used a previous method which was developed by the Nieman group. The procedure was used with 0.1 M and a 1.0 mM solutions of hydrogen peroxide. The 0.1 M Ce\(^{4+}\) solution was used as a titrant for the 0.1 M solution of hydrogen peroxide and a 1.0 mM solution of Ce\(^{4+}\) was prepared as the titrant for the 1.0 mM solution of hydrogen peroxide. (see Figure 16 for the overall titration reaction.) A 5.0 mL aliquot was pipetted into a 50 mL beaker with 4 drops of ferroin indicator added. All titrations were preformed by swirling the aliquots while slow additions of titrant were made. The color change was easily observed from orange to blue.

B. UV/visible method

B.1 Instrumentation

A Hewlett-Packard diode array spectrophotometer, model 8450A was used in this experiment. Quartz cuvettes were used in all experimentation.
\[
\frac{1}{2}H_2O_2 \rightleftharpoons \frac{1}{2} O_2 + H^+ + e^- \quad E = -0.341 \text{V}
\]
\[
Ce^{4+} + e^- \rightleftharpoons Ce^{3+} \quad E = +1.44 \text{V}
\]

\[
\frac{1}{2}H_2O_2 + Ce^{4+} \rightleftharpoons \frac{1}{2} O_2 + Ce^{3+} + H^+ \quad E = +1.099 \text{V}
\]

Figure 16: The redox reaction for the titration of hydrogen peroxide with cerium.
B.2 Reagents

Two reagents were used, a 30% hydrogen peroxide solution (Mallinckrodt), and millipore high purity water.

B.3 UV/visible procedure

The hydrogen peroxide solutions were scanned from 200 nm to 800 nm to find the maximum absorbance wavelength. 0.1 M and 1.0 mM solutions of hydrogen peroxide solutions were used. Once the maximum absorbance was found the range was narrowed between 240-260 nm. Millipore water was used as the reference. All cuvettes were triple rinsed before each scan.

C. Amperometric method

C.1 Instrumentation

The flow injection system used for the detection of hydrogen peroxide is shown in Figure 17. A Rainin peristaltic pump with 4 channel capability was used. Only 1 channel was used for this experiment, to carry the solution of potassium nitrate buffer of 0.1 M at 1.5 mL/min. A Rheodyne Model 5020 sample injection valve with an 80 µL sample loop
Figure 17: Flow injection system 2.
was used to inject the solutions of hydrogen peroxide. A BAS electrochemical thin layer flow cell (See Figure 18) was used along with a BAS down stream reference cell. The electrochemical cell used was a single glassy-carbon electrode (Model LC-19) version as the working electrode and the stainless steel backing containing the inlet and outlet orifices as the counter electrode (Model TL-5). The flow cell used a teflon spacer which gave the flow cell a total volume of 5 µL. The reference compartment (Model RC-2) was supplied from BAS and was placed 8.0 cm down stream from the thin layer cell. A Ag/AgCl reference electrode (Model RE-1) also supplied from BAS was used. The plumbing used in the system contained fittings (with 1/4-28 threads) and tubing supplied by Altax. The current generated by the reduction of hydrogen peroxide was amplified by an IBM model EC/230 potentiometer and output to a Wescon stripchart recorder.

C.2 Reagents

The reagents used all were readily available in the laboratory. They are as listed: potassium nitrate (Mallinckrodt); and hydrogen peroxide (Mallinckrodt) as 30 %, i.e. 10 M, solution stored in a plastic container under refrigeration.

All reagents used were of reagent grade quality and were used without further purification or preparation. All solutions were prepared using
Figure 18: Amperometric thin-layer flow cell used in flow injection system 2.
water purified by a Millipore Continental purification system.

C.3 Procedure

Since hydrogen peroxide oxidises at positive potentials it can be detected using amperometric methods of analysis. I chose flow injection using thin layer electrochemical cells due to their fast analysis times. The procedure used the flow injection system in Figure 17. The potentiometer was allowed to warm up at least five minutes. Millipore water was used to purge the system before and after each used. Once purged, the 0.1 M solution of potassium nitrate was allowed to flow through the system until a stable baseline was reached. Once a stable baseline was established the injections of hydrogen peroxide were made and the signal recorded. All injections were made in triplicate or until a reproducible signal was obtained. All containers used were triple acid rinsed and triple washed with millipore water. The flow cell was frequently checked for air bubbles. This was more of a problem at times since air permeable tubing was not used in this experiment. The syringes were triple rinsed between each injection for each solution of hydrogen peroxide.
DETERMINATION of HYDROGEN PEROXIDE CONCENTRATION

RESULTS AND DISCUSSION

Hydrogen peroxide, being the most widely used oxidant in the luminol oxidation reaction is known to decompose in aqueous solutions. The decomposition reaction products are water and oxygen. (see Figure 19)

In this experiment the stability of hydrogen peroxide was to be investigated in solutions of only water and hydrogen peroxide. The rate at which hydrogen peroxide decomposes with respect to time was of primary interest to the Nieman group members.

A. The titration method

Two concentrations of hydrogen peroxide were initially studied using the titration method. A 0.1 M and a 1.0 mM concentration were prepared. Four parameters were implemented into the experiment. Hydrogen peroxide solutions were stored in plastic and glass containers approximate 100 mL. Also the hydrogen peroxide solutions were stored at room temperature (i.e. 25 degrees celcius) and refrigerated (i.e. approx. 0 degrees celcius). Therefore 4, solutions of each hydrogen peroxide concentration were prepared. Figures 20 and 21 shows the plot of mL titrant added vs time. Figure 20 shows the 0.1 M hydrogen peroxide
\[ \text{H}_2\text{O}_2 \rightleftharpoons \text{H}_2\text{O} + \frac{1}{2} \text{O}_2 \]

**Figure 19:** The decomposition reaction of hydrogen peroxide in aqueous solution.
Figure 10: Plot of milliliters titrant added vs time for the 0.1M solutions of hydrogen peroxide.
Figure 21: Plot of milliliters titrant added vs time for the 1.0mM solutions of hydrogen peroxide.
solution behavior. The data show considerable scatter but demonstrate that a 0.1 M solution, i.e., a high concentration for use in the Nieman Group, decomposes very little over a 10 day period. Figure 21 shows the 1.0 mM solution behavior. This solution, also unstable, shows a general trend of decomposition over the 3 day experimentation period. In addition to downward trend, one can also make the generalization that a hydrogen peroxide solution stored in glass decomposes at a faster rate than a hydrogen peroxide solution stored in plastic. It is also observable that unrefrigerated solutions of hydrogen peroxide decompose at a faster rate than refrigerated solutions. This titration procedure lacked the sensitivity and precision necessary for study of the decomposition of more dilute solutions of hydrogen peroxide. It was for this reason that I went on to find a more accurate method.

B. The spectrophotometric method

This method for hydrogen peroxide determination is capable of determining its presence only. The reproducibility was observed to be extremely poor in the first runs on the instrument. It is for this reason that the experiments were not continued further.

C. The amperometric method
Figures 22-24 show the plots of current vs time for 3.0, 30, and 300 μM solutions of hydrogen peroxide varying temperature and the type of storage container. The 3.0 and 30 μM solutions show identical behavior patterns. It is clear from Figures 22 and 23 that the type of container is relatively insignificant in the rate at which hydrogen peroxide decomposes. One can make the observation that plastic is slightly better than than glass but not to a significant degree. It is very clear that temperature has a substantial effect upon the decomposition of hydrogen peroxide. The refrigerated solutions from Figures 22 and 23 show that an unrefrigerated solution of hydrogen peroxide decomposes at a rate almost twice that of a refrigerated solution at the same concentration. Figure 24 shows the plot of current vs time for a 300 μM solution of hydrogen peroxide. This plot appears much like that of Figure 21 where 1000 μM decomposition of hydrogen peroxide was studied using the titration method. The rate of decomposition in Figure 24 appears to be at similar rates for all the solutions. The observation of the data in Figure 24 however, shows that temperature affects the rate of decomposition. Table 1 shows the percent of hydrogen peroxide decomposed over a 48 hour period. This table better shows the differences between the container parameters. It is seen here that much less hydrogen peroxide is decomposed in a plastic container as opposed to a glass container.
Figure 22: Plot of current vs time at 3.0 μM hydrogen peroxide concentration.
Figure 23: Plot of current vs time at 30 µM hydrogen peroxide concentration.
Figure 24: Plot of current vs time at 300 μM hydrogen peroxide concentration.
<table>
<thead>
<tr>
<th>concentration $\text{H}_2\text{O}_2$ (µM)</th>
<th>3.0</th>
<th>30</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic, refrig.</td>
<td>31.4</td>
<td>28.1</td>
<td>16.2</td>
</tr>
<tr>
<td>Glass, refrig.</td>
<td>35.5</td>
<td>35.2</td>
<td>37.7</td>
</tr>
<tr>
<td>Plastic, unrefrig.</td>
<td>76.5</td>
<td>78.5</td>
<td>39.1</td>
</tr>
<tr>
<td>Glass, unrefrig.</td>
<td>83.4</td>
<td>77.7</td>
<td>39.8</td>
</tr>
</tbody>
</table>

Table 1: Percent of hydrogen peroxide degraded after 48 hours.
References


