A practical and versatile instrument has been developed both to optimize the chemistry of bio- and chemiluminescence reactions and to measure ultra-trace quantities of associated analytes. The instrument consists of a pneumatic flow injection system linked to a detection cell and a signal processing and readout system. The disk-like cell is defined by a pair of transparent polycarbonate plates and an "O-ring" seal. The lower plate is fitted with a reflective surface. Relative to conventional cells, this design affords a high light collection efficiency due to the large volume element viewed by an end-on photomultiplier detector. Rapid mixing of reagents within the cell is brought about by injection through concentric ports of a commercial burner assembly at a point immediately below the detector. The signal processing and readout system is interfaced to an IBM compatible personal computer and appropriate software was written to automate the instrument and to acquire, store and manipulate luminescence data.

With this instrumentation, the chemistry of marine bacteria bioluminescence was optimized for the determination of cis-11-hexadecenal and, ostensibly, for both the quantification of aldehyde insect pheromones and
potential use in the control of insect pests. With the optimized conditions, cis-11-hexadecenal was determined to 7 fmol. This value is more than an order of magnitude lower than detection limits for aldehyde pheromones reported in the literature. In this research, the less ideal substrates undecanal and heptanal were determined to 570 fmol and 65 pmol, respectively.

Marine bacteria bioluminescence was used to quantify several epoxide analytes derivatized to aldehydes. 1,2-epoxyhexadecane and 1,2-epoxytetradecane were determined to 55 and 51 fmol, respectively. 1,2-epoxyoctane and cis-7,8-epoxy-2-methylloctadecane were determined to 100 and 3 pmol, respectively. The latter compound is the sex pheromone of the gypsy moth \(\textit{Lymantria dispar}\), a well-known and serious agricultural pest. Epoxides have not been quantified previously with either chemi- or bioluminescence.

The instrument was modified for use with corrosive solutions and for possible interfacing with a high performance liquid chromatograph. Lophine chemiluminescence was optimized for the analysis of Cr(VI) samples. With the optimized conditions, aqueous solutions of Cr(VI) were determined to 50 \(\mu\)g/L. A plausible explanation is offered for the dependence of lophine chemiluminescence on the concentration of the chromium species.
Development of Automated Flow Injection Apparatus
and a Novel Flow Cell for Chemi- and Bioluminescence Determinations

by

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DEVELOPMENT OF AUTOMATED FLOW INJECTION APPARATUS
AND A NOVEL FLOW CELL FOR CHEMI- AND
BIOLUMINESCENCE DETERMINATIONS

CHAPTER 1

INTRODUCTION

Chemiluminescence (CL) occurs when a chemical reaction yields a
species in an excited electronic state which emits radiation (UV, visible or IR)
as it decays to its ground state. The excited species may be either a reaction
product or an intermediate. Two general categories of CL reactions are
recognized. In "direct CL", the excited species is the emitter (Equations 1.1
and 1.2) (1, 2),

\[ A + B \rightarrow C^* \quad (1.1) \]

\[ C^* \rightarrow C + hv \quad (1.2) \]

whereas in "sensitized CL" the excited species transfers energy to another
molecule called an "acceptor". The acceptor then undergoes a similar
process of decay with emission (Equations 1.3 and 1.4) (2, 3):

\[ C^* + F \rightarrow F^* + C \quad (1.3) \]
Three conditions must be satisfied for CL to occur. First, the reaction must be sufficiently exothermic to supply the energy for emission. For a species emitting in the visible spectrum, this corresponds to energies between 170 and 300 kJ/mol. Second, there must be an efficient chemical pathway leading to the excited, luminescing species compared to pathways leading to other, non-excited products. Finally, photoemission must be a favorable deactivation process relative to decay by quenching, internal conversion or other radiationless transitions (3, 4, 5).

In direct CL, the chemiluminescence quantum efficiency, $\varphi_{\text{cl}}$, is related to both the efficiency of the production of the excited species, $\varphi_{\text{ex}}$, and to the luminescing efficiency, $\varphi_{\text{l}}$, (Equations 1.5 and 1.6) (1, 3) where $R$ is a reactant species. The observed chemiluminescence intensity, $I_{\text{cl}}$, depends on the chemiluminescence quantum efficiency, the number of molecules

$$
F' \rightarrow F + hv \tag{1.4}
$$

$$
\varphi_{\text{cl}} = \varphi_{\text{ex}} \varphi_{\text{l}} \tag{1.5}
$$

$$
\text{photons emitted} = \frac{\text{(no. excited molecules)}}{\text{(no. molecules R reacted)}} = \frac{\text{(photons emitted)}}{\text{(no. excited molecules R reacted)}} \tag{1.6}
$$
reacting per unit time, and the volume element viewed (Equations 1.7 and 1.8) (1, 6).

\[ I_d(t) = \phi_d(t) \left[ \frac{dC^*}{dt} \right] V \] (1.7)

\[
\frac{\text{photons}}{s} = \left[ \frac{\text{photons}}{\text{molecules reacted}} \right] \left[ \frac{\text{molecules}}{L \ s} \right] L \] (1.8)

In the course of a CL reaction the intensity of the emission is generally observed to increase initially, pass through a maximum, then decrease with time as the reactants are consumed (Figure 1.1). The analytical utility of CL is realized by arranging the reaction conditions so that the analyte concentration is related to either the emission intensity or to the photon yield. Possible experimental conditions for a determination include those in which the analyte is both a necessary reactant and the limiting reagent, and conditions in which the analyte concentration affects the rate of reaction. Other analyses are based on conditions that impact the luminescence efficiency. An example of the latter case occurs in sensitized CL when suitable acceptor molecules are added to the reaction mixture.

Analyte determinations are made with respect to CL intensity versus time curves. Generally, these are obtained by either measuring the CL intensity at some time after mixing the CL reagents and sample, or by integrating intensity over time. In the first method, measuring the maximum
Figure 1.1. A Hypothetical CL Intensity versus Time Curve. The shape of the curve is influenced by the rate of mixing, the kinetics of the reaction, and any change in $\varphi_{\text{cl}}$ as the reaction proceeds. From: Seitz, W. R., "Chemiluminescence and Bioluminescence Analysis: Fundamentals and Biomedical Applications" in "CRC Analytical Reviews in Analytical Chemistry, Vol. 13", CRC Press, Boca Raton, FL, 1981, 6.
intensity is convenient and provides, often, the most favorable detectability. However, this method may not be appropriate for very fast reactions due to imprecision caused by variations in the mixing process. For fast reactions, integrating along the descending portion of the luminescence curve is preferred since this region is less dependent on the mixing rate (1).

Fortunately, the instrumentation required for CL measurements is both modest and relatively inexpensive. The necessary components include a sample cell and a light detector housed within a light-tight compartment. A device to mix reagents in the cell may be included. The light detector is linked to a signal processing and readout system. When the instrument is designed specifically for CL measurements, it is referred to as a "photometer". However, with the proper instrumental conditions, CL analyses may also be carried out with liquid scintillation counters, spectrophotometers or fluorimeters.

Usually, photometers are designed for use with either batch or continuous flow sampling methods. In the batch (discrete sampling) method, the sample and other CL reagents are added to the detection cell. Generally, the reaction is initiated by the addition of a final reagent necessary to the reaction. Often, this is carried out by rapid injection which may also provide sufficient agitation of the solution for efficient mixing. In the continuous flow (flow injection) method, separate streams of CL reagents and sample are mixed. Provision is made so that the reaction takes place within the view of the detector. Depending on the design, mixing of the streams may take place by either passive (hydrodynamic) or active processes. Passive mixing depends primarily on the process of diffusion, and to a lesser extent on frictional drag as the solution passes over a surface. To promote passive mixing, CL detection cells are often coiled. Since this type of mixing is relatively slow, it is used often in studies of fast CL reactions. Active mixing is usually carried out in the detection cell
by means of a vibrator, magnetic mixer, or by rapid injection. Centrifugal force has also been used for this purpose (7). A brief review of some of the CL flow cells and mixing methods has been presented by Seitz (1). Some of these are illustrated in Figure 1.2.

The design of the flow cell and the efficiency of the mixing of the CL reagents and sample are important factors related to the light collection efficiency for a CL analysis. If the reagents are not well mixed at the time of detection, or if well mixed reagents exit the cell before a large portion of the signal is collected, then sensitivity is reduced. The geometry of the cell and the placement of both the detector and any light focusing elements are important, as these factors influence the portion of light collected by the detector.

The thesis research presented in subsequent chapters covers broad areas integrated from several disciplines including biochemistry, entomology, insect pest management, automated flow injection instrumentation and analytical chemistry. Chapter 2 is historical, providing much of the background information necessary to a full appreciation of the experimental work presented later. The first subsection of this chapter discusses the chemistry of marine bacteria bioluminescence and analytical applications of this reaction. The second subsection reviews insect pheromone determinations and applications of pheromone sex attractants to the control of insect pests. Particular emphasis is placed on the sex pheromone of the gypsy moth, and a brief review of the damage caused by this exotic North American species is presented.

Instrumentation developed specifically for the determination of aldehyde insect pheromones with marine bacteria bioluminescence is discussed in Chapter 3. The automated flow injection photometer described includes a novel type of flow cell. This cell permits the collection of a large fraction of the light produced by either chemiluminescence or biolumi-
escence reactions.

Experimental work is presented in Chapters 4, 5 and 6. Much of Chapter 4 is focused on the optimization of the chemistry involved in bacteria bioluminescence for the determination of aldehydes. Aldehyde insect pheromone determinations achieved with the flow injection instrument are compared with results obtained with a discrete sampling apparatus and with other results presented in the literature. A scheme to determine ultra-trace quantities of epoxides with bacteria bioluminescence is presented in Chapter 5. This class of compounds has not been quantified previously with either chemi- or bioluminescence. Of particular interest, this method permits the determination of minute quantities of the gypsy moth sex pheromone.

Modification of the flow injection system to accomodate the chemistries of a broad variety of chemi- and bioluminescence reactions is discussed in Chapter 6. The modified instrument is used to determine Cr(VI) with lophine chemiluminescence. A mechanism is proposed to account for the reaction. The results of optimization work and determinations of Cr(VI) are compared with those obtained previously by researchers using discrete sampling instrumentation.
REFERENCES


Background

Bioluminescence has been defined as "the ecologically adaptive and energetically efficient production of visible light by a living organism or by components extracted from a living system" (1). About a dozen distinct bioluminescence chemistries have been identified. These differ from inorganic and organic chemiluminescence reactions in that the quantum yields are generally two to three orders of magnitude higher, in some cases approaching unity. Other differences lie in the requirement of oxygen, either molecular or as a metabolite, and the involvement of an enzyme in the generation of the excited chromophore.

Although the phenomenon of bioluminescence has been known since antiquity (2, 3), the significant advancements in knowledge of the chemistry of bioluminescence reactions have occurred within the last century. In an early experiment (1887) Dubois demonstrated the emission of light with the mixture of hot and cold water extracts from Pholas dactylus, a type of luminescent clam. The hot water extract destroyed a factor in the tissue, whereas another factor necessary to light emission was left intact. The heat-labile factor, an enzyme, was termed a "luciferase"; the factor stable to heat, and presumed to be oxidized with the production of light, was called a "luciferin".
Bacteria Bioluminescence

Currently, five genera including nine species of luminescent bacteria are recognized. Of these seven are marine, one terrestrial and one fresh water aquatic. Three species in particular, *Vibrio harveyi*, *Photobacterium phosphoreum* and *Vibrio fischeri* have been considered as chemiluminescence sources for biomedical, environmental and research applications (4, 5).

Investigations of bacteria bioluminescence date from the early part of this century. Although Harvey was unable to isolate either luciferin or luciferase from luminescent bacteria, his experiments of 1915 did show that oxygen is a necessary substrate (6). Strehler and Cormier added reduced flavin mononucleotide (FMNH₂) to the list of substrates in 1954 (7). Because such a small amount of long-chain aldehyde was required to activate the bacterial system, these researchers suggested that this factor played a catalytic rather than a stoichiometric role. Subsequent work has shown that the aldehyde is, in fact, a substrate and that it is oxidized to the corresponding fatty acid (8-11). Largely through the work of Hastings and his coworkers, the stoichiometry and much of the mechanism involved in the *in vitro* reaction have been established. Direct chemiluminescence is presumed to be operational in this system, although Gast and Lee have proposed that *in vivo* an indirect, or sensitized, mechanism is involved with species of *Photobacterium* (12, 13).

Studies of reaction kinetics, luciferase structures, properties of intermediates, analytical applications and a host of other subjects have been the focus of several research groups in the last thirty years. Fortunately, periodic reviews (4, 14, 15) have helped to organize the current extensive body of literature that relates to bacteria bioluminescence. The more immediate objective of this writing is to highlight the well documented
aspects of the in vitro reaction to facilitate a clear description of the thesis research.

**In Vitro Reaction Chemistry**

In all species of luminescent bacteria an enzyme catalyzes the oxidation of both flavin mononucleotide and a long-chain aliphatic aldehyde (16). The stoichiometry (Reaction 2.1) first proposed by Cormier and Totter (17) is supported by considerable experimental evidence (16, 18).

\[
\text{FMNH}_2 + \text{O}_2 + \text{RCHO} \xrightarrow{\text{luciferase}} \text{RCOOH} + \text{FMN} + \text{H}_2\text{O} + \text{hv} \quad (2.1)
\]

A reaction mechanism that proceeds in several steps to the emission of light has been described by Hastings et al. (14, 19-22). With reference to Fig. 2.1, this may be outlined as follows.

Enzyme (E) reacts rapidly with FMNH\(_2\), yielding a reduced flavin-enzyme complex. This intermediate then combines with oxygen to form a relatively stable luciferase-bound reduced flavin hydroperoxide. Reaction of flavin hydroperoxide with aldehyde, producing a peroxyhemiacetal, is postulated (23). A hydride shift (Baeyer-Villiger reaction) yields a fatty acid and an excited enzyme-bound flavin 4a-hydroxide. Subsequent to decay with emission, the ground-state luciferase-flavin hydroxide dissociates to water, flavin mononucleotide (FMN, riboflavin 5'-phosphate) and free enzyme (20).

*In vitro*, at 25\(^\circ\) C and with tetradecanal substrate, the emission follows
Figure 2.1. Reaction Sequence for In Vitro Bacteria Bioluminescence. Structures are for the Flavin Moieties.
first order kinetics with a half-life of about 0.5 s (19). Maximum intensity occurs near 490 nm with an overall quantum yield of about 0.10 (14). To populate the excited state of the emitter, luminescence at this wavelength requires approximately 240 kJ mol\(^{-1}\), a condition more than satisfied by the oxidation of FMNH\(_2\) (170 kJ mol\(^{-1}\)) and the peroxidation of aldehyde (ca. 320 kJ mol\(^{-1}\)) (11, 17, 24). Although the formation of the early intermediates is well documented (19, 21, 24), less experimental evidence is available to support the later steps of the reaction. Other possible mechanisms and proposed structures for the emitting chromophore occur in the literature (8, 13, 25, 26).

In addition to the light emitting path, two side reactions are known to compete either for substrate or for intermediate. In one of these (Reaction

\[
\text{FMNH}_2 + O_2 \rightarrow \text{FMN} + H_2O_2
\]  

2.2) unbound reduced flavin is oxidized rapidly by oxygen. Because the luciferase binds reduced flavin in a one-to-one ratio (24), and because excess reduced flavin is removed by the side reaction, enzyme activity is restricted. Turnover does not occur. Consequently, as noted by Hastings and Gibson (19), the luciferase may be treated as a reactive participant, discounting the catalytic function.

In the absence of aldehyde, enzyme-bound reduced flavin 4a-hydroperoxide decays via a low quantum yield (\(\varphi_{\text{cl}} < 0.001\)), or "dark", path:
For a given set of reaction conditions the decay rate for the hydroperoxide is constant and it determines the light intensity via the high quantum yield route (Reaction 2.1) when aldehyde is added. This intermediate is stabilized by high concentrations of luciferase or bovine serum albumin which act presumably via a protective mechanism to slow the rate of oxidation (19).

Although suggestions have been made to account for the weak luminescence by the dark path, the origin of this emission has not been established. The emission intensity is reduced significantly by hydroxylamine, an aldehyde scavenger, with no apparent effect on the dark path decay constant (27). This result supports the interpretation that the low quantum yield may arise from aldehyde impurity in the enzyme. However, this hypothesis is countered by the distinctly different exponential decay that occurs when aldehyde reacts with the stabilized intermediate (19). In batch type analytical determinations of substrate, the blank signal corresponds to the measurement of the endogenous luminescence from the low quantum yield pathway.

**Factors Affecting Analytical Determinations**

Physical and chemical parameters that impact either the maximum initial emission intensity ($I_0$) or the quantum yield are pertinent to substrate determinations. These include temperature, pH and phosphate concentration. Equally useful is a discussion of the enzyme(s), individual substrates, and reaction inhibitors.
**Temperature**  
In the range of 0 - 40°C and with enzyme from *V. fischeri*, the maximum light intensity increases with increasing temperature to a peak at about 28°C (28). Decreased luminescence at more elevated temperatures may be related to heat denaturation of the enzyme (19). Because the emission decay rate also increases with increasing temperature, the relative quantum yield does not change appreciably at stable enzyme temperatures (19).

**pH**  
With luciferase from *V. fischeri*, maximum luminescence occurs near pH 7.0. The experimental curve shows a broad profile within half a pH unit of the neutral point (28). This condition has been associated with the favorable formation of the enzyme-flavin hydroperoxide intermediate (29). Enzymes from different luminescent bacteria display different optimum pH profiles; for example, the MAV strain of *V. fischeri* has a maximum light yield in the pH range 5.6 - 6.8 (29). With *V. harveyi*, the optimum yield is nearly constant between pH 6.5 and 8.5 (15).

**Phosphate**  
Luciferase stability and activity is enhanced by phosphate, sulfate and other anions. This has been associated with both a decreased rate of proteolytic inactivation (16) and a flavin-to-enzyme binding dependence on ionic strength (30). Although there is evidence that phosphate can bind to the same luciferase site as does the phosphate ester of FMNH2, competitive inhibition of the substrate does not occur at relatively high phosphate concentration (0.5 M) (30). In other research (31) phosphate has been associated with improved conformation of the aldehyde binding site on the reduced luciferase-flavin hydroperoxide.

**Luciferase**  
Although the light emitting mechanism is believed to be similar in all species of luminescent bacteria (13), the individual luciferases differ in
composition, substrate specificity and binding, and stability in various chemical and physical environments (29, 32, 33). Structurally, the luciferases are (αβ) heterodimers with molecular weights of about 80,000. Experiments with both chemically modified and hybrid enzymes suggest that the kinetically active center resides on the larger α subunit (32, 34). Both subunits appear to be involved in the binding of FMNH₂ (15, 32).

Luciferase stock solutions are protected against denaturation from heat, urea and other chemical agents by both storage at low temperature (-14°C) and the presence of dithiothreitol (DTT), a mild reducing reagent. Phosphate or ammonium sulfate buffers at pH 7.0 stabilize the enzyme from possible rapid deactivation by proteases from bacterial contaminants (14, 33). Ethylene-diaminetetra-acetate (EDTA) at 1 mM may be used to slow the oxidation of the thiol reagent and to sequester metal ion inhibitors such as Ca²⁺ and Zn²⁺ (35, 36).

**Aldehyde** The initial light intensity and the rate of emission decay depend on the chain length of the aldehyde substrate (37, 38). With saturated aqueous solutions the initial emission intensity increases sharply for aldehydes with 12 to 16 carbons. Maximum initial intensity occurs in the middle of this range (37); generally, little activity occurs with chain lengths less than six or greater than eighteen carbons. Because the enzyme intermediate is consumed rapidly with aldehydes that produce high I₀ values, the reaction decay constant is also higher with these substrates. Quantum yields follow the trend in I₀ and indicate the relative binding strength between the aldehyde and enzyme complex. With luciferase from *P. phosphoreum*, Michaelis (K_m) constants decrease as the number of aldehyde carbons increases from ten to thirteen (18). A similar pattern occurs for K_m values with other bacterial luciferases. Typical values are in
the µM range (15).  

As a function of chain length, aldehyde solubilities vary considerably in water solution. At 25°C saturated concentrations range from about 0.1 M to 1 µM as the carbon chain increases from 5 to 18 atoms (37). Autoxidation of aldehydes occurs readily in aqueous media. Although decay half times are measured in hours, reaction rates depend on the aldehyde and experimental conditions (17, 18, 37). Organic solvents such as carbon disulfide, dimethylformamide (DMF), and ethanol dramatically improve the solution stability of this substrate (18, 37, 39).

Relative to aldehydes with saturated, unbranched structures, those with side chains or unsaturated bonds proximal to the aldehydic group are poor substrates with reduced bioluminescence (28, 40, 41). This is explained by the enhanced stability of the aldehyde to oxidation. Functionalities remote from the aldehydic moiety do not affect the luminescence yield (41).

In studies in which luciferase was isolated from *V. harveyi* or the MAV strain of *V. fischeri*, high concentrations of aldehyde were found to be inhibitory (29, 33). Inhibition by long-chain alcohols, alkenes, ketones, esters, thiols, and amines occurs because these cognate molecules compete with aldehyde for binding sites on the hydroperoxide intermediate (27, 41).

**Oxygen**  
Oxygen limits the bacteria bioluminescence reaction only at very low concentration, at about 5 µM or less (19). This concentration is near aqueous solution saturation for a 14-carbon aliphatic aldehyde. At ambient temperatures the solubility of oxygen in air saturated media is about two orders of magnitude higher concentration. Therefore, this substrate does not normally limit *in vitro* bacteria bioluminescence (36).
Reduced flavin mononucleotide substrate may be obtained by either hydrogenation of flavin mononucleotide with platinized asbestos catalyst or photochemical reduction of FMN in the presence of EDTA (19, 33). Another convenient preparation of the substrate occurs by reaction of FMN with sodium dithionite (33). Dithionite also reduces dissolved oxygen rapidly (42). With excess reductant bioluminescence is decreased because insufficient oxygen remains to saturate the reduced flavin-enzyme complex. The stability of the known bioluminescence reaction intermediates to dithionite has been demonstrated (19).

Applications of Bacteria Bioluminescence

With bacteria bioluminescence, the maximum initial light intensity and total emission depend on the concentration of the limiting reactant. This relationship is linear over several orders of magnitude, forming a broad basis for analytical determinations of the enzyme and substrates. Assays in the femtomole range have been reported for *V. harveyi* luciferase ($10^{-16}$ mol), FMNH$_2$ ($10^{-14}$ mol) and aldehyde insect pheromones ($10^{-13}$ mol) (28, 33, 40). Using a suspension of intact *V. fischeri* as a probe, Lloyd et al. have determined dissolved oxygen to 35 nM (35).

Analytical applications of bacteria bioluminescence are extended significantly by linkage to reactions that produce substrate. Both fatty acids and alcohols and their respective reductases and dehydrogenases may be determined by this method (43, 44). However, broader applications are achieved by coupling reactions of pyridine nucleotides to the formation of FMNH$_2$ (4, 45):
Since biochemical reactions that involve either NADH or NADPH are common, numerous additional enzymes and substrates may be determined. These include several dehydrogenases, glucose, testosterone, ammonia, lactate, and glycogen among others. Generally, detection limits are in the fmol - pmol range (45-47).

Potential use of bacteria bioluminescence in immunoassay has been demonstrated by the determination of trinitrotoluene (TNT) (48). Glucose-6-phosphate dehydrogenase, an enzyme with high turnover (77,000) in the production of NADH, is conjugated to trinitrobenzene sulfonic acid. Both the conjugate and analyte are recognized as antigen by TNT antibody bonded to Sepharose. Varying the amount of free TNT involved in competitive binding limits both subsequent NADH production by the Sepharose-antibody bound enzyme-antigen and the amount of light produced in the coupled bioluminescence assay. A detection limit of 10 attomoles of TNT was achieved from the developed calibration curve.

Analysis by bacteria bioluminescence immunoassay is both rapid and extremely sensitive, as illustrated above. In addition, because they are safe and stable, luciferase-antibody and other chemiluminescent labels offer a possible alternative to the use of radioisotopes prevalent in this type of analysis. Other bacterial luciferase-antibody conjugates for the immunoassay of estriol, biotin and 2,4-dinitrofluorobenzene have been reported (49, 50).
SUBSECTION ON INSECT PHEROMONE DETERMINATIONS

Insect pheromones belong to a broad class of chemical stimuli known as semiochemicals. Pheromones differ from allomones and kairomones, other groups of this class, in that the chemical communication is intraspecies specific; and generally, by the more diverse range of behavioral response mediated by pheromone stimuli. As of 1982, about 700 pheromones had been identified (51); these are of several types. Substances used for defensive purposes include molecules that act as alarms or repellents; password-like compounds allow members of a colony to recognize one another, and specialized secretions define territorial boundaries. In some societal species the location of a food source triggers the release of trail-following, or "recruiting", pheromones. Commonly, sex attractants are used to bring together potential mates, either in pairs or in groups.

Of the categories mentioned above, the sex attractants have received the most attention. Frequently, these compounds are alcohols, esters, acids, lactones, aldehydes, ketones, alkanes or olefins of 10 to 17 carbons, with molecular weights ranging to about 300 (52, 53). Considerable diversity in the structure of pheromones occurs both among and within the orders of insects that use them (54). More often than not, the pheromone consists of a blend of compounds, sometimes in a specific ratio, which helps to maintain the reproductive isolation of the species (55). Often, the biological activity has been traced to specific positional isomers; to the enantiomer with proper chirality; or, critically, to functional groups on the pheromone molecule (56-60). This, in turn, is related to the selectivity of the thousands of chemosensory receptors located on the sensilla of insect antennae (61, 62).

Vapor pressures for insect sex attractants are moderate, typically about 10 μTorr (56), in contrast with the shorter carbon chain, more volatile and fast-acting alarm pheromones (53, 54). Nevertheless, sex pheromones
have remarkable potency. Response thresholds for some species of Lepidoptera extend to a few thousand molecules per cubic centimeter of air (56). In the case of the cabbage looper (*Trichoplusia ni*), this corresponds to about two parts per trillion on a wt. / wt. basis. Instances have been reported in which male moths were attracted from substantial distances, up to half a mile, to locate "calling" females (56, 63, 64).

Historically, the discovery and investigation of insect pheromones were delayed until sufficiently powerful analytical tools and techniques became available to resolve and measure the ultra-trace quantities produced by even large numbers of a single species. This is well illustrated by the time and effort expended for the first successful isolation and identification of an insect pheromone. In 1959, Butenandt and his associates reported the separation of 12 mg of "bombykol" [(E,Z)-10, 12-hexadecadien-1-ol] attractant from extracts of a half-million virgin female silkworm moths, *Bombyx mori* (53, 54, 64). In another study (1960), Jacobson et al. (64) reported the identification of the sex pheromone of the gypsy moth, *Lymantria dispar*. Again, a half-million moths were sacrificed to obtain 20 mg of the attractant -- the culmination of decades of research. However, the identity of the chemical structure proved to be incorrect. Correct characterization and a successful synthesis of the compound were not achieved until 1974 (64-66).

The extremely low natural concentrations and the multi-component blend of many insect sex attractants define two of the major difficulties involved in both the determination and characterization of these compounds. Added to this scope is the extensive sample clean-up ordinarily required. Also, many pheromones are labile, which can limit both the number and kinds of manipulations that may be involved in an analysis (67). Therefore, from sample collection and throughout the analysis, great care is exercised to prevent losses and to preserve the integrity of the biologically active
Generally, pheromone sample collection and work-up follow either of two routes. One of these involves the extraction of the attractants from relatively large homogenized samples of insect body parts or waste products. Since at the onset of an analysis the pheromone structure is generally unknown, the solvent of choice is determined by comparing the results of extractions with media of different polarity. Commonly, hexane, carbon disulfide, benzene, ether, methylene chloride, methyl formate, acetone, and methyl and ethyl alcohols are selected for this purpose (67). Excess solvent is removed by either steam or vacuum distillation. Further clean-up is effected through gas or liquid chromatography. In the other method, the volatile pheromones are recovered from an air stream swept over large numbers of sexually mature insects. The pheromones are either condensed from the air current or they are adsorbed onto a porous bed (Tenax, Porapak Q, charcoal, XAD resin) from which they are subsequently extracted (56, 68). Frequently, the extracts are separated into classes of compounds on polar columns (silica gel, alumina, florisil) with a mobile phase of gradually increasing polarity. The saturated hydrocarbons, olefins, ethers and ketones elute in sequence, followed by aldehydes, alcohols and acids (67).

Gas chromatography, in combination with a variety of techniques, is often used for final diagnostic purposes. For example, the familiar method of comparing retention times for pheromones with those of model compounds has helped to elucidate both the carbon chain lengths and functional groups present in sex attractants. Kovat's retention index and data obtained from trials on several different GC columns have been used to identify attractants that are n-alkanes, alcohols, esters and aldehydes. This method has been particularly useful in identifying the pheromones of Lepidoptera (67).

Pheromone carbon chain lengths may be made apparent through
"carbon-skeleton" chromatography (69). This is a form of reaction chromatography in which hydrogen gas and a heated catalyst are used in a column to saturate multiple bonds and to cleave functional groups present in the test compound of unknown structure. The retention value obtained for the resulting hydrocarbon is an indicator of the length of the carbon chain.

Another form of reaction chromatography helpful in the characterization of pheromones involves subtraction loops. With this method the mixture containing the species of unknown structure is passed through a reagent immobilized on a support. The support either precedes the analytical column or is placed between the column and the detector. The type of immobilized reagent determines which type of functional group will be retained and, hence, "subtracted" from the chromatogram. As an illustration, aldehyde pheromone that encounters immobilized o-dianisidine is retained as the Schiff base. The absence of the aldehyde peak on the chromatogram is noted by comparison with a trial chromatogram obtained by injecting a sample of the original mixture onto a column fitted with a blank subtraction loop. With appropriate subtraction loops this type of chromatography may be used to help identify alcohols, ketones and epoxides in addition to aldehyde pheromones (69). As with carbon-skeleton chromatography, detection limits are generally in the low μg range.

When sufficiently concentrated pheromone is available, gas chromatographs fitted with flame ionization detectors (FIDs) are used. For example, Ma and Schnee (70) trapped sex pheromone of the female gypsy moth on Porapak Q adsorbent. The preconcentrated attractant was then extracted and samples of the extract were chromatographed using a fused silica capillary column fitted with a FID. Results indicated that the moths produce 2.4 to 4.0 μg of pheromone per day.

On occasion, where greater sensitivity is required, adult insects have been used as GC detectors (64, 66). Characteristic behavior of the insects
signals whether or not eluting fractions contain active compounds. This has proven valuable in both the elucidation of pheromone structures and to illustrate whether or not the pheromone in a mixture has survived a particular manipulation in the course of an analysis.

The electroantennogram (EAG) bioassay (57, 71) has been interfaced to a gas chromatograph (72). Here the detector is an excised insect antenna connected via electrodes to a high impedance amplifier and a recorder. GC effluent in a stream of humidified air is passed over the antenna. By recording simultaneous chromatograms using both EAG and FID detectors it is possible to identify and quantitate major and minor pheromone components. Several studies focused on the characterization of pheromone blends of Lepidoptera have employed this technique (72).

Quantitative analysis of insect sex pheromones by electron-capture GC occur in the literature (73-76). With this method an appropriate derivative of the subject pheromone is prepared and isolated via chromatography with packed capillary columns. Typical detection limits are in the pico- to nanomole range for the derivatives; air concentrations have been measured to a few nanograms per cubic meter.

Many insect pheromones do not lend themselves to convenient derivatization, and rarely do they contain strong electron-capturing groups. Therefore, direct analysis of ultra-trace levels of insect pheromones using conventional chromatographic detectors is not always possible. In these instances a useful alternate method is detection by GC-mass spectrometry, with sub-nanogram determinations achievable. This technique can be hampered, however, by the co-elution of geometric and positional isomers present in insect extracts. The problem may be accentuated by the spectral similarity of these compounds. To overcome these difficulties Buser and Am (77) used high resolution capillary columns in combination with mass specific detection to determine sex pheromones in extracts of the coddling
and European grapevine moths. Ions both upfield in the spectra and
representative of each pheromone were monitored, avoiding much of the
interference from co-eluting isomers. Values of 3.5 and 1.6 ng of
pheromone were obtained per adult female for the respective species.

Chromatography in conjunction with liquid scintillation counting has
been used to determine radiolabeled pheromones. By this method,
Schneider, Kassang and Kaissling (62) determined about $10^9$ molecules
($10^{-6}$ to $10^{-7}$ µg) as a lower limit of detection for tritium-labeled bombykol.
Other studies report the use of high performance liquid chromatography
(HPLC) (78) and thin layer chromatography (TLC) (79), respectively, in the
determination of radio-carbon and tritium-labeled attractants.

Several spectrometric methods have been used to determine
pheromones. Of these, ultraviolet absorption is the least applied because
conjugated pheromone structures occur infrequently (67). Nevertheless, this
use is reported in the literature (80, 81). If sufficiently pure sample is
available, infrared (IR) and proton nuclear magnetic resonance (PNMR)
spectra can provide detailed structural information. Multiple scans on
Fourier transform instruments have made possible the determinations of
pheromones in the mid-nanogram and low microgram ranges for each of
these respective techniques (82). In addition, PNMR may be used to deduce
optical purity and to distinguish between pheromone enantiomers in a chiral
environment (83).

Applications of Insect Pheromone Technology

The isolation and characterization of hundreds of insect pheromones
represents an important advancement in the understanding of insect
behavior. In addition, both the synthesis of insect sex attractants in large
quantity and the application of these compounds for the control of insect populations has sparked considerable research interest in the field of integrated pest management. This interest stems from several perceived advantages of pheromone-based strategies. First, since many insects have evolved highly specific means of chemical communication, synthetic sex attractants may be used to interfere with the reproductive cycle of individual species. This occurs without affecting the natural predators of the target species or other wildlife. Second, even at levels much exaggerated relative to natural concentrations, insect pheromones are not considered to be environmentally hazardous (78). Life spans of pheromones released to the environment are relatively short. Half-lives for the attractants, either in water or soil, are measured in hours or days (56). This may be contrasted with numerous conventional pest control chemicals of high toxicity and long environmental persistence. Furthermore, increased resistance by target species to these chemicals has led to both heavier applications of pesticides and a costly search for new and more effective insecticides (84).

Three pheromone-based methods leading to the suppression of insect pest populations have been described (55, 78, 85-88). These include detection and survey, mass trapping and air permeation.

In the survey method, insect counts from traps baited with pheromone lures permit rough estimates of pest population densities. These, in turn, determine where and when measures of control should be applied. By detecting and limiting incipient infestations, the savings on projected resource damage and eradication costs are substantial, with estimates measured in millions of dollars (87). This method has been used extensively by the United States Department of Agriculture, several research groups and private enterprise.

The mass trapping and air permeation methods are aimed at the direct control of insect populations. With mass trapping the intent is to reduce the
target population to economically acceptable levels. Baited traps equipped with toxicants, pathogens, adhesives, or one-way mechanical baffles are used for this purpose. Several studies and reviews of this approach occur in the literature (57, 63, 84, 89).

With air permeation, pheromone lures of potency competitive with (or orders of magnitude stronger than) those of an emitting insect are distributed in sufficient numbers and, often, in a grid-like pattern to suffuse an infested area with attractant. Confused, or possibly habituated, males are unable to locate calling females. Disruption of the mating cycle leads to population reduction (60, 84, 85, 89, 90).

**Controlled Pheromone Release Dispensers**

Pivotal to integrated pest management research has been the development of analytical methods to determine levels of airborne pheromone and release rates from artificial dispensers of attractants. Though simple in concept, the dispensers should satisfy a number of criteria. Ideally, the design should provide for the release of pheromone at a constant, optimum rate and the emission should extend over the calling period. These factors vary with the target species. Further, labile pheromones require protection from oxidation, hydrolysis and sunlight. Successful designs include those in which the lure formulations are encased in microcapsules, laminated plastics and hollow fibers (77, 85, 86, 91).

Published methods to determine pheromone lure performance characteristics follow either of two routes: measurement of total pheromone remaining in the dispenser, or measurement of the pheromone released as a function of time. As has been pointed out by Golub and Weatherston (68),
both analyses should be carried out whenever possible to achieve mass balance. Not surprisingly, both individual studies (81, 92, 93) and a review (68) of these techniques indicate that they are similar to those used to determine pheromones from natural sources.

A Brief History of the Gypsy Moth in North America

An Eurasian species, the gypsy moth (*Lymantria dispar*) was introduced to the North American continent in an unsuccessful attempt to develop a new strain of silk-spinners (64, 94). Since 1869 the species has been expanding its range from the Boston area at a prodigious rate. In little more than a decade the area of infestation was about 360 mi.²; by 1974 this range had increased to more than 200,000 mi.², including lands in Canada (60). As of 1982 the area to which the species had spread extended south to Virginia and west to Michigan, Wisconsin and Nebraska (85). Aided by transport from passenger and recreational vehicles, significant numbers of gypsy moths have been trapped in California, Oregon and Washington in the last decade (95).

In the late instars, individual gypsy moth caterpillars consume an estimated 24 in.² of foliage per day. Combined with a feeding habit so broad as to include hundreds of species of vegetation, damage caused by gypsy moth larvae can be extensive. In 1981 roughly half of the three million acres of Connecticut were defoliated; elsewhere in the East another ten million acres were stripped in that year (85, 96).

Attempts to control the gypsy moth date from the late nineteenth century, as by this time the insect was already a serious pest of fruit trees and hardwoods. Live virgin female moths were used as bait in mass trapping efforts in 1893; however, this control proved unsuccessful and was
abandoned (60, 63). In the early part of the twentieth century dozens of species of the moth's natural enemies were imported from Europe and the Soviet Union. Few of these survived, although one in particular, conidia (spores) of a Japanese fungus, released 79 years ago, appears to have made a significant recent contribution to the suppression of a major outbreak in New England (96, 97). Several pesticides have been used against the larvae, including those with the generic names Sevin, Orthene and Dimilin (85, 98). The naturally occurring *Bacillus thuringiensis* (B.t.) has been used against the early instars. Sprayed on foliage and once ingested, this bacterium crystallizes in the digestive tract of the larvae. Generally, this measure is preferred by environmentalists because toxicity to fish, mammals, birds and other wildlife is low (99). Other control programs include the release of sterile male moths and the use of pheromone baits in mass trapping and mating disruption strategies (57, 60, 63, 85, 87, 90). Estimates of total expenditures by state and federal governments to manage this exotic insect range into hundreds of millions of dollars, making the gypsy moth the most costly American agricultural pest (60, 85).

**Sex Pheromone of the Gypsy Moth**

The sex pheromone of the gypsy moth was first reported as cis-7-hexadecene-1,10-diol 10 acetate. This compound was given the common name "gyptol". The 18-carbon homolog, "gyplure", cis-9-octadecene-1,12-diol 12 acetate, was used by the Department of Agriculture for trapping of the gypsy moth (64). Subsequent work indicated that these compounds were inactive, prompting a re-investigation. In 1970 Bierl et al. (66) characterized the structure as cis-7,8-epoxy-2-methyloctadecane. The activity of the synthesized attractant was checked both by laboratory bioassay and by
Figure 2.2. Enantiomers of Disparlure: (+) (7R,8S)- and (-) (7S,8R)-cis-7,8-epoxy-2-methyloctadecane.
trapping tests in the field. Activity was reported for as little as 2 picograms in the bioassay; trap catches were significant compared with those made with extracts of the natural pheromone. Used in survey, mass trapping and mating disruption studies, the synthetic attractant was called "disparlure".

A further advancement, establishing the stereochemistry of the natural pheromone, was achieved via the synthesis of the enantiomers of disparlure (see Figure 2.2). Iwaki et al. (100) demonstrated that the (+) (7S,8R) isomer had a significantly lower response threshold than either the racemic mixture (used by Bierl et al.) or the (-) (7S,8R) enantiomer. As little as 100 pg/mL of (+)-disparlure was detected by male moths in a bioassay. The considerable difference in response thresholds suggested that the male moth antennae have chiral receptors, with differential preference for the (+)-enantiomer. Data from an electroantennogram bioassay (57) suggested that the receptor system consists of different neurons having affinity for either (+)- or (-)-disparlure. Moreover, (-)-disparlure was found to be inhibitory, as indicated by both laboratory and field studies comparing the efficacy of various mixtures of the enantiomers (57, 58, 101). Although this work supports the suggestion that the female gypsy moth emits (+)-disparlure exclusively, this hypothesis has not been confirmed (94). Interestingly, (-)-disparlure has no effect on the nun moth (Lymantria monacha), a sibling species of the gypsy moth, yet male nun moths are also attracted by (+)-disparlure. Reproductive isolation of the two species appears to be mediated by the (-)-enantiomer in the 10:90 blend emitted by the female nun moth (54, 95).
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Overview

The apparatus developed for use in this research consists of a pneumatic flow injection system and a novel type of flow cell for chemi- and bioluminescence determinations. A general schematic is shown in Figure 3.1. With compressed air as a pump, a stream of mixed reagents and a carrier solution are delivered to the flow cell. A valve directs the flow of the carrier through a sample loop, sweeping a plug of reaction initiator to the detection cell. Mixing of the two reagent streams occurs within the cell at a confluence point focused by the narrow concentric ports of an injector. The resulting chemiluminescence is detected by a photomultiplier tube (PMT). After signal processing, a voltage proportional to the chemiluminescence intensity is displayed by the chart recorder. The apparatus is interfaced to an IBM compatible personal computer (PC) fitted with a Scientific Solutions LabMaster input / output board. With appropriate software, this arrangement is used both to actuate a pump for the filling of a sample loop and to switch a valve from sampling to injection modes. In addition, luminescence data may be acquired, stored and manipulated.

Reagent and Sample Delivery System

A schematic for the flow injection network is shown in Figure 3.2. With reference to this diagram, house air reduced to about 50 psi is used both as the drive for the valve actuator, discussed below, and to provide pressure to a regulator connected to the reagent flow system. The regulator (Matheson 2403N4) steps the house air down to 8.2 psi, a pressure compatible with the strength of the tubing junctions and sufficient for full apparatus performance.
Figure 3.1. General Schematic of Flow Injection and Signal Processing Apparatus.
Figure 3.2. Reagent and Sample Delivery System.
This pressure is maintained in both a Clippard (MAN-12) 12-port manifold and a 500-mL glass Erlenmeyer flask. The substantially larger reservoir of the flask cushions any sudden pressure demands that might occur within the system, effectively reducing reagent flow surges.

Several ports on the manifold are linked to sidearm flasks. Check valve units (Clippard MNV-1s joined to MCV-2s) spliced into these connections both bring individual flasks "on-line", as needed, and prevent back-flow. The flasks contain reagents necessary for chemi- or bioluminescence analyses. As can be seen in Figure 3.2, five sidearm flasks are linked to the manifold, representing the number of reagents required for a marine bacteria bioluminescence reaction. Fewer, or a greater number of flasks may be used for experiments with a different type of reaction.

The arrangement of the flasks into two groups in Figure 3.2 indicates the separate paths that the reagents follow. In the branch leading to the outer conduit of the injector, reagents flow from 125-mL sidearm flasks through Gilmont flow meters. Model numbers for these meters and a listing of representative water flow rates, to full capacity, are presented in Table 3.1. Flows from these meters are combined in a Clippard (MRM-6) 6-port rotary manifold (Figure 3.3 A). With two opposed ports blocked, and each adjacent port at an angle of 60°, the geometry of this manifold permits both merging zone and turbulent mixing of the reagents. Each open port input is fitted with a check valve (Clippard MCV-2), preventing reagent back-flow. The combined flow exits through a port located on the underside of this manifold and is linked, via Tygon tubing, to a miniature magnetic mixer (Figure 3.3 B). The mixer is a glass envelope, the size and shape of a 1.0-mL volumetric flask, which provides containment for a "pea"-size stirbar. Ports located at the top of the vertical stem of the envelope and out the side, near the bottom flat, provide an "L"-shaped path through the unit that includes the turbulence of the spinning magnetic rotor. The stirbar is rotated by a Cole-Parmer No.
Table 3.1. Flow Rates for Gilmont Flow Meters.

<table>
<thead>
<tr>
<th>Flowmeter Designation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Gilmont No.</th>
<th>Scale Reading at Center of Float&lt;sup&gt;a&lt;/sup&gt;</th>
<th>10</th>
<th>25</th>
<th>40</th>
<th>55</th>
<th>70</th>
<th>85</th>
<th>93</th>
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<tbody>
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<td>1 A7972</td>
<td>0.075</td>
<td>0.38</td>
<td>0.82</td>
<td>1.45</td>
<td>2.32</td>
<td>3.25</td>
<td>4.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 A5403</td>
<td>0.065</td>
<td>0.32</td>
<td>0.81</td>
<td>1.44</td>
<td>2.31</td>
<td>3.31</td>
<td>4.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 A7967</td>
<td>0.075</td>
<td>0.38</td>
<td>0.82</td>
<td>1.44</td>
<td>2.30</td>
<td>3.28</td>
<td>3.98</td>
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</tr>
<tr>
<td>4 A7984</td>
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<td>0.35</td>
<td>0.82</td>
<td>1.46</td>
<td>2.31</td>
<td>3.28</td>
<td>3.98</td>
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</tr>
<tr>
<td>5 H376</td>
<td>0.80</td>
<td>4.4</td>
<td>11.2</td>
<td>18.4</td>
<td>25.8</td>
<td>33.1</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Glass floats are supplied with these meters. Accuracy is within 2% of a reading (mL / min) or one division, whichever is greater.

<sup>b</sup> As shown in Figure 3.1.
Figure 3.3. Mixer Apparatus. (A) Check Valve and Manifold Assembly and (B) Connection to Miniature Mixing Apparatus.
4800 stirring unit, centered below the flat bottom of the glass housing.

Solution from the mixer is passed to a 6-to-1 rotary valve (Rheodyne Model 5012) that selects either the mixed reagent flow, or a gravity flow from one of two detection cell wash solutions: deionized water, or a 1/100 (v/v) solution of Triton X-100 in water. The selected flow passes through a 44-cm length of 1.5-mm bore Teflon tubing, en route to the outer conduit of the injector.

The other branch emanating from the air-pressurized manifold is used to drive the carrier stream from a 1000-mL sidearm flask and through flow meter 5, also characterized in Table 3.1. This stream, directed by the valve, provides transport of the reaction initiating reagent to the inner conduit of the injector.

**Sample Injection Valve**

A pair of Dionex 4-way valves (Cat. No. 030520), configured as a sample loop injection valve, is used either to pass the carrier directly to the injector or to first shunt this stream through a reagent filled sample loop. The valves are switched in tandem by the pneumatic, piston-like action of a rectangular sliding bar. Air pressure for this operation is provided by a pair of 3-way solenoid-driven valves exterior to the Dionex housings. When the sliding piece has reached full travel in either direction, channels bored through the bar complete a required flow configuration.

In the sampling mode (Figure 3.4 A), a PC automated pump (Pharmacia Paristaltic P-3, No. 90626) draws reagent into the sample loop and flushes this volume several times. Excess reagent wash is passed to a waste reservoir. Simultaneously, the carrier stream flows through a bypass loop, then continues on a path to the injector. In the injection mode (Figure 3.4 B), the contents of the sample loop are flushed to the injector by the carrier. Ideally, the reaction initiating reagent is transported as a plug.
A. SAMPLING MODE

Sample Loop

Reaction Initiator Solution

To Waste Reservoir

Bypass Loop

Carrier Stream

To Injector

B. INJECTION MODE

Sample Loop

Carrier Stream

To Injector

Figure 3.4. Operation of Sample Injection Valve. (A) Sampling Mode; (B) Injection Mode.
A few additional notes regarding the modes of the sample injection valve are pertinent. First, in the sampling mode, the carrier stream provides a continuous wash to the detection cell via the flow from the injector inner conduit. This stream is made from the same solvent or solution, less the reaction initiator, as that contained in the sample loop. Therefore, when this flow is mixed in the cell with the other chemiluminescence reagents, the resulting flow constitutes a continuous blank solution wash within the cell. Second, during the injection mode, reagent used to fill the sample loop does not continue through the bypass loop and on to the waste reservoir; rather, the pump that controls this flow is switched off by the computer prior to the switching of the Dionex valves. Although the pump is stopped at the end of the sampling mode, the inertia of the reaction initiator within the tubing could cause a minor flow into the bypass loop. If this occurs, and the bypass is flushed subsequently by the carrier, a "ghost" signal may be recorded by the detection system. Signals of this type may be reduced or eliminated by the introduction of a delay between the switching of the pump and valves. The length of the delay may be mediated by software.

**Injector**

The injector is a Beckman burner assembly (No. 4020), used for atomic absorption (AA) or emission (AE) spectrometry. The burner unit, less the outer cowl and upper arm fuel port, is shown in Figure 3.5. This section consists of a brass tapered nozzle, a brass body fitted with an arm-like oxidant port, and a stainless steel shaft, sheathed with a platinum atomizer pipe. The orifice at the end of the nozzle taper has inner and outer dimensions of 0.030 and 0.065 in., respectively. This opening is made concentric with that of the platinum pipe by three set screws located in the nozzle shaft and positioned at 120° with respect to one another. The tip of the platinum pipe has an inner diameter of 0.010 in. and a wall thickness of
Figure 3.5. Beckman Burner Assembly, Modified for Use as a Chemiluminescence Injector.
For this research application, a chemiluminescence reaction mixture is pushed through the port normally reserved for the oxidant. A final reagent, required to initiate a chemi- or bioluminescence reaction, is passed through the solution port. Typically, this reagent is the analyte.

Collimation of the flows from the injector nozzle may be checked by observing the angle of deflection of the net flow from 90° with respect to the horizontal plane of the lower cell plate (see Figure 3.6). It is helpful to carry out this procedure at relatively high flow rates (ca. 9 and 12 mL/min, respectively, through the outer and inner conduits) and to catch the fountain-like net flow on the bottom of a small inverted glass beaker, held several inches above the nozzle. By switching the rotary valve alternately between a null position and pressurized flow to the injector, it is possible to observe poor collimation as a displacement of the point at which the flow spout strikes the bottom of the beaker.

Detection Cell

The 0.83 cm³ volume of the detection cell (see Figures 3.6 and 3.7) is defined by a pair of transparent polycarbonate plates and an "O"-ring seal. Plate separation is maintained by a set of eight flat washers, each 0.016 in. (0.41 mm) thick. These are positioned at a distance of 1.78 in. (4.52 cm) and in 45° arcs with respect to the center of either plate. The two concentric reagent streams, flowing under pressure from the injector nozzle, strike the underside of the upper cell plate. The individual flows are deflected into one another by this impact and turbulent mixing ensues. Mixing occurs at a point centered in the volume element viewed by an end-on photomultiplier tube (PMT). From the point of injection, the chemiluminescence mixture flows radially, between the cell plates. At a radius of 1.0 in. the flow encounters a spent reagent gutter, cut into the lower cell plate. Twelve equally spaced exhaust ports bored through this groove allow the mixture to
Figure 3.6. Detection Cell Assembly with Mounted Beckman Burner Injector and Photomultiplier Tube (PMT). The mirrored surface shown is a film thin lamina attached to the backside of the Lower Cell Plate.
Figure 3.7. Detection Cell Lower Plate, Topview, with Injector Ports Visible. Dimensions (inches) in terms of consecutive diameters measured from the center of the plate are as follows: injector inner conduit o.d., 0.017; injector outer conduit o.d., 0.065; spent reagent groove, inner wall, 2.00; spent reagent groove outer wall, 2.12; Buchner funnel waste catch groove, inner wall cut into the plate underside, 2.96; waste catch outer groove, 3.17; outer diameter, 4.00. Other dimensions: plate thickness, 0.245; spent reagent groove depth, 0.125; spent reagent port diameter, 0.063; threaded mounting ports, 4-40 NC.

Dimensions (inches) for cell upper plate (not shown): "O"-ring groove, inner diameter, 2.05; groove width, 0.12, depth, 0.086, (accepts a 2-138 size "O"-ring); outer diameter, 4.00. Other dimensions: plate thickness, 0.234; mounting ports, 0.125.
exit the cell. Effluent from the cell is collected by a plastic Buchner funnel, fitted to a groove cut into the underside of the lower cell plate (Figure 3.8).

The cell is suspended within a 5-in. diameter black vinyl cylinder by screws that pass through an aluminum plate. This plate both caps the upper end of the cylinder and serves as the mounting bracket for the PMT housing. Holes bored through the wall of the cylinder provide entrance and exit ports for tubing carrying chemiluminescence and spent reagents. Another aluminum plate caps the bottom of the cylinder. When fully assembled, including the PMT housing and a shroud of black cloth, the apparatus is light-tight. This is indicated by no apparent difference in the baseline signal, at high gain, when the apparatus is exposed alternately to room light and near total darkness.

Detection, Signal Processing and Readout Systems

The PMT, an RCA type C31059, has spectral responsivity in the 300 to 660 nm range, with peak response at about 400 nm. Typical anodic dark current is 0.8 nA at 22°C and with an operating potential of 1000 V. Expected gain with these conditions is 2.9 x 10^6. The 30-mm diameter head-on tube is supported in a Pacific Precision model 62 housing. Potential to the detector is supplied by a Keithley 244 High Voltage Supply.

The photoanodic signal is converted to a voltage and amplified by a Keithley 427 Current Amplifier. A Spectrum 1021 Amplifier and Filter provides discrimination against noise and smaller, non-decade amplification steps. Readout is given by a Heath SR-255B chart recorder and both the screen monitor and line printer connected to the PC.

Normally, a recorder full-scale setting of 100 to 500 mV and an analog-to-digital converter (ADC) with a 10-V bipolar range were used. To provide adequate resolution in the digitized signal it was necessary to further amplify the output from the amplifier filter. For this purpose an Analog Devices
Figure 3.8. Detail of Reagent Ports in the Cell Housing.
AD522 instrumentation amplifier was configured for PC programmable gain (Figure 3.9). The AD522 chip is designed for high accuracy, linearity and common mode rejection (CMR), as well as low voltage drift and low noise. Since the CMR is large (> 80 dB), the output of this chip may be approximated by Equation 3.1, where $R_G$ is the gain resistor and $(E_1 - E_2)$ is the potential difference between the amplifier inputs.

$$E_o = \left(1 + \frac{200 \, k\Omega}{R_G}\right) \left(E_1 - E_2\right)$$  \hspace{1cm} (3.1)

With the LabMaster Daughter Board jumpered for 10-V, bipolar, full range response, values for $R_G$ were calculated so that a full scale signal on the recorder yielded +10 V into the ADC. For recorder settings of 100, 200, and 500 mV full scale, the required respective gains are 100, 50 and 20. From Equation 3.1, the corresponding values for $R_G$ are 2.02, 4.08 and 10.5 kΩ.

The required $R_G$ value is selected by a set of logic signals transmitted from the LabMaster Daughter Board to inputs of an Analog Devices AD7510 CMOS quad analog switch. The circuit in Figure 3.9 was calibrated by bringing a measured potential to the signal input of the AD522, selecting an $R_G$ line with the appropriate logic signals, and by adjusting $R_G$ on a trimpot until the output of the amplifier registered proper gain. The resulting $R_G$ value is a combination of the resistance of the adjusted trimpot and the ca. 75 Ω impedance of the selected SPST switch of the AD7510. This calibration was carried out with several test potentials to confirm the required gain for each recorder range. Resolution allowed by the LabMaster 12-bit ADC is 4.9 mV.
Figure 3.9. AD522 Instrumentation Amplifier with Programmable Gain Interface. AD7510 Switch "ON" for Address "HIGH".
Software Control

A chart describing the automation of the flow injection / measurement cycle is shown in Figure 3.10. A brief review of the software operation follows; the code listing appears in Appendix I.

The selection of the chart recorder range is made compatible with the PC data acquisition system by typing a response to the prompt on the PC monitor. Input of the value 1, 2 or 3 results in the transmission of a logic 001, 010 or 100 out port B of the LabMaster Daughter Board, closing the appropriate AD7510 switch. With this input the software initiates a flow injection cycle.

The cycle begins with the switching of the sample injection valve. A HIGH signal sent from port C, bit 3, of the Daughter Board triggers the power circuit to a solinoid valve. Air pressure is directed to one face of the slide bar, switching the valve to the sampling mode. The carrier stream flows to the detection cell via the bypass circuit. A second HIGH signal from port C, bit 0, actuates the power circuit to the pump. This signal is held for 30 s, a duration sufficient to pass 2.3 mL of solution through the sample loop. In this period, a 0.5-mL loop is filled with reagent, following a 3.6 equivalent volume rinse. As discussed earlier, a brief delay is required after the pumping cycle to ease any reagent pressure within the tubing and to prevent a subsequent residual flow into the bypass loop. The delay required varies with the viscosity of the solution and may be altered by the operator, as desired, in the "Switch.Valves.and.Pump" subroutine of the program.

When the injection valve is switched to the injection mode (LOW at port C, bit 3), the contents of the sample loop are transported to the detection cell via the carrier stream. Since a 40-cm length of 0.8-mm i.d. Teflon tubing separates the valve port from the detection cell, data acquisition is delayed briefly. Given a nominal carrier flow rate of 8.0 mL / min, and considering the 0.20-mL volume of the tubing, the time required to transport the reagent plug
Figure 3.10. Software Control Flowchart. Delay Times listed are for a Marine Bacteria Bioluminescence Reaction.
to the cell is 1.5 s. Therefore, a 1.0 s delay in data collection is appropriate. The remaining 0.5 s is sufficient for background signal (baseline) averaging with a moderate data acquisition rate.

The LabMaster 9513 Timer, counter number 5, is programmed to collect luminescence data through one channel of the LabMaster analog-to-digital converter, ADC0. The code is written for a collection rate of 77 Hz, providing 459 data points in 6 s. Although these parameters are altered easily, this rate and time frame are compatible with the kinetics of a bacteria bioluminescence reaction. Following the data acquisition, the flow of the carrier is continued through the sample loop for 30 s, rinsing this volume several times.

The HIGH and LOW bytes of the data are stored temporarily in two arrays. Subsequently, this information is written to disk as a file with a unique name, based on the date and time-of-day. A profile of the chemiluminescence peak is displayed briefly on the monitor, as well as the results of the maximum voltage and peak area calculations. Hardcopy in the form of a report is made available via the line printer, including the information listed in Table 3.2.

This completes the software cycle. A new cycle is initiated immediately, beginning with the query to the operator regarding the match between the chart recorder range and the gain value to be used with the instrumentation amplifier. This arrangement economizes on the use of potentially expensive chemiluminescence reagents. If the experiment is planned carefully, no other software parameters require adjustment. Between measurements it is helpful to check the Gilmont meters for drift from predetermined flow rates.

**Apparatus Start-up and Operation**

Normally, at the start of an experiment, the PMT housing has been removed from the apparatus and stored in a holster for safe-keeping. Both
Table 3.2. Chemiluminescence Information Output to the Line Printer.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>peak number</td>
<td>integer</td>
</tr>
<tr>
<td>chart recorder range</td>
<td>mV</td>
</tr>
<tr>
<td>maximum potential(^a)</td>
<td>V</td>
</tr>
<tr>
<td>avg. baseline(^b)</td>
<td>V</td>
</tr>
<tr>
<td>peak signal(^c)</td>
<td>V</td>
</tr>
<tr>
<td>peak area(^d)</td>
<td>V x s</td>
</tr>
</tbody>
</table>

\(^a\) Largest signal voltage sorted from the data array.
\(^b\) Mean of the first ten data potentials.
\(^c\) Difference between the maximum voltage and the averaged baseline.
\(^d\) Difference between the sum of the data points and the product of the averaged baseline and the data acquisition time.
the reagent flasks and flow lines are filled with deionized water. The presence of the water in the flow system is due to a rinse cycle conducted at the end of a previous experiment. This is a precaution against salt deposit or bacteria build-up and subsequent blockage of small bore regions within the flow system. The water in the flasks is discarded and each of these containers is rinsed with a small amount of a designated reagent or analyte solution. The flasks are then filled with the respective solutions and the flow lines are bled through to the detection cell.

In the course of the bleed process it is inevitable that air bubbles will be introduced to the cell. Most bubbles are passed through the spent reagent ports; however, some may become trapped between the cell plates and in the region viewed by the PMT. The presence of bubbles will disrupt the radial flow of the luminescence solution within the cell and, even worse, migration of air pockets in the area viewed by the detector will lead to the imprecision of experimental results. Bubbles are removed most easily by switching the rotary valve in Figure 3.2 so that flow of the aqueous Triton X-100 solution (1:500, v/v) is directed through the injector outer conduit. Often, the flow and reduced surface tension of this solution are sufficient to dislodge bubbles. Higher solution flow rates are achieved by introducing additional flow with the carrier stream. If this fails, a gentle tapping on the exposed surface of the upper cell plate with a gloved finger may prove helpful. Throughout this procedure the cell plates should be plumb. This may be checked with the aid of a small level placed on the exposed surface of the upper cell plate. In extreme cases in which bubbles fail to dislodge, it is probable that they are clinging to irregularities on the cell plate surfaces. In such circumstances the cell should be dismantled, the plates should be washed with deionized water, and then polished carefully with lens paper. Once the cell is made clear with the Triton X-100 solution, the cell is then rinsed with deionized water by switching the rotary valve to the
appropriate position (Figure 3.2).

The PMT housing is mounted and screwed in place. In this procedure care should be taken not to expose the photocathode to ultraviolet radiation present in fluorescent room light. According to literature accompanying the detector, an exposure of this kind can cause an increase in the anode dark current by as much as three orders of magnitude. The temporary increase may last up to 48 hours and could occur even though no voltage was applied to the detector at the time of the exposure.

After the PMT housing has been mounted, a shroud of black cloth is wrapped around both the detector housing and the cell assembly encasement. Leakage of stray light may be checked by observing any difference in the baseline established at high gain when room light intensity is switched from high to low levels. Proper wrapping of the shroud effectively eliminates stray light.

The carrier stream and chemiluminescence reagent flow rates are adjusted to predetermined levels on the Gilmont meters. When these solutions are mixed in the detection cell, the signal on the chart recorder will be observed to rise and become constant. Ostensibly, this is the blank signal. However, the injection of a matrix-matched blank should be included as a standard part of the analysis procedure with this apparatus. Any signal different from the established baseline and due to the injection of the matrix is traced, usually, to contamination of either the prepared blank or the tubing used in the flow system.
CHAPTER 4

INSTRUMENT OPTIMIZATION AND LONG CHAIN ALDEHYDE DETERMINATIONS WITH BACTERIA BIOLUMINESCENCE

OVERVIEW

This chapter is concerned with aldehyde substrate determinations with the bacteria bioluminescence reaction reviewed in Chapter 2. Preliminary studies were conducted with a discrete sampling system to confirm that results presented in the literature could be duplicated, and to assess the stability of dilute aldehyde solutions. Other work involves optimization of the bioluminescence signal of cis-11-hexadecenal with the flow injection system described in Chapter 3. Once optimum conditions were established, calibration curves and detection limits for three aldehyde substrates were obtained.

EXPERIMENTAL

**Discrete Sampling Studies**

**Reagents and solution preparation.** Dithiothreitol and glycerol were obtained from U. S. Biochemical Corporation; dibasic potassium phosphate was acquired from Allied Chemical; monobasic sodium phosphate was obtained from Mallinkrodt; all other reagents and solvents were purchased from Sigma. Aqueous solutions were prepared with in-house deionized water passed through a Millipore Milli-Q system, a double deionization process.
Solution preparations were similar to those presented in previous discrete sampling studies (1, 2). A principal difference was that the dithionite reductant was not used as a solid; rather, it was prepared as a solution. This approach is both convenient and reproducible and has been suggested previously (3, 4). The sodium salt (Na₂S₂O₄) was dissolved in 1.25 M phosphate buffer (pH 7.0) and used promptly following preparation.

*V. harveyi* luciferase primary stock solution, 10.0 mg protein / mL, was prepared in 10 mM dithiothreitol, 35% glycerol and 63 mM phosphate buffer (K₂HPO₄ / NaH₂PO₄), pH 7.0. This solution was stored at -140 °C and used within a week. Secondary enzyme stock solution, 0.50 mg protein / mL, was obtained by dilution of the primary stock in a solution of 1.0 mM hydroxylamine, 50 mM β-mercaptoethanol, and 63 mM phosphate buffer (pH 7.0). Flavin mononucleotide (FMN) solution, 5.0 mM, was also prepared in the buffered medium. Cis-11-hexadecenal was dissolved in dimethylformamide (DMF) and stored as primary stock solutions at 4 °C. Secondary (assay) solutions were prepared by water dilution.

**Instrumentation** For the discrete sampling measurements, the chemiluminescence photometer described by Hoyt (5) was employed. A PMT bias voltage of -680 V and a filter cutoff frequency of 0.1 Hz were used. With Eppendorf pipets, chemiluminescence reagents were added to the detection cell, a cuvette, with the volumes and concentrations specified in Table 4.1. The reaction was initiated by the rapid injection of 0.996 mL of an aqueous aldehyde sample solution. The resulting peak-shaped chemiluminescence signal was monitored on a chart recorder. Analytical signals were taken as the difference between peak heights measured for water blanks and the aqueous aldehyde samples. Generally, three or more injections were made for each aldehyde concentration. After each peak was recorded, the sample
Table 4.1. Reagent Deliveries to the Detection Cell used with Discrete Sampling Apparatus.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
<th>Reagent Conc. (mM)</th>
<th>Amount (nmol)</th>
<th>In-Cell Conc. a (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>10</td>
<td>5.0</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>luciferase</td>
<td>25</td>
<td>0.063 b</td>
<td>0.16</td>
<td>0.15</td>
</tr>
<tr>
<td>dithionite</td>
<td>25</td>
<td>57 c</td>
<td>1440</td>
<td>1360</td>
</tr>
<tr>
<td>buffer d</td>
<td>1000</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

a Prior to the injection of 0.996 mL of an aqueous aldehyde sample.  
b Equivalent to 0.5 mg / mL.  
c Equivalent to 10 mg / mL.  
d 1.0 mM hydroxylamine, 50 mM β-mercaptoethanol and 63 mM phosphate buffer (pH 7.0).
cuvette was rinsed three times with deionized water. Similarly, each time a fresh solution was used in an analysis, the sample injection syringe and delivery tubing were rinsed several times with the new solution. This procedure was followed prior to the initiation of any injection and measurement cycles.

**Aqueous Aldehyde Solution Stability**  It is well known that aldehydes are both volatile and subject to autoxidation. In the air, the oxidation occurs in two steps. The initial product is a peracid, formed from the addition of molecular oxygen to the aldehydic C-H bond (Reaction 4.1). Disproportionation of the peracid with a second aldehyde molecule yields the final acid product (Reaction 4.2). Free radical mechanisms are proposed to account for atmospheric aldehyde oxidations. In acidic, alkaline and neutral solution, ionic processes yield carboxylic acids from aldehyde substrates (6).

![Chemical reactions](attachment:reactionImages.png)

Other researchers report the use of aqueous aldehyde sample preparations within 20 min (1, 2). To better assess the stability of cis-11-hexadecenal in this medium, three experiments were conducted. These were designed to indicate, qualitatively, the temporal effects of volatile and oxidative losses.
In the first experiment (EXP 1), a solution of 12 nM cis-11-hexadecenal was prepared in water solvent that had been sparged with air for 20 min. Approximately 125 mL of this solution was added to an open, 3.75-in. diameter Kimax dish and 71 cm$^2$ of the solution surface was exposed to the air. Samples taken from the dish were analyzed as a function of time.

In the second experiment (EXP 2), an identical aldehyde solution was divided into seven portions of approximately 110 mL each. Each portion was stored in a 100-mL volumetric flask and care was taken to exclude air bubbles from the capped vessels. The numbered flasks were sampled in sequence. Generally, three samples were taken from each opened flask before sampling the next container.

In the third experiment (EXP 3), 12 nM cis-11-hexadecenal was prepared in water that had been sparged with helium for 20 min. The solution was drawn into a set of four 20-mL capacity glass syringes. The solutions in the numbered syringes were sampled sequentially. Generally, five samples were drawn from each container prior to sampling the next syringe in the sequence.

Flow Injection Studies

Reagents and solution preparation. The reagents used in the flow injection experiments were the same as those used in the discrete sampling studies, described above, with the following exceptions and additions. Exhausted supplies of the phosphate buffer salts were replaced with similar reagents from Sigma. The aldehydes used to develop calibration curves, undecanal and heptanal, were acquired from Aldrich; tetradecanal was purchased from Sigma.

Flow injection experiments were conducted with aqueous solutions,
buffered at pH 7.0 and prepared with the water solvent equilibrated to ambient temperature, generally 19-24° C. The pH condition is optimum for luminescence response with *V. harveyi* luciferase (7), and the temperature conditions are similar to those used in other studies (1, 2).

As in the discrete sampling experiments, aldehydes were dissolved in dimethylformamide (DMF) and stored as primary stock solutions at 4° C. Secondary (assay) solutions were prepared by water dilution and used immediately. Typical aqueous dilution factors were 10<sup>3</sup> to 10<sup>5</sup>. In similar proportion, carrier solution was prepared from DMF and water. The concentration of the phosphate buffer (pH 7.0) was 1.5 M in the sodium dithionite solution preparation and 0.75 mM in the FMN and luciferase assay solutions. Otherwise the solution preparations were the same. In the optimization experiments, the "buffer" solution was 1.0 mM hydroxylamine, 50 mM β-mercaptoethanol and 75 mM phosphate buffer (pH 7.0).

**Instrumentation.** Flow injection measurements were conducted with the apparatus discussed in Chapter 3. A PMT bias voltage of -680 V and a filter cut-off frequency of 0.1 Hz were used. To review, the dithionite, buffer, FMN and luciferase solutions were mixed and delivered to the outer conduit of the injector. Aldehyde sample solution was transported by the carrier stream to the injector inner conduit.

**Bacteria bioluminescence assay optimization.** For the assay optimization, a fixed FMN concentration, 500 μM, and a 1.22 mL / min flow rate were used. The latter setting is mid-scale (i.e., 50) on the Gilmont A7967 meter. With a factor of twenty dilution in the detection cell, after mixing with other reagents, the flavin concentration is in sufficient molar excess for in-cell aldehyde substrate determinations upward to low micromolar concentrations. In aqueous
solution, micromolar concentrations of long chain aldehydes approach the saturation limit (8).

To identify "optimum" peak signal conditions, flow rates for the dithionite, luciferase, buffer and carrier stream solutions were varied throughout the range of each dedicated Gilmont meter. In general, three measurements were made at each flow condition. As each flow was adjusted and an "optimum" condition deduced, this parameter value became fixed and was used in subsequent flow optimizations. Given the scope of this research, the univariate method presented here was followed for practical purposes and does not lead necessarily to global optimization of the chemiluminescence signal.

Aldehyde calibration curves. As indicated previously, aldehyde primary stock was prepared in DMF. Serial dilutions of this stock in DMF extended the aldehyde concentration to the nanomolar range. Subsequent dilutions in water reduced this level further to picomolar concentrations. Both calibration curve abscissas (moles of aldehyde) and detection limit determinations are based on calculations involving the 0.565-mL sample loop.

RESULTS AND DISCUSSION

Discrete Sampling Studies

A calibration curve for cis-11-hexadecenal is presented in Figure 4.1. The fit to the data has slope 1.02, indicating linearity. The detection limit (DL), calculated as the concentration yielding an analytical signal equal to
Figure 4.1. Analytical Calibration Curve for Cis-11-hexadecenal Obtained with Discrete Sampling Apparatus. The linear least squares fit to the data is from 0.69 pmol to 1.4 nmol.
twice the standard deviation in the blank signal, is 0.74 pmol. A blank signal equivalent to 6.1 pmol of aldehyde was observed. Since this substrate is ideal for determinations with bacteria bioluminescence (1, 9), the DL presented here may be compared with the successful detection of 100 fmol of aldehyde substrate reported in the literature (1, 2). Peak profiles reproduced from the strip chart and representative of aldehyde quantities at levels both near the detection limit and in the upper linear range are presented in Figure 4.2. The kinetics of the bioluminescence reaction are rapid as the signal returns to the baseline within about 5 s after injection. The duration of the peak is limited somewhat by the time for injection and the mixing rate.

**Aqueous Aldehyde Solution Stability** Results for the aldehyde solution stability studies are shown in Figure 4.3. To better illustrate the general trends, the data for each experiment were averaged over ten minute intervals. The curve fit to the data of EXP 1 indicates that the chemiluminescence signal is attenuated significantly within 20 min of the preparation time. The log of the chemiluminescence signal is linear as a function of time, indicating that the aldehyde loss follows first order kinetics. This trend could be explained by either oxidative or volatile processes, or both. However, comparison of this curve with those of the two other experiments indicates that the aldehyde losses are due, primarily, to volatility.

The linear fits in the data of EXPs 2 and 3 illustrate aqueous aldehyde stability with respect to oxidation, since volatile losses were limited. The slight negative slopes of these curves, -0.0012 and -0.0010 nA/min, respectively, may be due to aldehyde oxidation proceeding at a slow rate. However, if this is the case, the rate does not appear to be influenced significantly by the presence of dissolved oxygen. This gas was present in the solutions of EXP 2, but was excluded from the solutions of EXP 3. The
Figure 4.2. Peaks Obtained with Cis-11-hexadecenal and the Discrete Sampling Photometer. (A) Signals obtained with 1.4 pmol of aldehyde, a gain of $10^9$ V/A and 50 mV/in (ordinate). (B) Signals obtained with 340 pmol, a gain of $10^7$ V/A and 10 mV/in. The chart speed (abscissa) was 1 in/min in each case. Half-widths for both sets of peaks are 1.9 s.
Figure 4.3. Results of the Aqueous Aldehyde Solution Stability Studies. (●) EXP 1: open vessel, volatility / oxidation experiment. (○) EXP 2: closed vessel with water solvent sparged with air for 20 min. (▲) EXP 3: closed vessel with water solvent sparged with helium for 20 min.
initial difference in the analytical signal for these curves, extrapolated to time \( t = 0 \), could be explained by imprecision in the solution preparations. Differences in slopes may be due to volatile losses possible when each flask used in EXP 2 was opened and samples were withdrawn over several minutes of time.

With regard to proper aqueous aldehyde solution preparation and handling, it is evident from these experiments that the procedure followed by previous researchers (1, 2) is adequate. In EXP 2, analytical signals measured 20 min after the aldehyde sample preparation were within 97% of the initial value extrapolated along the curve to time, \( t = 0 \). Results presented here may be compared with those of a similar study with decanal (8).

Flow Injection Studies

Bacteria Bioluminescence Assay Optimization. Results for the assay optimization studies are shown in Figures 4.4-4.10 and Figures 4.12-4.13. In interpreting the data, several points should be considered. First, the observed chemiluminescence signal depends on the kinetics of the reaction and the mixing efficiency. Any factor affecting the rate, such as a change in the in-cell concentrations of the reactants, will influence the maximum intensity. Second, the amount of light collected by the detector and the peak duration depend on both the time to deliver the sample into the cell \( (t_d) \) and the residence time \( (t_r) \) of the sample in the cell. If the mixing efficiency is poor or the kinetics are relatively slow, unreacted aldehyde will exit the volume element viewed by the detector and only a fraction of the photons emitted will be observed. Although the diameter of the detection cell is 50 mm and was designed for use with a variety of PMTs, the diameter of the photocathode used in these studies is 23.1 mm. Thus, as a first approxima-
tion, the effective cell volume is 0.17 cm$^3$. The sample loop volume is 0.565 cm$^3$. Therefore, the minimum total time ($t_t$) for the aldehyde plug to pass into and through the detection cell is given by Equation 4.3, where $F_i$ is the flow rate through the injector inner conduit and $F_t$ is the total flow rate. Due to dispersion of the aldehyde plug before it reaches the detection cell, the actual total time is expected to be greater than the calculated value.

When the flow rate for a given reagent is increased, the in-cell concentration of the reagent increases, in proportion to the reagent flow rate divided by $F_t$. Simultaneously, the in-cell concentrations of the other reagents decrease, the residence time of the aldehyde decreases, and the mixing efficiency may change.

**Carrier stream optimization.** The H376 meter, used for the carrier stream, has the greatest flow range. Since a wide variation in the carrier flow rate might have a significant affect on the reaction kinetics, mixing efficiency or total observation time, this parameter was considered first in the optimization sequence. For this experiment, nominal flow rates for the other reagent solutions were set near mid-scale on the respective flow meters. Results for this trial are presented in Figure 4.4. As indicated by the curve fit to the chemiluminescence peak heights, the signal increases steadily with increasing flow rate. This trend continues to the maximum flow allowed by either the pressure drive to the carrier or the flow impedance through the injector. Given this constraint, a nominal rate of 8.5 mL / min (scale = 34.5) was adopted for the carrier. With the meter valve not quite fully open, this setting

$$t_t = t_d + t_r = \frac{0.565 \text{ cm}^3}{F_i} + \frac{0.170 \text{ cm}^3}{F_t} \quad (4.3)$$
Figure 4.4. Carrier Solution Flow Rate Optimization.

<table>
<thead>
<tr>
<th>reagent</th>
<th>meter</th>
<th>scale setting</th>
<th>(mL/min)</th>
<th>conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>carrier</td>
<td>H376</td>
<td>variable</td>
<td>variable</td>
<td>100 µL DMF / 500 mL H₂O</td>
</tr>
<tr>
<td>dithionite</td>
<td>A5403</td>
<td>60.0</td>
<td>1.70</td>
<td>1.00 mg / mL</td>
</tr>
<tr>
<td>luciferase</td>
<td>A7972</td>
<td>50.0</td>
<td>1.23</td>
<td>0.10 mg / mL</td>
</tr>
<tr>
<td>buffer</td>
<td>A7984</td>
<td>50.0</td>
<td>1.22</td>
<td>50 mM, pH = 7.0</td>
</tr>
<tr>
<td>FMN</td>
<td>A7967</td>
<td>50.0</td>
<td>1.22</td>
<td>500 µM</td>
</tr>
</tbody>
</table>

aldehyde = 2.18 nM cis-11-hexadecenal; 500 µL DMF in 500 mL H₂O
air temp. = 18.0 C sample loop = 0.565 mL; fill / flush = 30 s.
allowed minor adjustment near the maximum flow rate.

Over the flow range studied, the peak chemiluminescence signal increases by a factor of 5.1. The in-cell aldehyde concentration increases by a factor of 7.2, whereas the luciferase, dithionite and flavin concentrations are decreased by a factor of 0.42. Thus, the observed trend appears to be due primarily to the increased in-cell aldehyde concentration.

Peaks reproduced from the strip chart and representative of the extreme high and low carrier flow rates are shown in Figure 4.5. The peaks recorded with the high flow rate are flash-like; whereas, the signal profiles recorded at the low flow rate are characterized by relatively flat crests. The corresponding peak half widths are 4.8 and 37 s. These values may be compared with both the respective minimum total times (4.7 and 70 s) and delivery times (4.0 and 68 s), calculated from Equation 4.3. Therefore, the peak duration is influenced primarily by the time interval of aldehyde delivery within the cell. Flash-like peak profiles are recorded at higher flow rates because the more transient and higher in-cell aldehyde concentrations yield more photons per second.

If the light collection and mixing efficiencies were similar for the peaks recorded in Figures 4.5 A and B, then the ratio of the peak areas should approximate unity. An adequate comparison of the two areas cannot be presented from data collected by the computer, since the software was written for a 6-s integration time. Calculations based on the product of the peak heights and half-widths are misleading because the peak shapes are quite different. The peak areas were compared by photocopying the strip chart, cutting the profiles from the paper, and then weighing the paper profiles on an analytical balance. Respectively, the weights recorded for the signals reproduced at the high and low carrier flow rates were 17.9 and 16.7 mg or, roughly, 1 : 1. These data suggest that the mixing efficiencies were similar at all of the carrier stream flow rates and that the chemiluminescence
Figure 4.5. Peak Profiles Obtained from the Carrier Optimization Strip Chart. Carrier flow rates: (A) 8.5 mL/min; (B) 0.5 mL/min. The chart speed (abscissa) was 1 in/min. Both sets of peaks were recorded with a gain of $10^9$ V/A and 50 mV/in (ordinate).
reaction kinetics are fast relative to the aldehyde sample plug residence times. At the highest flow rate $t_r$ is 0.74 s, indicating that the majority of the chemiluminescence reaction is complete by the time that the sample plug has passed from the volume element viewed by the PMT photocathode.

**Buffer optimization.** Results for the buffer optimization study are shown in Figure 4.6. The inverse relation between the signal and flow rate suggests that this solution is not required for optimum signal conditions and, in subsequent flow studies, the buffer flow was set to zero. As the buffer solution flow rate was decreased through the range illustrated, both the in-cell reactant concentrations and the residence time increased by a factor of 1.3. The total time increased slightly, by a factor of 1.03. Concurrently, the signal increased by a factor of 2.4 and the peak half-widths decreased slightly, from 4.8 to 4.2 s. Peak areas increased by a factor of 2.2, as determined by the weighing of signal profiles reproduced from the strip chart. This ratio is in good agreement with the increase in signal intensity. These data suggest that the increase in signal is not related solely to an increased in-cell aldehyde concentration; rather, the quantum efficiency of the bioluminescence reaction appears to increase with higher in-cell reagent concentrations.

**Luciferase optimization.** Results for the enzyme solution flow experiment are presented in Figure 4.7. Relative to previous flow rate optimization studies, the enzyme solution concentration was increased by a factor of five to allow a broader range of in-cell concentrations for this reagent. Significant scatter about the linear fit to the plot suggests that the precision of the results is related closely to the amount of luciferase delivered to the cell. Similar observations are reported in discrete sampling studies (9-11). Thus, in the plot, the scatter may be related to the reproducibility of the luciferase flow rates.
Figure 4.6. Buffer Solution Flow Rate Optimization.

<table>
<thead>
<tr>
<th>reagent</th>
<th>meter</th>
<th>scale setting</th>
<th>(mL/min)</th>
<th>conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>carrier</td>
<td>H376</td>
<td>34.0</td>
<td>8.2</td>
<td>100 µL DMF / 500 mL H₂O</td>
</tr>
<tr>
<td>dithionite</td>
<td>A5403</td>
<td>60.0</td>
<td>1.70</td>
<td>1.00 mg / mL</td>
</tr>
<tr>
<td>luciferase</td>
<td>A7972</td>
<td>50.0</td>
<td>1.23</td>
<td>0.10 mg / mL</td>
</tr>
<tr>
<td>buffer</td>
<td>A7984</td>
<td>variable</td>
<td>variable</td>
<td>50 mM, pH = 7.0</td>
</tr>
<tr>
<td>FMN</td>
<td>A7967</td>
<td>50.0</td>
<td>1.22</td>
<td>500 µM</td>
</tr>
</tbody>
</table>

aldehyde = 2.18 nM cis-11-hexadecenal; 500 µL DMF in 500 mL H₂O
air temp. = 19.8 C sample loop = 0.565 mL; fill / flush = 30 s.
Figure 4.7. Luciferase (*V. harveyi*) Flow Rate Optimization.

<table>
<thead>
<tr>
<th>reagent</th>
<th>meter</th>
<th>scale setting</th>
<th>(ml/min)</th>
<th>conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>carrier</td>
<td>H376</td>
<td>35.0</td>
<td>8.7</td>
<td>100 µL DMF / 500 mL H₂O</td>
</tr>
<tr>
<td>dithionite</td>
<td>A5403</td>
<td>60.0</td>
<td>1.70</td>
<td>1.00 mg / mL</td>
</tr>
<tr>
<td>luciferase</td>
<td>A7972</td>
<td>variable</td>
<td>variable</td>
<td>0.50 mg / mL</td>
</tr>
<tr>
<td>FMN</td>
<td>A7967</td>
<td>50.0</td>
<td>1.22</td>
<td>500 µM</td>
</tr>
</tbody>
</table>

aldehyde = 2.18 nM cis-11-hexadecenal; 500 µL DMF in 500 mL H₂O

air temp. = 19.4 C    sample loop = 0.565 mL; fill / flush = 30 s.
In Figure 4.7 the chemiluminescence signal increases by a factor of 13.6 as the in-cell enzyme concentration ratio increases by a factor of 140. The concurrent in-cell aldehyde concentration decreases by a factor of 0.75. Clearly, the signal dependence is not first order with respect to the aldehyde concentration and the quantum efficiency of the reaction appears to increase with increasing amounts of enzyme entering the cell. A possible explanation for this trend is the reported enhanced stability of the enzyme-bound reduced flavin hydroperoxide intermediate with high luciferase concentrations (12). This intermediate decays via a low quantum yield path from the time of its formation. The maximum initial light intensity achieved with the addition of the aldehyde substrate is related directly to the intermediate concentration. Since the hydroperoxide intermediate is formed by the mixture of the FMN, dithionite and luciferase solutions (see Figure 3.2), increased chemiluminescence signal is expected with both the more rapid transport of the intermediate to the detection cell and increased enzyme concentration. Generally, these effects are more difficult to observe with discrete sampling apparatus since the enzyme concentration is fixed and the intermediate is formed in the detection cell just prior to the injection of the aldehyde sample.

The nonlinear relationship between the chemiluminescence signal and the in-cell enzyme concentration accounts for difficulties in relating the signal dependence to changing in-cell aldehyde concentrations in the previous flow rate optimizations. Apparently, the relationship between the signal and aldehyde concentration is first order for a fixed enzyme concentration. Presumably, this relationship applies to the other substrates when each substrate is reaction limiting.

As the luciferase flow rate was increased throughout the range illustrated, peak half-widths were relatively constant (4.8 s). Peak areas increased by a factor of 13.4 and are in good agreement with the increase in peak signal intensities. These data support the hypothesis that most of the
chemiluminescence occurs during the cell residence time.

In Figure 4.7, the chemiluminescence signal rises rapidly to about 1.0 mL/ min; thereafter, the signal doubles for a quadrupled increase in the flow rate. Because the enzyme is expensive ($400/ g) and since the increase in signal response slows appreciably above the 1.00 mL/ min flow rate, this flow condition was accepted as a compromise "optimum" value.

**Reductant optimization.** Flow optimization with dithionite was conducted with the previously established flow conditions for FMN, the carrier and luciferase. Although significant scatter is present at lower flow rates (Figure 4.8), it is clear that the signal is not a strong function of the reductant delivery rate over the range tested. Optimum signal occurs at about 0.64 mL/ min, at a scale reading of 35 on the meter. At higher flow rates the shape of the curve may be influenced by dilution of other in-cell reagent concentrations (e.g., aldehyde or enzyme) or a loss of dissolved oxygen needed to saturate the reduced flavin-enzyme complex. Signal attenuation at lower flow rates might be related to decreased in-cell concentrations of FMNH₂.

To clarify these ambiguities, the dithionite concentration was varied at the optimum flow rate suggested by Figure 4.8. A slightly higher signal was obtained at a 1.25 mg/mL concentration (Figure 4.9), suggesting that oxygen substrate had not been depleted in the previous experiment. The optimum signal occurs at a dithionite to FMN ratio of 7.5 (mol/mol). This result is supported by the maximum signal achieved with a reagent ratio of 7.9 (mol/mol) using dithionite solution and the photometer described by Hoyt (5) (Figure 4.10). In another discrete sampling study (13), constant chemiluminescence response was observed with the addition of 0.2 to 1.0 mg of solid sodium dithionite to 0.05 μmol FMN in the detection cell. The equivalent reductant to FMN ratios range between 23 and 115 (mol/mol). These ratios are not expected to illustrate simple stoichiometry since dithionite also
Figure 4.8. Dithionite Solution Flow Rate Optimization.
Figure 4.9. Dithionite Concentration Optimization.

<table>
<thead>
<tr>
<th>reagent</th>
<th>meter</th>
<th>scale setting</th>
<th>(mL/min)</th>
<th>conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>carrier</td>
<td>H376</td>
<td>31.5 (max.)</td>
<td>7.0</td>
<td>100 µL DMF / 500 mL H₂O</td>
</tr>
<tr>
<td>dithionite</td>
<td>A5403</td>
<td>35.0</td>
<td>0.64</td>
<td>variable: 0.5 - 1.5 mg / mL</td>
</tr>
<tr>
<td>luciferase</td>
<td>A7972</td>
<td>45.0</td>
<td>1.00</td>
<td>0.50 mg / mL</td>
</tr>
<tr>
<td>FMN</td>
<td>A7967</td>
<td>50.0</td>
<td>1.22</td>
<td>500 µM</td>
</tr>
</tbody>
</table>

aldehyde = 2.18 nM cis-11-hexadecenal; 500 µL DMF in 500 mL H₂O

air temp. = 18.2 ºC sample loop = 0.565 mL; fill / flush = 30 s.
Figure 4.10. Optimization of the Reductant with Discrete Sampling Apparatus. Additions to the detection cell: 10 μL of 5.0 mM FMN (50 nmol); 25 μL of 0.5 mg / mL *V. harveyi* luciferase; 1000 μL buffer solution (1.0 mM hydroxylamine, 50 mM β-mercaptoethanol and 63 mM phosphate buffer (pH 7.0)). Sodium dithionite in phosphate buffer (pH 7.0) was added to the reaction cuvette in quantities ranging from 10 μL of 0.2 mg / mL (57 nmol) to 25 μL of 100 g / mL (14 μmol). The bioluminescence reaction was initiated by the rapid injection of 0.996 mL of 6.9 nM cis-11-hexadecenal aqueous solution. Optimum was obtained with the addition of 69 μg (ca. 400 nmol) of dithionite, at a reductant to FMN ratio of 7.9.
reduces dissolved oxygen present in the prepared chemiluminescence solutions. Excess dithionite is required to maintain the flavin in the reduced state prior to the initiation of the reaction since significant overlap occurs between the bioluminescence emission spectrum and the absorption spectrum of FMN (11).

**Phosphate concentration optimization.** Since other research (11) with *V. fischeri* luciferase indicates a significant signal dependence on phosphate concentration (Figure 4.11), this parameter was addressed in the dithionite solution preparation (Figure 4.12). Because the total flow through the injector decreases the in-cell dithionite concentration by an order of magnitude, the phosphate concentration range of Figure 4.12 is matched to the region of most significant signal dependence in Figure 4.11. The chemiluminescence signal was found to vary little through a five fold dilution of the phosphate buffer prepared with the optimum reductant concentration. The curve indicates nearly constant *V. harveyi* luciferase activity. To summarize, 1.25 mg/mL dithionite in 1.50 M phosphate buffer was accepted both as an optimum condition and a convenience in the reductant solution preparation.

**Outer conduit flow rate optimization.** The total flow rate through the injector outer conduit is considered in Figure 4.13. Flows for the dithionite, enzyme and FMN solutions were either reduced or increased in proportion to the settings presented in Figure 4.12. Factors of 1/2, 1, 2 and 3 multiplied by the optimum, now standard, flow rates for these solutions were used in this experiment. Highest chemiluminescence intensity and the greatest peak areas were recorded at the standard and half standard flow rates. These trends may reflect either the increased in-cell aldehyde concentration or the increased sample residence time with the lower flow rates. An increase in the sample residence time may result in the collection of a greater portion of
Figure 4.11. Effect of Phosphate Buffer Concentration (M), pH 7.0, on Bacteria Bioluminescence with *V. fischeri* luciferase. From Stanley, P. E. *Anal. Biochem.* 1971, 39, 447.
Figure 4.12. Phosphate Optimization in the Dithionite Solution.

<table>
<thead>
<tr>
<th>reagent</th>
<th>meter</th>
<th>scale setting</th>
<th>(mL/min)</th>
<th>conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>carrier</td>
<td>H376</td>
<td>35.0</td>
<td>8.7</td>
<td>100 µL DMF / 500 mL H₂O</td>
</tr>
<tr>
<td>dithionite</td>
<td>A5403</td>
<td>35.0</td>
<td>0.64</td>
<td>1.25 mg / mL</td>
</tr>
<tr>
<td>luciferase</td>
<td>A7972</td>
<td>45.0</td>
<td>1.00</td>
<td>0.50 mg / mL</td>
</tr>
<tr>
<td>FMN</td>
<td>A7967</td>
<td>45.0</td>
<td>1.00</td>
<td>508 µM</td>
</tr>
</tbody>
</table>

aldehyde = 2.18 nM cis-11-hexadecenal; 500 µL DMF in 500 mL H₂O

air temp. = 17.3 C sample loop = 0.565 mL; fill / flush = 30 s.
<table>
<thead>
<tr>
<th>reagent</th>
<th>meter</th>
<th>scale setting</th>
<th>(ml/min)</th>
<th>conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>carrier</td>
<td>H376</td>
<td>35.0</td>
<td>8.7</td>
<td>100 µL DMF / 500 mL H₂O</td>
</tr>
<tr>
<td>dithionite</td>
<td>A5403</td>
<td>variable</td>
<td>variable</td>
<td>1.25 mg / mL</td>
</tr>
<tr>
<td>luciferase</td>
<td>A7972</td>
<td>variable</td>
<td>variable</td>
<td>0.50 mg / mL</td>
</tr>
<tr>
<td>FMN</td>
<td>A7967</td>
<td>variable</td>
<td>variable</td>
<td>500 µM</td>
</tr>
</tbody>
</table>

aldehyde = 2.18 nM cis-11-hexadecenal; 250 µL DMF in 500 mL H₂O

air temp. = 18.3 C

sample loop = 0.565 mL; fill / flush = 30 s.

Figure 4.13. Injector Outer Conduit Flow Optimization.
the total light yield should the chemiluminescence reaction continue beyond
the zone viewed by the detector. The half standard set of parameters is pre-
ferred since these flow values economize further on reagent consumption.

The optimum flow conditions and reagent concentrations, deduced from the experiments above, are reviewed in Table 4.2.

Aldehyde Calibration Curves  The optimum conditions specified in Table 4.2 were used to develop calibration curves for aldehyde substrates. Two of these, cis-11-hexadecenal and undecanal are pheromone attractants for the artichoke plume (*Platyptilia carduidactyla*) and greater wax (*Galleria mellona*) moths, respectively. Other species in the order Lepidoptera use these molecules as pheromone components (14). A calibration curve for heptanal is presented to illustrate reduced detectability with this shorter carbon chain, less ideal, substrate.

A calibration curve for cis-11-hexadecenal is shown in Figure 4.14. The fit to the data has a slope of 0.92, indicating that the chemiluminescence signal is nearly linear with aldehyde substrate in the femto- and picomole range. The negative deviation above ca. 200 pmol of aldehyde substrate suggests a limiting factor. Relatively constant chemiluminescence signal response at high aldehyde concentrations has been reported previously (2) and has been attributed to saturation of the enzyme binding sites. From a comparison of reagent concentrations in the detection cell (Table 4.3), it is evident that the luciferase concentration and restricted turnover become reaction limiting as the amount of aldehyde exceeds 200 pmol. Possibly, the useful calibration range could be extended by increasing the enzyme concentration in the detection cell.

Flattening of the curve with atto- and femtomole substrate levels suggests that the kinetics of the reaction have changed with respect to a first-
Table 4.2. Optimum Flow Rates and Reagent Concentrations for Bioluminescence Analyses with the Flow Injection Apparatus\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Gilmont Meter</th>
<th>Scale Setting</th>
<th>Flow Rate (mL / min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>carrier</td>
<td>100 µL DMF in 500 mL H$_2$O</td>
<td>H376</td>
<td>35.0</td>
<td>8.5</td>
</tr>
<tr>
<td>dithionite</td>
<td>1.25 mg / mL</td>
<td>A5403</td>
<td>25.0</td>
<td>0.33</td>
</tr>
<tr>
<td>luciferase</td>
<td>0.50 mg / mL</td>
<td>A7972</td>
<td>30.0</td>
<td>0.50</td>
</tr>
<tr>
<td>FMN</td>
<td>500 µM</td>
<td>A7967</td>
<td>30.0</td>
<td>0.50</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Determined with cis-11-hexadecenal substrate.
Figure 4.14. Cis-11-hexadecenal Calibration Curve. The linear least squares fit to the data is from 60 fmol to 0.60 nmol.
Table 4.3. Comparison of Reagent Concentrations in the Detection Cell.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Prepared Conc. (µM)</th>
<th>Detection Cell Dilution Factor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Detection Cell Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>luciferase</td>
<td>6.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05</td>
<td>0.31</td>
</tr>
<tr>
<td>FMN</td>
<td>500</td>
<td>0.05</td>
<td>25</td>
</tr>
<tr>
<td>aldehyde</td>
<td>0.40</td>
<td>0.87</td>
<td>0.35&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>dithionite</td>
<td>7180</td>
<td>0.05</td>
<td>359</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reagent flow rate divided by the total flow rate through the injector.

<sup>b</sup> Based on a 0.50 mg/mL solution preparation and 80,000 Daltons.

<sup>c</sup> Corresponds to a 226 pmole sample plug with a 0.565-mL sample loop.
order dependence on the aldehyde concentration. Other researchers (12) have reported that second-order kinetics occur with extreme dilution, and that the chemiluminescence signal becomes dependent on the concentrations of the enzyme, substrates and, possibly, transient intermediates.

In the non-linear region of the calibration curve theoretical and practical detection limits may differ. With analytical curves of this type, the practical detection limit is preferred since this method yields more accurate analyte determinations at limiting signal-to-noise ratios (15). In Figure 4.14 it is not possible to distinguish between chemiluminescence signals for substrates at attomole levels. Practically, these signals are blanks. Ten averaged peak intensities for sample sizes of 150 to 600 amol yield a mean, extrapolated blank signal \( S'_{bk} \) of 11.7 pA. The standard deviation \( sbk \) of these signals is 1.2 pA. The practical detection limit is calculated from Equation 4.4 in

\[
SDL = S'_{bk} + ksbk = 14.1 \text{ pA}
\]  

(4.4)

which the confidence factor, \( k \), is 2. A signal of this magnitude corresponds to 11 fmol of substrate.

Additional evidence supporting this calculation is illustrated in Figure 4.15, a calibration curve emphasizing the DL region. Data sets plotted for the two lowest substrate levels correspond to 2 and 20 femtomoles of aldehyde.

Calibration curves for undecenal and heptanal are illustrated in Figures 4.16 and 4.17. Theoretical detection limits and the linear range determined for both of these substrates and cis-11-hexadecenal are reviewed in Table 4.4. Practical detection limits are presented in Table 4.5.

The results tabulated in Tables 4.4 and 4.5 may be compared to values
Figure 4.15. Cis-11-hexadecenal Calibration Curve Emphasizing the Region of the Detection Limit. For this experiment the dark current signal, dark current noise and background signals were 74, 0.3 and 21 pA, respectively.
Figure 4.16. Undecanal Calibration Curve. The slope of the linear least squares fit is 0.98 for data from 5.3 pmol to 5.3 nmol.
Figure 4.17. Heptanal Calibration Curve. The slope of the linear least squares fit is 0.84 for data from 80 pmol to 80 nmol.
Table 4.4. Linear Ranges and Theoretical Detection Limits (DLs) of Aldehyde Substrates Determined with Bacteria Bioluminescence.

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Linear Range&lt;sup&gt;a&lt;/sup&gt; (log fmol)</th>
<th>Baseline Std. Dev. (pA)</th>
<th>Slope (pA / pmol)</th>
<th>Theoretical DL&lt;sup&gt;b&lt;/sup&gt; (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-11-hexadecenal</td>
<td>2.0 -- 5.7</td>
<td>1.3</td>
<td>220</td>
<td>0.012</td>
</tr>
<tr>
<td>undecanal</td>
<td>3.5 -- 6.7</td>
<td>1.4</td>
<td>3.3</td>
<td>0.86</td>
</tr>
<tr>
<td>heptanal</td>
<td>5.5 -- 7.5</td>
<td>1.6</td>
<td>0.072</td>
<td>44</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimated from the calibration curves.

<sup>b</sup> Calculated from $2s_{bk} / m$; where the slope, m, is measured in the most linear region of the calibration curve. The standard deviation in the baseline, $s_{bk}$, was measured with the most sensitive amplifier gain used to collect data.
Table 4.5. Practical Detection Limits (DLs)\textsuperscript{a} of Aldehyde Substrates Determined with Bacteria Bioluminescence.

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Baseline Std. Dev. ($\text{pA}$)</th>
<th>Linear Fit Slope ($\text{pA/}\text{pmol}$)</th>
<th>DL (pmol)</th>
<th>Second Order Fit Slope ($\text{pA/}\text{pmol}$)</th>
<th>DL (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-11-hexadecenal</td>
<td>1.3</td>
<td>340</td>
<td>0.007</td>
<td>330</td>
<td>0.007</td>
</tr>
<tr>
<td>undecanal</td>
<td>1.4</td>
<td>5.2</td>
<td>0.54</td>
<td>4.9</td>
<td>0.57</td>
</tr>
<tr>
<td>heptanal</td>
<td>1.6</td>
<td>0.050</td>
<td>62</td>
<td>0.048</td>
<td>65</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Calculated from $2s_{bk}/m$, where values of the slope, $m$, were obtained from both linear and second order least squares fits to selected data points. For these fits only the data from the two or three most dilute aldehyde samples yielding baseline resolved chemiluminescence signals were used. Bacteria bioluminescence reactions with very dilute aldehyde substrate solutions are expected to follow second order kinetics (124).

Equations obtained from linear least squares fits yield slope values directly. With second order fits, the equations are of the form:

$$S_x = ax^2 + bx + c$$

where $S_x$ is the chemiluminescence signal obtained with $x$ moles of aldehyde. The derivative of this equation, given below, may be used to

$$S'_x = 2ax + b = m_x$$

calculate the slope ($m_x$) of the calibration curve near the detection limit for suitable values of $x$. The value of $x$ was selected by choosing the aldehyde datum (moles) closest to the the detection limit obtained from $2s_{bk}/m$, with $m$ equal to the slope value obtained from the linear least squares fit. The standard deviation in the baseline, $s_{bk}$, was measured with the most sensitive amplifier gain used to collect data.
presented in the literature. Meighen, Slessor and Grant (2) report the determination of 100 fmol of trans-11-tetradecenal in 1 mL of sample solution. This aldehyde substrate is considered ideal for use in bacteria bioluminescence assays. The signal response was linear from the detection limit to 3 nmol. Discrete sampling and V. harveyi luciferase were used in this determination.

With flow injection apparatus the calibration curve for cis-11-hexadecenal is linear from about 100 fmol to 0.5 nmol. As little as 7 fmol of the substrate was detected. Similar bioluminescence response is expected for this aldehyde and trans-11-tetradecenal (2). The lower detection limit achieved with the flow injection apparatus may be related to an improved light collection efficiency, due to both the geometry of the detection cell and additional optimization of the chemistry involved. Probably, the signal is enhanced by both the collection of light from a thin, disk-like solution lamina and the elimination of the buffer solution from the reaction medium.

With reference to Table 4.4, it is evident that heptanal is a poor substrate for use with bacteria bioluminescence. The detection limit is considerably higher and the linear range is more restricted relative to the other values listed. The results in the table support the general conclusion that aliphatic aldehydes of 12 to 18 carbons are more suitable for use with this assay (1, 2, 8). However, with flow injection, femtomole quantities of undecanal were detected. The linear range for this substrate is broad, 3 to 4 orders of magnitude, and well suited to analytical determinations. In a study involving V. fischeri luciferase, reported $K_m$ (Michaelis constant) values for undecanal and tridecanal were similar (140 and 120 µM, respectively) and lower than those of other aliphatic aldehydes with 9 to 13 carbon chain lengths (16).
CONCLUSIONS

The flow injection apparatus and detection cell described in Chapter 3 were used to optimize the chemistry of bacteria bioluminescence. For analysis, the maximum initial light intensity was related to the concentration of the aldehyde substrate. Levels of aldehyde to 7 fmol were detected, a significant improvement relative to measurements achieved with both the photometer described by Hoyt (5) (740 fmol) and values reported in the literature (1, 2) (100 fmol). The prototype flow injection apparatus combines convenience of operation with a method of analysis that is both rapid and highly sensitive.
REFERENCES


CHAPTER 5

EPOXIDE DETERMINATIONS

OVERVIEW

Bacteria bioluminescence and the flow injection apparatus described previously (Chapters 3 and 4) were used to detect ultra-trace quantities of aliphatic epoxides. A convenient method to convert cis-7,8-epoxy-2-methylloctadecane (disparlure) to undecanal and heptanal is presented first. The kinetics of the conversion are studied for this species and three homologs: 1,2-epoxyhexadecane, 1,2-epoxytetradecane and 1,2-epoxyoctane. Subsequently, calibration data and detection limits are presented for the four epoxides.

BACKGROUND

Analytical applications of bacteria bioluminescence are extended significantly by linkage to reactions that produce one of the substrates. Often, this is accomplished by coupling reactions of pyridine nucleotides to the formation of FMNH₂ (1, 2). Because biochemical reactions involving these nucleotides are common, numerous additional enzymes and substrates may be determined (1, 3, 4). Similarly, reactions that produce aldehydes may be linked to bacteria bioluminescence. This approach permits determinations of both the reactants and enzymes involved in conversions of long chain fatty acids, alcohols and esters (5, 6). Convenient, high-yield derivatizations of other compounds to aldehydes may extend further the range of analyses possible with this method.
Aldehyde Products Derived from Epoxides

Epoxide Hydrolysis Vic-glycols may be prepared from epoxides by either acid or base catalyzed hydrolysis (Reaction 5.1).

\[
\begin{array}{c}
\text{O} \\
\text{C} - \text{C} \\
\mid \\
\text{H}_2\text{O} \\
\text{H}^+ \\
\text{or OH}^- \\
\end{array}
\rightarrow
\begin{array}{c}
\text{HO} \\
\text{C} - \text{C} \\
\mid \\
\text{OH} \\
\end{array}
\] (5.1)

In hot alkaline solution, diols are obtained from sterically hindered cyclic epoxides with 0.3 M KOH in 85% dimethylsulfoxide-water solvent. Under such conditions (6 hr at 100°C), diol yields of 60% from 1-phenylcyclohexane oxide have been reported (7). Faster reactions with greater yields may be achieved with aliphatic epoxides.

Mineral acids are used for epoxide hydrolysis. Usually perchloric acid is preferred, both for its strength and because the use of HCl or H₂SO₄ may lead to the formation of chlorohydrin or sulfate ester by-products (8). Often, reactions are carried out in aqueous acetone or dioxane to solvate organic substrates.

Oxidative Cleavage of Diols Oxidation of glycols to aldehydes is quantitative, or nearly so, by a variety of methods. For example, aldehydes are produced by anodic cleavage of 1,2-diols. The oxidation is effected at carbon electrodes dipping into a supporting electrolyte mixed in methanol solvent. Based on current measurements, carbonyl compound yields as
high as 96% have been reported (8).

Commonly, in an organic solvent (benzene, dimethyl sulfoxide, pyridine, acetic acid, dioxane, acetonitrile, or tetrahydrofuran), lead tetraacetate is used for oxidative cleavage of glycols (Figure 5.1 A). The slow addition of lead tetraacetate to the reaction mixture preserves selectivity, since products susceptible to further oxidation do not encounter excess oxidant (10). With moisture, lead tetraacetate is hydrolyzed to the hydroxide; also, reactions involving this oxidant are inhibited by oxygen and phenols. Therefore, highest aldehyde yields are achieved with pure, dry solvents and with reaction mixtures degassed with nitrogen (11, 12).

In aqueous solution, oxidation of glycols is usually carried out with periodate (Figure 5.1 B). Common sources of this anion include sodium and potassium metaperiodate and paraperiodic acid (H₅IO₆). Of these, the sodium salt (NaIO₄) is preferred, both for its solubility and because it can be obtained in high purity. Normally, as a precaution against loss of the oxidant specificity, periodate oxidations are conducted at ambient or sub-ambient temperatures. In a determination of ethylene diol, quantitative oxidation to formaldehyde was achieved in 1 hr at room temperature (13).

**EXPERIMENTAL**

**Reagents**

Bacteria bioluminescence reagents and places of origin are identical to those presented previously for the determinations of aldehydes with the flow injection apparatus (Chapter 4). Dimethylformamide (DMF) and sodium metaperiodate were purchased from Sigma. Epoxide compounds were obtained from Aldrich. Reagent grade sulfuric acid was acquired from
Figure 5.1. Diol Oxidations to Aldehyde Products. (A) Reaction with Lead Tetraacetate; and (B) with Periodate.
Preliminary Nuclear Magnetic Resonance (NMR) Studies

Both acid catalyzed hydrolysis of racemic cis-7,8-epoxy-2-methyl-octadecane and oxidative cleavage of the diol products were attempted by the admixture of the necessary reactants in a 1.00-mL volumetric flask. Undecanal and heptanal were expected as major products of the reaction sequence. Both the addition order and amounts of reagents transferred to the flask are presented in Table 5.1. Mixing was continued for 1 hr at room temperature. Subsequently, a 1.00-mL sample of the reaction mixture was used to obtain a proton NMR spectrum.

Epoxide Derivatization Rates Monitored with Bacteria Bioluminescence

Bacteria bioluminescence and the flow injection apparatus were used to follow the rate of increase of aldehyde products derived from four epoxides: cis-7,8-epoxy-2-methyloctadecane, 1,2-epoxyhexadecane, 1,2-epoxytetradecane and 1,2-epoxyoctadecane. Bioluminescence reagent concentrations and flow rates were adjusted to previously determined conditions of optimum chemiluminescence response for cis-11-hexadecenal (Table 4.2). Sample reaction mixtures were prepared according to the listing in Table 5.2 and stirred continuously in 1.00-mL volumetric flasks at room temperature. At 15 min intervals, 50-μL aliquots pipetted from the flasks were added to 500 mL of water. These well mixed solutions were analyzed immediately.

Epoxide Calibration Curves

Reaction mixtures were prepared using the procedure and reactants given in Tables 5.1 and 5.2. Following 1 hr of combined acid hydrolysis and oxidation with periodate, 100-μL samples were pipeted from the reaction
Table 5.1. Reaction Mixture for the Derivatization of Disparlure to Aldehydes.

<table>
<thead>
<tr>
<th>Addition Order</th>
<th>Reagent</th>
<th>Vol. (µL)</th>
<th>Wt. (mg)</th>
<th>Quantity (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1000</td>
<td>--</td>
<td>13.7</td>
</tr>
<tr>
<td>2</td>
<td>disparlure&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
<td>107.8</td>
<td>0.370</td>
</tr>
<tr>
<td>3</td>
<td>conc. H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>100</td>
<td>--</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>NaI&lt;sub&gt;O&lt;/sub&gt;4</td>
<td>--</td>
<td>208.6</td>
<td>0.975</td>
</tr>
</tbody>
</table>

<sup>a</sup> This highly polar aprotic solvent was used to solvate both periodate and the aldehyde reaction products.

<sup>b</sup> cis-7,8-epoxy-2-methyloctadecane.
Table 5.2. Epoxide Derivatization Reaction Mixtures and Expected Products.

<table>
<thead>
<tr>
<th>Epoxide</th>
<th>Wt. (mg)</th>
<th>Purity (%)</th>
<th>Quantity (µmol)</th>
<th>Expected Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-7,8-epoxy-2-methyleneoctadecane (disparlure)</td>
<td>85.1</td>
<td>90</td>
<td>271</td>
<td>undecanal, 6-methylheptanal</td>
</tr>
<tr>
<td>1,2-epoxy-hexadecane</td>
<td>63.9</td>
<td>85</td>
<td>245</td>
<td>pentadecanal, formaldehyde</td>
</tr>
<tr>
<td>1,2-epoxy-tetradecane</td>
<td>76.1</td>
<td>85</td>
<td>305</td>
<td>tridecanal, formaldehyde</td>
</tr>
<tr>
<td>1,2-epoxy-octane</td>
<td>81.7</td>
<td>97</td>
<td>618</td>
<td>heptanal, formaldehyde</td>
</tr>
</tbody>
</table>

\(^{a}\) Reaction mixtures were prepared with 1000 µL DMF, 100 µL H\textsubscript{2}SO\textsubscript{4} (conc.), and 200 mg Na\textsubscript{2}O\textsubscript{4}, added in the order listed in Table 5.1.
flasks and added to 10.0 mL of DMF. Serial dilution of these solutions in DMF extended the equivalent unreacted epoxide concentrations into the nanomolar range. Aqueous dilutions of samples pipetted from the DMF solutions were analyzed immediately with bacteria bioluminescence.

RESULTS AND DISCUSSION

NMR Studies

Results of the preliminary proton NMR studies are shown in Figure 5.2. Unresolved doublets at δ2.6 (3H) and δ2.8 (3H) and the unresolved multiplet at δ7.8 (1H) may be assigned to the hydrogens of the DMF solvent. Aldehydic protons are evident in the peak at δ9.9. Comparison of this spectrum with a spectrum of disparlure in DMF (Figure 5.3) indicates that a significant portion of the epoxide was converted to aldehydes.

Conversion Kinetics

Plots of chemiluminescence intensity as a function of time are presented in Figures 5.4 and 5.5. Times of maximum luminescence response, deduced from the plots, indicate that the shorter carbon chain epoxides are oxidized more rapidly to aldehydes. Reduced signal with longer reaction times may be related to either side reactions involving aldehydes or volatile losses. The head space within the reaction vessel increases with the removal of each successive sample.

Since the aldehyde product yields are unknown, the information presented in Table 5.2 and Figures 5.4 and 5.5 cannot be used to deduce relative chemiluminescence quantum yields from aldehyde substrates of
Figure 5.2. Proton NMR Spectrum of Racemic cis-7,8-epoxy-2-methylloctadecane (disparlure) Derivatized to Aldehydes in DMF solvent. Spectrometer: Varian EM-60 (60 MHz).
Figure 5.3. Proton NMR Spectrum of Racemic cis-7,8-epoxy-2-methyloctadecane (disparlure) in DMF solvent. Spectrometer: Varian EM-60 (60 MHz).
Figure 5.4. Progress of Epoxide Derivatizations as Determined by Bacteria Bioluminescence. Substrates are 1,2-epoxyhexadecane and Racemic cis-7,8-epoxy-2-methyloctadecane (disparlure).
Figure 5.5. Progress of Epoxide Derivatizations as Determined by Bacteria Bioluminescence. Substrates are 1,2-epoxytetradecane and 1,2-epoxyoctane.
different chain lengths. Although the profile of the signal response for disparlure is additive for two aldehydes, undecanal and heptanal, greater light intensity is expected from an equal yield of pentadecanal from 1,2-epoxyhexadecane (Figure 5.6).

**Calibration Data**

Calibration curves relating the chemiluminescence signal to moles of epoxide are presented in Figures 5.7 and 5.8. Flattening of the curves at the highest epoxide levels may be related to saturation of the enzyme binding sites (Chapter 4).

Theoretical detection limits, slopes and linear ranges are reviewed in Table 5.3. Practical detection limits are presented in Table 5.4. The detection limits achieved with disparlure and 1,2-epoxyoctane may be compared with the results presented earlier for undecanal and heptanal (Tables 4.4 and 4.5). These aldehydes are the oxidation products expected to limit the luminescence signal response in the respective epoxide determinations. The practical first order DL for heptanal is lower than that of 1,2-epoxyoctane by a factor of 1.8. The corresponding DL for undecanal is lower than that of disparlure by a factor of 5.6. Comparing slopes from the most linear portions of the calibration curves, the slope for 1,2-epoxyoctane is about 19% of that for heptanal; that for disparlure is about 14% of the slope of undecanal. These results suggest that the aldehyde yields were in the range of 10-40% from the combined epoxide hydrolysis and oxidation reactions.
Figure 5.6. Relative Light Intensities as a Function of Aldehyde Chain Length for 100 pmol of the Saturated Aldehyde with *V. harveyi* (●) and *P. phosphoreum* (○) Luciferases (14).
Figure 5.7. Calibration Curves Determined with Bacteria Bioluminescence for 1,2-epoxyhexadecane and Racemic cis-7,8-epoxy-2-methyloctadecane (disparlure) Substrates Derivatized to Aldehydes. For data ranging from 2.5 to 250 pmol the slope of the least squares fit to the plot of 1,2-epoxyhexadecane is 1.00. The slope of the fit to the disparlure plot is 0.83 for data from 22 to 550 pmol.
Figure 5.8. Calibration Curves Determined with Bacteria Bioluminescence for 1,2-epoxytetradecane and 1,2-epoxyoctane Substrates Derivatized to Aldehydes. For data ranging from 3 pmol to 3 nmol the slope of the least squares fit to the plot of 1,2-epoxytetradecane is 0.96. The slope of the fit to 1,2-epoxyoctane is 0.55 for data from 460 to 46 nmol.
Table 5.3. Linear Ranges and Theoretical Detection Limits of Epoxide Substrates Oxidized to Aldehydes and Determined with Bacteria Bioluminescence.

<table>
<thead>
<tr>
<th>Epoxide</th>
<th>Linear Range&lt;sup&gt;a&lt;/sup&gt; (log fmol)</th>
<th>Baseline Std. Dev. (pA)</th>
<th>Slope (pA / pmol)</th>
<th>Theoretical DL&lt;sup&gt;b&lt;/sup&gt; (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-epoxy-hexadecane</td>
<td>3.0 − 6.0</td>
<td>4.3</td>
<td>400</td>
<td>0.02</td>
</tr>
<tr>
<td>1,2-epoxy-tetradecane</td>
<td>3.0 − 6.5</td>
<td>4.9</td>
<td>95</td>
<td>0.10</td>
</tr>
<tr>
<td>cis-7,8-epoxy-2-methyloctadecane (disparlure)</td>
<td>3.5 − 5.5</td>
<td>2.3</td>
<td>0.47</td>
<td>9.9</td>
</tr>
<tr>
<td>1,2-epoxy-octane</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.6</td>
<td>0.014</td>
<td>360</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimated from the calibration curves.

<sup>b</sup> Calculated from $2s_{bk} / m$; where the slope, $m$, is measured in the most linear region of the calibration curve. The standard deviation in the baseline, $s_{bk}$, is measured with the most sensitive amplifier gain used to collect data.

<sup>c</sup> Not determined. A linear range was not apparent from either the log-log or a linear scale plot of the data for this calibration curve.
Table 5.4. Practical Detection Limits (DLs) of Epoxide Substrates Oxidized to Aldehydes and Determined with Bacteria Bioluminescence.

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Baseline</th>
<th>Linear Fit</th>
<th>Second Order Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Std. Dev.</td>
<td>Slope (pA / pmol)</td>
<td>DLa (pmol)</td>
</tr>
<tr>
<td>1,2-epoxy-hexadecane</td>
<td>4.3</td>
<td>190</td>
<td>0.045</td>
</tr>
<tr>
<td>1,2-epoxy-tetradecane</td>
<td>4.9</td>
<td>190</td>
<td>0.050</td>
</tr>
<tr>
<td>disparlure</td>
<td>2.3</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td>1,2-epoxy-octane</td>
<td>2.6</td>
<td>0.046</td>
<td>110</td>
</tr>
</tbody>
</table>

a Calculated from \(2s_{bsk} / m\), where values of the slope, \(m\), were obtained from both linear and second order least squares fits to selected data points. Details of the methods used are identical to those presented in the caption of Table 4.5.
CONCLUSIONS

This research presents methodology developed for the determination of long chain aliphatic epoxides to pico- and femtomole levels. The determination of cis-7,8-epoxy-2-methyloctadecane to 3 pmol (0.85 ng) is of particular interest. This measurement, equivalent to 1 ng/mL of the gypsy moth sex attractant, may be compared with biological activity known to occur with as little as 100 pg/mL of (+) disparlure (15). In another study (16), male moths stationed at the effluent port of a gas chromatograph were activated by the injection of a 2 pg sample of synthetic pheromone.

In a multistep analysis, airborne levels of disparlure have been measured with GC-ECD (17). The pheromone is trapped by drawing air through a bed of molecular sieve adsorbent. Following extraction with 1:1 hexane-ether the recovered attractant is derivatized to a vic-dibromide with triphenylphosphine dibromide. Chromatographic clean-up of the product on activated Florisil yields the dibromo derivative in a hexane fraction. This fraction is then concentrated prior to the injection of an aliquot onto the analytical column. By this method, dilute levels of disparlure (0.2 ng/m³) have been measured in forest air.

The application of bacteria bioluminescence described above may reduce significantly the number of steps and labor involved in this type of analysis and may thereby prove useful to researchers concerned specifically with gypsy moth pest management. Bioluminescence may be used to determine both performance characteristics of disparlure dispensers, used in mass trapping and survey studies, and airborne levels of the natural attractant.

Only a small number of geographically well dispersed insect species use epoxide pheromones (18). In the analysis of complex pheromone
samples this circumstance is fortuitous and may be contrasted with the abundance of aldehyde attractants used by pest Lepidoptera. Lack of specificity in the determination of aldehyde components in multicomponent pheromone systems has been identified as a potential problem with the application of bacteria bioluminescence (14).

The success of the epoxide determinations with bacteria bioluminescence suggests that analytical use of this reaction may be extended further. Investigations of chemistries that yield suitable substrate are pivotal to this research. The approach leading to the epoxide determinations followed from reactions producing aldehydes. Other reactions that produce this substrate in high yield occur in the literature and may prove useful for specific applications.
REFERENCES


Ordinarily, blank signal limited analyte detectability is not improved through improved instrumentation alone. In these circumstances, further optimization of the chemistry involved may lower the detection limit, or better detectability may be achieved if the origin of the blank signal is determined and if this cause is either partially masked or eliminated. As illustrated in the discrete sampling experiments of Chapter 4, the determination of aldehyde substrate with bacteria bioluminescence is blank signal limited. The successful determinations of aliphatic aldehydes and epoxides to low femtomole quantities with bacteria bioluminescence (Chapters 4 and 5) suggested that detection limits achieved with previous chemiluminescence analyses may be lowered through the use of the apparatus described in Chapter 3. To this end, the application of lophine (2,4,5-triphenylimidazole) chemiluminescence to the determination of Cr(VI) in aqueous solution was re-investigated. This analyte is a known carcinogen and, therefore, the analysis of drinking water for Cr(VI) is an important analytical problem. Also, extensive in-house investigations of this problem with discrete sampling apparatus provide ample basis for comparisons and discussion.

In this chapter, discrete sampling results are presented first and are compared with results achieved with previous investigations. The discussion includes a study of the lifetime of an intermediate believed to be
involved in the chemiluminescence reaction. Subsequently, preliminary optimization studies with the flow injection system are described. These experiments led to a number of important modifications to the injector and flow injection system to accommodate harsh chemical conditions. Details of these modifications are discussed. Finally, optimization work and a calibration curve achieved with the more chemically inert flow injection system are presented.

BACKGROUND

The chemiluminescence reaction mechanism of lophine (Figure 6.1) has been characterized by White and Harding (1). Intermediate (II) is formed by both the attack of oxygen on the imidazole (I) and abstraction of the hydroperoxide proton in alkaline solution. The peroxide anion acts as a nucleophile, leading to the formation of the four-membered ring of the cyclic peroxyde (III). Hydrogen bonding at the nitrogen atoms to molecules of the solvent promotes rearrangement of the structure (IV) and leads to the excited singlet of the diaroylamidine (V). The proposed reaction scheme is supported by the observed chemiluminescence of several lophine derivatives and evidence that reactions of lophine peroxides with strong base yield light at the same wavelength maximum as the parent lophines. In ethanol, chemiluminescence maxima of several lophines and lophine peroxides occur between 485 and 530 nm. Relative to luminol ($\phi_{cl} = 0.01$), quantum yields range from about $10^{-7}$ to $10^{-3}$, depending on the derivative. The light yield is improved slightly with increased reaction temperatures (1).

Lophine-based chemiluminescence determinations of Cr(VI) were carried out previously by both Marino and Waddle (2, 3). In these studies, solutions of lophine, hydrogen peroxide and sample were added to a
R = phenyl

Figure 6.1. Lophine Chemiluminescence Reaction Mechanism
detection cell. The reaction was initiated by the rapid injection of strong base. However, no mechanism was proposed to account for the dependence of lophine chemiluminescence on the concentration of the chromium species. In the section that follows, such a mechanism is presented.

**Proposed Lophine-Cr(VI) Reaction Mechanism**

In acid solution, Cr(VI) species exists as two oxo-anions in equilibrium with one another. This is expressed in reactions 6.1 and 6.2 below (4).

\[
\begin{align*}
H^+ + CrO_4^{2-} & \rightleftharpoons HCrO_4^- \\
2HCrO_4^- & \rightleftharpoons Cr_2O_7^{2-} + H_2O
\end{align*}
\]

Both species are reduced by peroxide in acid solution (4, 5).

\[
\begin{align*}
2HCrO_4^- + 3H_2O_2 + 8H^+ & \rightarrow 2Cr^{3+} + 3O_2 + 8H_2O \\
Cr_2O_7^{2-} + 3H_2O_2 + 8H^+ & \rightarrow 2Cr^{3+} + 3O_2 + 7H_2O
\end{align*}
\]

However, reactions 6.3 and 6.4 represent net chemistry. Several inter-
mediate species may be formed depending on the reaction conditions (5). At room temperature reaction 6.5 also occurs:

\[
HCrO_4^- + 2 \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{CrO(O}_2)\text{2} + 3\text{H}_2\text{O} \tag{6.5}
\]

The blue \(\text{CrO(O}_2)\text{2}\) species is unstable and decomposes to \(\text{Cr}^{3+}\).

With regard to the lophine-based chemiluminescence determination of \(\text{Cr(VI)}\), an important aspect of the lophine reaction mechanism (Figure 6.1) is the requirement of oxygen for the emission of light. It is proposed that the determination of \(\text{Cr(VI)}\) is linked to lophine chemiluminescence by arranging the reaction conditions such that the reduction of this species limits the amount of oxygen available for reaction with lophine. Chemiluminescence does not occur prior to the addition of the base because the lophine solution is prepared in an acidic medium.

**EXPERIMENTAL**

**Discrete Sampling Studies**

**Reagents and solution preparation.** Lophine was obtained from Aldrich; (ethylenedinitrilo)-tetraacetic acid disodium salt (EDTA) was acquired from MCB Manufacturing Chemists, Inc. Reagent grade potassium hydroxide was acquired from Baker. Analytical grade potassium dichromate, hydrogen peroxide (30%), HPLC-grade methanol and concentrated nitric acid were purchased from Mallinkrodt.

Chromium solutions used to develop calibration curves were prepared in deionized water by dilution of a 1.003 g/L standard solution. All other
solutions were prepared in concentrations used previously (2). To review, 2.0 mM lophine was prepared in 0.2 M HNO₃ with methanol solvent. The base, 4.0 M KOH, and the solution of EDTA and hydrogen peroxide (10 mM and 0.20 M, respectively) were prepared with in-house deionized water. Deionized water was used as the blank solution. Prior to use, all solutions were equilibrated to room temperature.

Instrumentation, procedure and calibration curves. For the discrete sampling measurements, the chemiluminescence photometer described by Hoyt (6) was employed. With Eppendorf pipets, reagents were added to the detection cell in the following volumes: sample or blank (1.00 mL), H₂O₂/EDTA (0.50 mL), lophine/HNO₃ (0.50 mL). The reaction was initiated by the rapid injection of 0.80 mL of KOH into the cell, while the in-cell solution was mixed with a magnetic stir bar. The injection was controlled by the manual activation of a solenoid linked to the pneumatic drive of a syringe. As in a previous study (2), the detection cell was rinsed once with 1 M HNO₃ in methanol and then twice with deionized water between measurements. Unless otherwise stated, the noise filter cutoff frequency was 0.1 Hz and the PMT bias was -680 V in each experiment.

Intermediate lifetime study. Since it is known that the CrO(O₂)₂ species is both unstable and related to the solution concentration of dissolved oxygen, an experiment was conducted to investigate what impact the lifetime of this or other intermediates might have on the precision of the lophine-based Cr(VI) determination. In this study the peroxide, lophine and a 100 mg/L sample of Cr(VI) were mixed in the cell. The chemiluminescence intensity of the reaction was measured as a function of the delay time of injection of the KOH solution.
Flow Injection Studies

Reagents and solution preparation. The reagents and solution preparations used in the flow injection experiments were identical to those used with discrete sampling, with the following exceptions and additions. Standard solutions of Cr(VI) were prepared with fresh K$_2$Cr$_2$O$_7$ reagent purchased from EM Science. Potassium hydroxide solution was prepared with fresh reagent obtained from High Purity Chemical. Carrier solution was prepared from a 50:50 (v/v) mixture of methanol and water. Inclusion of methanol in the carrier proved essential. Without methanol the solubility of lophine was exceeded. Invariably, this led to blockage of the cell near the ports of the injector. Ten drops of Triton X-100 (alkylaryl polyether alcohol) surfactant (Baker) were added to each 100 mL of the carrier solution. The presence of the surfactant reduced the surface tension of the mixture within the detection cell and this helped to clear the cell of bubbles.

Procedure. As in the bacteria bioluminescence experiments (Chapters 4 and 5), each flask coupled to a Gilmont flow meter was dedicated for use with a specific solution. With reference to Figure 3.2 and Table 3.1, the solutions and flow meters were paired as follows: lophine (meter 1, Gilmont A7972); H$_2$O$_2$/EDTA (meter 2, Gilmont A5403); deionized water blank (meter 3, Gilmont A7967), Cr(VI) samples (meter 4, Gilmont A7984); carrier (meter 5, Gilmont H376). The KOH solution (reaction initiator) was drawn from a reservoir into the sample loop by the pump, as illustrated in Figures 3.1 and 3.2. For all studies, the PMT bias voltage was set to -680 V and the noise filter cutoff frequency was adjusted to 0.1 Hz.

In preliminary experiments, an unexpected difficulty arose with the mixture of the solutions prepared in methanol and those prepared with deionized water. Degassing occurred. This caused both the passage of gas
bubbles to the detection cell, and nucleation and growth of bubbles within the cell. As discussed in Chapter 3, the presence of bubbles in the detection cell leads to imprecision in the experimental results. These problems were addressed by bubbling helium through the prepared solutions for at least 20 min., a common practice used to degas HPLC solvents. The degassing served a double purpose, since the concentration of dissolved oxygen in the prepared solutions was lowered significantly.

At the start of an experiment, the gas lines of the pneumatic flow injection system were both flushed of air and pressurized with helium. Similarly, the sidearm flasks on-line to the manifold (Figure 3.2) were flushed with helium before solutions were added to them. The general procedure for conducting optimizations and analyses was described previously in Chapter 4.

Preliminary Studies with the Beckman Burner Injector

Modifications to the instrument. Initial experiments with lophine-Cr(VI) chemiluminescence and with the flow injection system indicated that modifications to the apparatus were necessary. Much of this need was attributed to the significant difference in the chemistry of the lophine reaction compared to that of marine bacteria bioluminescence (Chapter 2). Optimum bacteria bioluminescence occurs near pH 7.0 and this reaction is highly specific for the aldehyde substrate. In the lophine reaction, a very large and rapid change in pH (about nine units) is required to initiate flash-like chemiluminescence. With these conditions, the likelihood of metal ions entering into solution and causing high blank signals is significant. Therefore, it was deemed necessary to replace the metal fittings in the flow system.
Replacement of the metal pieces involved boring and tapping of the ports of the Gilmont flow meters so that they could accept low pressure, Teflon tube end fittings (1/4-28). To insure leak-proof junctions at the meters, Teflon tubing inserts with flared ends were press-fit into the bored ports. Other parts of the flow system, the check valves and manifold assembly of Figure 3.3 A, were replaced with plastic reed-type valves and by milling a new manifold from Delrin. The manifold was tapped-out to accept low pressure, Teflon tube end fittings (1/4-28) and a bleed port was provided to remove trapped gasses. The new part has a smaller fluid volume, which speeds the transport of reagents to the detection cell, an important consideration when one or more unstable intermediates is involved in the light-producing reaction. The Beckman burner injector was sheathed in Teflon tubing, both in the inbound side port and in the brass nozzle of the injector head. This modification reduced the fluid volume inside the unit and helped to minimize contact of solution with the metal surfaces.

It was observed that the mirror used to enhance the collection of light by the photomultiplier tube (Figure 3.6) was dissolved slowly by the harsh chemical conditions. A new mirror was cut from Mylar film and this was applied to the underside of the lower plate of the detection cell. The mirror was sealed in place with an "O"-ring greased with silicone and supported by a backing plate machined from Delrin.

It was also observed that bubbles nucleated on the inner surfaces of the glass mixer, illustrated in Figure 3.3 B. Presumably, the bubbles grew as a result of catalytic decomposition of hydrogen peroxide at active sites on the glass. To prevent both the passage of these bubbles to the detection cell and to reduce any blank signal caused by excess oxygen production, the glass mixer was removed from the flow system. With 28 cm of 0.8-mm i.d. Teflon tubing, a direct connection was made between the exit port of the
Delrin manifold and the side port (outer conduit) of the injector. The rotary selection valve (illustrated in Figure 3.2) and the wash solutions of deionized water and of Triton X-100 dissolved in water were not used. An adequate and almost continuous wash of the detection cell was provided by the flow of the carrier solution.

**Optimization Experiments with the Beckman Burner Injector.** As a starting point for the chemiluminescence signal optimization experiments, a sample flow rate of 3.70 mL/min (scale = 90) was selected on the A7984 Gilmont flow meter. This setting insured that the quantity of Cr(VI) sample entering the detection cell would be about as large as possible given the limit of the total measurable flow through the meter (about 4.0 mL/min). Also, setting the flow rate at 90 allows the scale to be read should any upward drift of the flow rate occur between two measurements. The flow rates of the other solutions were varied in sequence on each dedicated Gilmont meter to determine the optimum signal response to a 1.00 mg/L Cr(VI) standard solution. A 0.486-mL sample loop was used for the delivery of the base solution.

Subsequent experiments were conducted to both reduce the magnitude of the blank signal and to improve the precision of this measurement. To this end, several factors were considered as possible contributors to the blank signal. In addition, studies were performed to identify the cause of the long duration of the peak halfwidths as part of an effort to reduce the analysis time with flow injection. These experiments involved further modifications of the apparatus, including the fitting of a new injector.

**Design of a new Injector.** Contact between the solution passing through the Beckman injector outer conduit and the surface of a stainless steel sleeve supporting the platinum-iridium inner conduit was considered as a possible source of the blank signal. Chromium ions leached from the stain-
less steel, or catalytic decomposition of the peroxide reagent at the metal surfaces could account for the high blank signals recorded. Therefore, a new injector was machined from Delrin, Teflon and nylon parts and this was fit to the lower cell plate. Details of this design are illustrated in Figure 6.2. The new inner conduit is a capillary, the height of which may be adjusted within the injector housing. Interchangeable capillaries were fabricated by cutting a section from a platinum syringe needle, and by cutting and polishing sections of fine glass capillaries used customarily for spotting in thin layer chromatography. The glass capillaries were susceptible to damage during the collimation of the flows through the injector, as was discussed in Chapter 3. In the experiments, the platinum inner conduit was used and this was set about 1 mm below the level of the tapered end of the outer conduit.

RESULTS AND DISCUSSION

Discrete Sampling

Calibration curves obtained with the discrete sampling apparatus are presented in Figures 6.3 and 6.4. Each data point is a peak height corrected for the mean height of the blank signal, averaged over 15 to 20 blanks. The slopes of the linear least squares fits to the log-log plots (0.960 and 0.884, respectively) indicate that the luminescence response is nearly linear over four orders of magnitude, from 10 µg/L to about 100 mg/L Cr(VI). Above 100 mg/L both curves show negative deviations from linearity. With reference to the stoichiometry presented earlier (Equations 6.3 and 6.4), the reduction of the two Cr(VI) oxo-anions yields either 1.5 or 3 molecules of oxygen. In the
Figure 6.2. Injector Schematic. The new injector is constructed from three sections: a nozzle, a body, and a capillary support. Dimensions in inches and additional information for each section are presented below.

Nozzle (Teflon): This part serves as the outer conduit. At the end of the taper the o.d. is 0.060, the i.d. is 0.039; (a) 0.39; (b) 0.19; (c) 0.29; (d) 4-40 nylon collimating set screw, one of three positioned at 120° with respect to one another; (e) 0.49; (f) 0.38; (g) 1/4-28 thread.

Body (Teflon): (h) 1/2-32 thread screws into the flange of the lower cell plate illustrated in Figures 3.6 and 3.8; (i) sidearm of the outer conduit (Delrin); (j) body length: 0.88, diameter 0.75; (k) outer conduit port is tapped to accept a 1/4-28 low pressure end fitting.

Capillary Support (Nylon): (l) inner conduit of platinum piping -- 0.016 i.d., 0.028 o.d. (0.8 mm-i. d. Teflon tubing slides over the capillary shaft); (m) 6-32 thread on a shaft 0.55 in. long; (n) 0.48 (o) the capillary support is tapped to accept a 1/4-28 low pressure end filling; (p) 0.45.
Figure 6.2. Injector Schematic.
Figure 6.3. Cr(VI) Calibration Curve Achieved with Lophine Chemiluminescence and Discrete Sampling Apparatus. The PMT bias voltage was -600 V.
Figure 6.4. A Second Cr(VI) Calibration Curve Achieved with Lophine Chemiluminescence and Discrete Sampling Apparatus.
chemiluminescence reaction, one oxygen molecule is required for each lophine. Thus, a negative deviation from linearity is expected as the ratio of the final in-cell concentrations of Cr(VI) and lophine approaches a ratio of 1 to 3. In the discrete sampling experiments, the ratio of the in-cell concentrations of Cr(VI) to lophine is 1.9, or roughly 2 to 1, when the sample concentration of Cr(VI) reaches 100 mg/L. When the Cr(VI) concentration exceeds that of lophine by a significant amount, both the lophine molecules and the reaction solution should become saturated with oxygen. Bubbles of gas are observed to effervesce from solution when 0.8 mL of the base is injected slowly into a test tube containing both a 0.5 mL sample of 200 mg/L Cr(VI) and the volumes and concentrations of the other solutions described previously.

At very low Cr(VI) concentrations (e.g., below 1 µg/L) the analytical signal becomes indistinguishable from the blank signal. With reference to Figure 6.1 and Equations 6.3 and 6.4, the blank signal limited detectability of Cr(VI) may be due to residual quantities of dissolved oxygen. The solutions used in both these experiments and in those conducted by previous researchers (2, 3) were not degassed. Also, the upper surface of the solution mixed in the detection cell is exposed to the air. A five-fold reduction in the blank signal has been observed with the addition of EDTA to the peroxide solution and this has been attributed to the complexation of metal impurities present in the hydrogen peroxide reagent (2). With reference to Figure 6.1, complexation of metal ions from any source that might otherwise catalyze the decomposition of the peroxide to water and oxygen molecules would tend to reduce the blank signal. However, without the degassing of the reagent solutions and other precautions to prevent the diffusion of air into the reaction solution, the residual concentration of dissolved oxygen may limit the concentration of Cr(VI) detectable by this analytical method.
Practical detection limits calculated from the data of Figures 6.3 and 6.4 are reviewed in Table 6.1. For each of the calibration curves, blank corrected data for the 10 µg/L standards had relative standard deviations (RSDs) ranging from 10 to 20% of the analytical signal.

Results of the intermediate lifetime study are presented in Figures 6.5 A and 6.5 B. The curve illustrated in Figure 6.5 A suggests that the early and consistent delivery of the base solution to the detection cell is essential to the attainment of the lowest possible detection limit. As can be seen in the figure, the strongest chemiluminescence signal is achieved with the shortest delays; also, the slope of the curve is steepest for small delay times. Imprecision in the data may be related to the timing involved in both of the pipetting of the necessary reagents to the detection cell and the delivery of the base. An effort was made to keep this timing as consistent as possible when the data were collected to develop the calibration curves. However, automation of the solution deliveries to the detection cell would probably lower the detection limit.

A plot of the natural logarithm of the chemiluminescence signal versus the time of delivery of the base is illustrated in Figure 6.5 B. The correlation coefficient of the linear least squares fit is 0.978. This relationship suggests that the kinetics of the decay are either first order or pseudo first order with respect to the intermediate (or intermediates) involved. The half-life, calculated from the slope value (-4.62 x 10^-3 s^-1) is 2.50 min. Marino (2) observed that the blue CrO(O2)2 species formed in the detection cell, prior to the delivery of KOH, persisted for about 2 min. as the solution was stirred.

The results presented in Table 6.1 may be compared with those obtained previously. Marino (2) reports a detection limit (DL) of 0.3 µg/L Cr(VI) by this method. This value is equivalent to a chemiluminescence (CL) signal equal to twice the SD in the blank divided by the slope of the calibration curve. The blank SD was taken as the irreproducibility in the
Table 6.1. Practical Detection Limits (DLs) for Cr(VI) Determined with Discrete Sampling Apparatus and Lophine Chemiluminescence.

<table>
<thead>
<tr>
<th>Calibration Curve</th>
<th>Blank Signal Std. Dev. (pA)</th>
<th>Linear Fit Slope pA / (µg/L)</th>
<th>DL&lt;sup&gt;a&lt;/sup&gt; (µg/L)</th>
<th>Second Order Fit Slope pA / (µg/L)</th>
<th>DL&lt;sup&gt;a&lt;/sup&gt; (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 6.3</td>
<td>3.8</td>
<td>3.3</td>
<td>2</td>
<td>3.6</td>
<td>2</td>
</tr>
<tr>
<td>Fig. 6.4</td>
<td>4.0</td>
<td>3.0</td>
<td>3</td>
<td>8.1</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated from 2sbk / m, where values of the slope, m, were obtained from both linear and second order least squares fits to selected data points. A discussion of the method used to obtain the detection limit using the second order fit is given in the caption of Table 4.5. Standard deviations (SDs) in the blank signals, sbk, were used to calculate the detection limits. SDs of the baseline was not used for these calculations because the blank signals were less precise and a sufficient number of blanks were measured to calculate the SDs reliably.
Figure 6.5. (A) Chemiluminescence Signal Dependence on the Delay in the Delivery of the Base (0.8 mL of 4.0 M KOH) to the Detection Cell and (B) Plot to Determine the First Order Rate Constant of the Chemiluminescence Signal Decay.
dark current, not as the irreproducibility in the blank signal. Chemiluminescence signals were taken as the difference in mean peak heights between five samples and five blanks. The RSD of the CL signal with 10 μg/L Cr(VI) samples was 2%. The reported calibration curve is linear from 10 μg/L to about 100 mg/L. Data points below 10 μg/L were not presented. Marino (2) reports that no curvature in the calibration plot occurs until the Cr(VI) concentration approaches that of lophine.

Using lophine chemiluminescence and apparatus consisting of a remote reaction chamber and a fiber optic cable to transmit the CL signal to a photomultiplier (PMT) detector, Waddle (3) obtained a Cr(VI) DL of 11 μg/L. The detection limit was calculated from the signal equivalent to twice the dark current noise divided by the slope of the calibration curve. Interestingly, no blank signal was observed in this study.

The difference in detectability achieved by Waddle (3) and that found by Marino (2) was attributed to both differences in the PMT detectors utilized in each study and the reduced light collection efficiency achieved with the fiber optic system. Using lophine chemiluminescence and the photometer described by Hoyt (6), Waddle (3) calculated a Cr(VI) DL of 3 μg/L. This value is in excellent agreement with the detection limits presented in Table 6.1.

Flow Injection

Optimization Experiments with the Beckman Burner Injector

Optimization data with the Beckman burner injector are shown in Figures 6.6, 6.7 and 6.8. In Figures 6.6 and 6.7, optimum conditions were taken as the signal maxima on the curves. In Figure 6.8, the optimum condition for the delivery of the
Figure 6.6. Chemiluminescence Signal Optimization as a Function of the Lophine Solution Flow Rate. Individual data points are peak heights measured from the baseline. Constant flow rates (mL/min): carrier/KOH, 3.0; H$_2$O$_2$/EDTA, 0.20; 1.00 mg/L Cr(VI) standard, 3.7. Air temp: 23.6° C; sample loop: 0.486 mL.
Figure 6.7. Chemiluminescence Signal Optimization as a Function of the H$_2$O$_2$/EDTA Solution Flow Rate. Individual data points are peak heights measured from the baseline. Constant flow rates (mL/min): carrier/KOH, 3.0; lophine, 2.6; 1.00 mg/L Cr(VI) standard, 3.7. Air temp: 23.6° C; sample loop: 0.486 mL.
Figure 6.8. Chemiluminescence Signal Optimization as a Function of the Carrier/KOH Solution Flow Rate. Individual data points are peak heights measured from the baseline. Constant flow rates (mL/min): lophine, 2.6; H₂O₂/EDTA, 0.30; 1.00 mg/L Cr(VI) standard, 3.7. Air temp: 22.8°C; sample loop: 0.486 mL.
base was taken as a trade-off between the best signal precision and the magnitude of the chemiluminescence signal. Since the signal varied by less than a factor of two throughout the range of the flow rates tested, the best compromise occurs between 3.0 and 4.5 mL/min. The optimum conditions so obtained are listed in Table 6.2 where they may be compared to optimum conditions deduced previously by Marino (2). The average of seven blank signals recorded at the optimum flow conditions was 2.1 nA, with a standard deviation of 0.5 nA. When the optimum flow conditions were used, peak half-widths recorded for 1.00 mg/L Cr(VI) samples averaged 13.4 s. Average half-widths of 1.0 s were recorded for identical samples with the discrete sampling apparatus.

**Optimization experiments with the new injector.** Several experiments were conducted with the new injector as part of the effort to improve the quality of the blank signal. The results of a few of these experiments are discussed below.

Since the magnetic mixer was removed from the apparatus, an investigation was conducted to determine whether or not the signal imprecision for both the blanks and the standard samples was related to incomplete mixing of the solution delivered to the outer conduit. To test this possibility, a mixing coil was placed on-line between the Delrin manifold and the injector port. The coil was prepared by wrapping 1.05 m of 0.8-mm i.d. Teflon tubing around a cylinder of Delrin machined to a diameter of one inch. However, no significant improvement in the precision of the blank signals was found through the use of the coil. Since the coil delayed transport of solution to the cell and, therefore, decreased the response time to manual adjustment of the flow rates, the mixing coil was not used in subsequent experiments.

Reducing the size of the sample loop at the injector reduced the peak
Table 6.2. Comparison of Optimum In-cell Reagent Concentrations for the Determination of Cr(VI) with Lophine Chemiluminescence by the Methods of Discrete Sampling and Flow Injection.

<table>
<thead>
<tr>
<th>Method</th>
<th>Reagent</th>
<th>Volume (mL)</th>
<th>Flow Rate (mL/min)</th>
<th>Pre-cell Conc. (M)</th>
<th>Cell Conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discrete Sampling</td>
<td>Cr(VI) sample</td>
<td>1.00</td>
<td>--</td>
<td>X</td>
<td>400X&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;/EDTA</td>
<td>0.50</td>
<td>--</td>
<td>0.20</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>lophine</td>
<td>0.50</td>
<td>--</td>
<td>0.0020</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>KOH</td>
<td>0.50</td>
<td>--</td>
<td>4.0</td>
<td>800</td>
</tr>
<tr>
<td>Flow System with the</td>
<td>Cr(VI) sample</td>
<td>--</td>
<td>3.70</td>
<td>X</td>
<td>390X&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Beckman Injector</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;/EDTA</td>
<td>--</td>
<td>0.30</td>
<td>0.20</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>lophine</td>
<td>--</td>
<td>2.6</td>
<td>0.0020</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>KOH</td>
<td>--</td>
<td>3.0</td>
<td>4.0</td>
<td>1250</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated by: (volume of reagent pipetted to the cell) x (reagent conc.) (total solution volume added to the cell)

<sup>b</sup> Calculated by: (flow rate of reagent to the cell) x (reagent conc.) (total flow rate to the cell)
half-widths. When a 0.072-mL sample loop was used in conjunction with a 1.00 mg/L Cr(VI) sample and the optimum flow conditions described above, peak half-widths averaged 10.2 s. Compared to results obtained with the 0.486-mL sample loop, the 25% reduction in peak half-width was related, presumably, to both the reduced volume of the base delivered to the cell and the time required to flush the sticky and viscous alkaline solution from the loop. A further significant decrease in the size of the sample loop was not practical given both the dimensions of Teflon tubing available and the 1.7 cm distance between the injector valve ports to which the loop was attached. The great difference in half-widths compared to those obtained with discrete sampling is related to the difference in the mechanism by which the base is delivered. As prescribed with discrete sampling, delivery of the base with a syringe is clearly the more rapid of the two methods.

The Hastelloy bubblers, used to degas the prepared solutions, were considered as a possible source of contaminating metal ions since these were dipped in solutions of both strong acid and base. Hastalloy is an alloy of nickel which may contain up to 20% chromium. However, this material is considered to be quite inert and is used commonly in chemical equipment. Replacement of the bubblers with similar devices made of porous glass did not reduce the magnitude of the blank significantly, nor did it improve the precision of this signal. However, as a precaution, use of the Hastelloy bubblers was discontinued.

Possible contamination of the glassware used in the flow injection system was investigated as a potential source of the blank signal. To remove any CrO$_4^{2-}$ adhering to the walls of the sidearm flasks, this glassware was rinsed with a 1.0 M solution of Pb(NO$_3$)$_2$. Since the solubility product of PbCrO$_4$ is $1.8 \times 10^{-14}$ (7), the chromate is precipitated to a very low residual level. Subsequently, each flask was rinsed several times with copious amounts of 0.1 M EDTA to complex the remaining lead (formation
constant = 10^{18}; (8)) and other cations. This washing was followed with several rinses with house deionized water. Eleven blank signals recorded at the optimum flow rates listed in Table 6.2 averaged 0.33 nA, with a standard deviation of 0.09 nA.

Blank chemiluminescence due to an unknown side reaction involving Triton X-100 was considered as a possible source of unwanted signal. However, a side-by-side comparison of blank signals recorded with carrier solution prepared with and without the surfactant suggested that this reagent was not involved to any measureable extent.

Flow rate optimization experiments with the new injector. The optimization experiments described earlier were repeated with the new injector. Results of these studies are presented in Figures 6.9, 6.10 and 6.11. The conditions of maximum CL response were similar to those deduced from the experiments with the Beckman burner injector. In Figure 6.9 the optimum flow condition was taken as the signal that gave both the highest chemiluminescence value for the standard and the greatest difference between the standard and blank curves. This condition occurs at about 2.5 mL/min. In Figure 6.10, the greatest difference between the standard and blank signals occurs at a flow rate of 4.5 mL/min. However, better precision in the data is obtained at a flow rate of 8.7 mL/min. Similarly, in Figure 6.11, a difference between the signals is evident at a flow rate of 0.30 mL/min; but, better precision and a greater signal difference is obtained at 0.62 mL/min. In Figures 6.10 and 6.11, optimum conditions were taken as the flow rates yielding the best signal precision.

It is interesting to note in Figure 6.11 that both the blank and standard solution signals are both finite and virtually indistinguishable from one another when the peroxide/EDTA flow rate is zero. Also, both signals
Figure 6.9. Chemiluminescence Signal Response as a Function of the Lophine Solution Flow Rate for Deionized Water Blanks, a 5 μg/L Cr(VI) Standard (A) and a 20 μg/L Cr(VI) Standard (B). In plot (A) the constant flow rates (mL/min) were: carrier/KOH, 4.5; H₂O₂/EDTA, 0.30; Cr(VI) standard or water blank, 3.7. Air temp: 23.2°C; sample loop: 0.272 mL. Plot (B) was developed with similar experimental conditions. The air temperature was 21.0°C.
Figure 6.9.
Figure 6.10. Chemiluminescence Signal Response as a Function of the Carrier/KOH Solution Flow Rate for both Deionized Water Blanks and Samples of a 20 µg/L Cr(VI) Standard. Constant flow rates (mL/min): lophine, 2.6; H₂O₂/EDTA, 0.30; Cr(VI) standard or water blank, 3.7. Air temp: 22.6° C; sample loop: 0.072 mL.
Figure 6.11. Chemiluminescence Signal Response as a Function of the H$_2$O$_2$/EDTA Solution Flow Rate for both Deionized Water Blanks and Samples of a 20 µg/L Cr(VI) Standard. Constant flow rates (mL/min): carrier/KOH, 8.7; lophine, 2.6; Cr(VI) standard or water blank, 3.7. Air temp: 22.6°C; sample loop: 0.072 mL.
increase initially with the increasing flow rate. Although it is possible that the weak signals recorded at the zero flow rate may result from an unknown chemiluminescence reaction, it is also presumable that this trend is related to the reaction of lophine with a residual amount of dissolved oxygen. As the peroxide flow rate is increased, the difference between the blank and sample signals increases to a nearly constant value as the in-cell concentration of peroxide increases. If the peroxide reacts with Cr(VI) in the sample, more oxygen becomes available for the chemiluminescence reaction. Above an optimum peroxide flow rate, the in-cell Cr(VI) concentration may no longer limit the signal difference, and both the blank and sample signals may fall as a result of the dilution of reagents in the detection cell.

**Calibration curve obtained with the new injector.** A Cr(VI) calibration curve (Figure 6.12) was developed using the optimum flow rates described above. The slope of the least squares fit to the log-log plot is 0.953 between 300 μg/L and 30 mg/L. A theoretical detection limit of 42 μg/L was calculated as the concentration yielding a signal equivalent to twice the standard deviation in seven blank signals (69 pA) divided by the slope value (3.3 nA/(mg/L)) obtained from the most linear portion of the calibration curve. Similarly, a practical detection limit of 54 μg/L was calculated by using the slope of a second order linear least squares fit to the data in the region of the detection limit.

The calibration curve in Figure 6.12 and Equation 4.4 were used to determine the blank equivalent concentration of Cr(VI). In the equation, values of 360 pA, 70 pA and 2 were substituted for the respective values of $S'_{bk}$, $S_{bk}$ and $k$. The calculated value of the blank signal, $S_{DL}$, was substituted into the equation of a second-order least squares fit to the calibration curve in the region of the detection limit. A blank equivalent value of
Figure 6.12. Cr(VI) Calibration Curve Developed with Lophine Chemiluminescence. Constant flow rates (mL/min): carrier/KOH, 8.7; lophine, 2.6; H2O2/EDTA, 0.30; sample/blank, 3.7. Air temp: 22.6° C; sample loop: 0.072 mL.
$190 \, \mu g/L \text{ Cr(VI)}$ was calculated by this method. This value is considerably higher than results obtained from the discrete sampling curves. Blank equivalent values of 5 and 9 $\mu g/L$ were obtained from Equation 4.4 and from the calibration curves of Figures 6.3 and 6.4, respectively. Using the discrete sampling apparatus, Marino (2) reported a blank equivalent value of $15 \, \mu g/L \text{ Cr(VI)}$.

The matter of the unexpectedly large blank signal obtained with the flow injection apparatus is of considerable interest, since this factor limits the lower concentration of Cr(VI) that may be determined by this method. Possible sources of interference include a residual concentration of oxygen dissolved in solution, and/or the presence of interfering cations causing an undesirable excess decomposition of the peroxide reagent. Residual dissolved oxygen might be caused by the pneumatic drive system. The drive is helium under pressure. However, the helium was transported to the apparatus through approximately 15 feet of Tygon tubing, a material known to diffuse small gas molecules through its walls. Were the experiment to be repeated, it might be useful to replace the Tygon gas line with one made of copper tubing.

Another possible source of the blank signal is the presence of active sites on walls of the sidearm flasks. Adsorbed metal ions might have sufficient catalytic power to cause a significant blank signal. Conceivably, this problem would be solved by silanization of the sidearm flasks and the other glass parts of the flow system. The platinum inner conduit of the injector is, potentially, a major factor contributing to the blank signal. The catalytic utility of this material is well documented and undesirable decomposition of excess peroxide reagent at the outer surface of the conduit is probable. Since hydrogen peroxide is common to chemiluminescence reactions (e.g., those of lucigenin and luminol), replacement of the platinum
conduit with one of silanized glass (or of another suitable material) is recommended for studies involving this reagent.

It is unfortunate that the blank signal was so high in the experiments described above that an adequate assessment of the potential performance of the instrument was not possible. Ultimately, the chemist electing to perform Cr(VI) determinations with lophine chemiluminescence may choose discrete sampling apparatus since the use of that equipment is facile. However, two important points that arise from the flow injection studies apply to the analysis with discrete sampling. First, it is suggested that the effect of degassing of the solutions used in the analysis should be tested. If the blank signal is reduced with the degassed solutions, and if the reaction is to be carried out in an open cell, then the atmosphere immediately above the cell should be free of oxygen to prevent the diffusion of this gas into the reaction solution. Both of these suggestions may be related to observations made by Radziszewski, the earliest investigator of lophine chemiluminescence. In Radziszewski’s experiments (1877), luminescence was observed when an alkaline and alcoholic solution of lophine was shaken with air. Brightest luminescence was observed at the solution-air interface. Luminescence was not observed when oxygen was excluded from the mixture (9).

CONCLUSIONS

A mechanism has been proposed to account for the determination of Cr(VI) with lophine chemiluminescence. In this proposal, the reduction of hydrogen peroxide to molecular oxygen by the chromium species limits the amount of light produced by the reaction when the other reagents are in excess. The origin of the blank signal is thought to be related to residual levels of dissolved oxygen. Unless precautions are taken to remove it,
dissolved oxygen is present naturally. In the flow injection system described above undesirable levels of dissolved oxygen may occur as a result of peroxide reduction by metal ion contaminants, by active sites on the glassware of the flow system, or by catalytic decomposition of the peroxide at the surface of the platinum inner conduit of the injector.

The optimum in-cell reagent concentrations for lophine-Cr (VI) chemiluminescence deduced previously (2) were confirmed by variation of the solution flow rates with the flow injection system. With flow injection, a detection limit of about 50 µg/L Cr (VI) was calculated. This value compares unfavorably with detection limits determined with discrete sampling apparatus, both in this study and previously (2, 3). However, the 50 µg/L value is significantly lower than the 120 µg/L minimum specified by the Safe Water Drinking Act (3). Because the detection limit was restricted by the blank signal, this does not reflect unfavorably on either the capabilities of the light collection system or the fundamental design of the flow injection apparatus.

The modifications to the instrumentation described in Chapter 3 are improvements that significantly broaden the equipment application. The original design was sufficient for use with bacteria bioluminescence. In its present form, the apparatus may be operated with corrosive solutions, if necessary, and provision has been made for direct coupling of the detection cell and the flow injection system to a high performance liquid chromatograph (HPLC). This arrangement should provide other researchers with an opportunity to study a variety of bioluminescence and chemiluminescence reactions.
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PC SOFTWARE FOR THE AUTOMATED FLOW INJECTION APPARATUS
"CLFLOINJ.BAS", a program to control pump and flow injection operation; also, to acquire and manipulate data for chemiluminescence (CL) measurements.

DEFINT A-Z
GOSUB Prepare

Flow.Injection.Cycle:

GOSUB Peak.Num.Update
GOSUB Full.Scale
GOSUB Switch.Valves.and.Pump
GOSUB Data.Acquisition
GOSUB Flush.Sample.Loop
GOSUB Sort.Max.V.and.Write.File
GOSUB Baseline.and.Peak.Signal.Calcs
GOSUB Plot.and.Print

GOTO Flow.Injection.Cycle

The Subroutines follow

Prepare:

address% = 1808  'LabMaster (I/O mapped) addr.
control = address% + 15  '8255 control port address
porta = control - 3  '8255
portb = control - 2  'input/output
portc = control - 1  'port addresses
OUT control, &H98  'Mode 0: port A inp., B outp.;
                   'C bits 0-3 output, 4-7 input.

DIM L(500), H(500)  'Arrays: LO, HI data bytes;
DIM D!(500), T!(500)  'potential (V) and time (s).
PEAK.NUM = 0

CLS : BEEP: BEEP
LOCATE 8, 5
PRINT "Enter delay times for operation of the pump"
LOCATE 9, 5
PRINT "and valves. Time intervals are in sec., except"
LOCATE 10, 5
PRINT "the CL initiator transport, for which intervals"
LOCATE 11, 5
PRINT "are multiples of 0.5 s. Recommended delay times"
LOCATE 12, 5
PRINT "in '( )' are for a bacteria bioluminescence"
LOCATE 13, 5
PRINT "(BBL) reaction and a sample loop of 0.5 mL."
LOCATE 15, 5
INPUT "Fill / flush sample loop (30 s, BBL): ", V1
LOCATE 17, 5
INPUT "Retard pump / reagent flow (5 s, BBL): ", V2
LOCATE 19, 5
INPUT "Transport CL initiator, (2 = 1 s, BBL): ", V3
LOCATE 21, 5
INPUT "Flush sample loop (30 s, BBL): ", V4
RETURN

*******************

Peak.Num.Update:

PEAK.NUM = PEAK.NUM + 1
LPRINT "Peak number is ", PEAK.NUM: LPRINT
RETURN

*******************

Full.Scale:

CLS : BEEP: BEEP 'Prompt for operator (need input).
LOCATE 12, 5
PRINT "Enter chart recorder full scale setting (mV)"
LOCATE 14, 5: PRINT "Enter (1) for 100 mV"
LOCATE 15, 5: PRINT "Enter (2) for 200 mV"
LOCATE 16, 5: PRINT "Enter (3) for 500 mV"
LOCATE 19, 5
PRINT "Press Ctrl-Break, then Alt-F, to exit program."
LOCATE 22, 5: PRINT "Choice (1-3) :": INPUT a$
DO
LOOP WHILE a$ < "1" OR a$ > "3"
SELECT CASE a$
CASE "1": OUT portb, 1
LPRINT "Full Scale is 100 mV"
CASE "2": OUT portb, 2
LPRINT "Full Scale is 200 mV"
CASE "3": OUT portb, 4
LPRINT "Full Scale is 500 mV"
END SELECT
LPRINT
RETURN

*******************
Switch.Valves.and.Pump:

OUT portc, 8  'Port C, bit 3, HI;  
              'Carrier to cell via bypass.
OUT portc, 9  'Port C, bit 0, HI;  
              'power pump via Grayhill switch,  
              'filling sample loop. Bit 3, HI;  
              'Carrier via bypass.

TOPSEC = V1
FIFTYmsPeriods = 20
MESSAGE$ = "Filling Sample Loop   "
GOSUB Delay  'Fill sample loop (30 s, BBL).

OUT portc, 8  'Sample loop filled, flushed. Port  
              'C, bit 0, LO; pump off. Bit 3, HI;  
              'Carrier to cell via bypass loop.

TOPSEC = V2  'Retard pump/reagent inertia.  
FIFTYmsPeriods = 20  '(5 s delay for BBL)
MESSAGE$ = "Delay to reduce pump/reagent inertia.   "
GOSUB Delay

OUT portc, 0  'Port C, bit 3, LO;  
              'sample injected via Carrier.

TOPSEC = V3
FIFTYmsPeriods = 10
MESSAGE$ = "DATA DELAY = 0.5 sec    "
GOSUB Delay  'Delay to transport sample (1s for BBL).

RETURN

'******************************************************************************

Delay:
'This Subroutine is adapted from a program presented by  
'Counter 2 of the LabMaster 8253A Programmable Interval  
'Timer (PIT) is used to produce delay times.

ControlAd = &H43  'Address of 8253A control port.
Timer2 = &H42    'Addr. of Timer 2 read/write port.
Modecommand = &HB4  'Control byte; 10110100:  
                   '10 = counter #2; 11 = read/load LSB,  
                   'MSB; 010 = mode 2 (free running);  
                   '0 = binary count(16 bits).

Enable = &H61    'Addr. of 8255 Programmable  
                 'Peripheral Interface (PPI),  
                 'Port B. Note: bit 0 gates Timer 2 on a rising edge.
Fiftymillisec\# = 59659  'Master crystal operates at
 '4.77 MHz/4 = 1.19316 MHz.
 'For 50 msec count, multiply by 0.05; get 59659.

Highbyte\# = Fiftymillisec\# \ 256  'HI and LO bytes of
Lowbyte\# = Fiftymillisec\# - Highbyte\# * 256  'variable.

FreezeValue = \&H80  '10000000 written to the 8253A
 'control port will freeze the
 'value in Timer 2, not stopping the down count.

OUT ControlAd, Modecommand  'Set mode.
OUT Timer2, Lowbyte\#  'Write LO, HI bytes of
OUT Timer2, Highbyte\#  '59659 to Timer 2.
OriginalPPI = INP(Enable)  'Store in 8255 Port B.
NewPPI = OriginalPPI OR 1  'Bit 0 = "1" of Timer 2,
 'starting timer.

StartingVal\# = Fiftymillisec\#  'When timer 2 crosses a
 '0 count, the counter
 'reloads for another count down. The program will
 'keep track of the number of count downs in TOPSEC.

CLS
LOCATE 10, 15
OUT (Enable), NewPPI  'PPI has value 1, a rising edge
 'turns "ON" Timer 2.

FOR I = 1 TO TOPSEC
 interval = FIFTYmsPeriods
 Cycle:
    OUT ControlAd, FreezeValue  'Freeze cnt.; Timer cont.
    CurrentLo\# = INP(Timer2)  'Read cntr. val. (LO/HI).
    CurrentHi\# = INP(Timer2)

 'Run through a 50 msec interval:
 IF (CurrentHi\# * 256 + CurrentLo\# < StartingVal\#) THEN
    StartingVal\# = CurrentHi\# * 256 + CurrentLo\#
 GOTO Cycle
 END IF
    StartingVal\# = CurrentHi\# * 256 + CurrentLo\#  'Reload.
    interval = interval - 1
    IF interval = 0 THEN PRINT MESSAGE$;  ELSE GOTO Cycle
 NEXT I
OUT Enable, OriginalPPI
RETURN
 '**************************
Data.Acquisition:

'This subroutine has been adapted from a program written by the Scientific Solutions people to demonstrate data collection (DEMO6, p68, of the LabMaster Installation Manual and User’s Guide).

'Counter 5 is set to trigger analog-to-digital conversion of input at channel 0 at a rate of 76.5 Hz. A jumper must be placed between T5 0 and STC of the Daughter Board connecting the output of counter 5 to the external start conversion input. The input range must be +/-10 V.

'I/O mapped mode.

CLS

channel = 0
X = INP(address% + 6) 'Read in ADC high 4 bits of data, resetting done flip flop.
OUT address% + 4, 128 'Write 10000000 to ADC control; disable auto-incrementing, ext.

'start converts, all interrupts. Once timer is started, ext. start coverts are enabled.

OUT address% + 9, 23 'Data pntr. = Master Mode reg.
OUT address% + 8, 0 'To timer data port; Master Mode
OUT address% + 8, 128 'reg. set: BCD division, incr.

'enabled, 8 bit bus, FOUT on,
'divide by 16, f1=source, comparitors and t-of-day off.

OUT address% + 9, 5 'Data pntr. set to timer control port, counter 5 mode reg.
OUT address% + 8, 49 'Cnter #5 set: No gating; cnt.
OUT address% + 8, 15 'rising edge of f5 (100 Hz);
'spec. gate OFF; reload from load;
'cnt. down repetitively w/BCD; active hi TC pulse.

OUT address% + 8, 2 'Load counter 5 load register w/ 2 BCD. The counter counts from 2 'to 0, producing a 76.5 Hz output. Note:if BCD 99 were 'used, "0099" =0000 0000 1001 1001 B = 153D; this count 'down would produce a 1 Hz output.

OUT address% + 8, 0 'High byte of (BCD 2="0002").
OUT address% + 9, 112 'Load conuter 5, start counting.
OUT address% + 4, 132 'ADC ctrl. byte; ext. cnvts."ON".
OUT address% + 5, channel 'Will use ADC channel 0.

LOCATE 10, 15
PRINT "Sample Injection and Data Collection"
TOPNUM = 459
FOR j = 1 TO TOPNUM
WHILE INP(address% + 4) < 128: WEND 'Wait here until
'10000000 = done with a conversion.

L(j) = INP(address% + 5) 'Read in low 8 bits of data.
H(j) = INP(address% + 6) 'Read in high 4 bits.
NEXT j
OUT address% + 9, 255 'Stop 9513 timer.
RETURN

Flush.Sample.Loop:

TOPSEC = V4
FIFTYmsPeriods = 20
MESSAGE$ = "Flushing Sample Loop "
GOSUB Delay 'Sample loop flushed (30 s, BBL).

OUT portc, 8 'Port C, bit 3, HI;
'Carrier switched thru bypass.
RETURN

Sort.Max.V.and.Write.File:

CLS
MaxV! = 0
D$ = DATE$
T$ = TIME$
P1$ = MID$(D$, 4, 2)
P2$ = MID$(T$, 1, 2)
P3$ = MID$(T$, 4, 2)
P4$ = MID$(T$, 7, 2)
FILENAME$ = P1$ + P2$ + P3$ + P4$ + ".DAT"
LOCATE 10, 5
PRINT "Writing data to disk with filename: ", FILENAME$
OPEN FILENAME$ FOR OUTPUT AS #1
TIMEDAT! = 0!
FOR I = 1 TO TOPNUM
TIMEDAT! = TIMEDAT! + .01307 '6/459 = 0.01307 s/pt.
T!(I) = TIMEDAT!
tot& = 256! * H(I) + L(I) 'Compose 12-bit data.
IF tot& > 32767 THEN tot& = tot& - 65536!
'Neg. value check; conv. to 16 bits.
D!(I) = tot& / 204.8  'Set bipolar 10 V range.
IF tot& / 204.8 >= MaxV! THEN MaxV! = tot& / 204.8
'PRINT #1, L(I), H(I), tot& / 204.8, T!(I)
'PRINT L(I), H(I), D!(I), T!(I)
NEXT I
CLOSE #1
RETURN
'*****************************************************************************

Baseline.and.Peak.Signal.Calcs:

CLS
LOCATE 10, 1
PRINT "Maximum Voltage is ", MaxV!, "V"
PRINT
LPRINT "Maximum Voltage is ", MaxV!, "V"
LPRINT
Baseline! = 0
FOR I = 1 TO 10
    tot& = 256! * H(I) + L(I)
    IF tot& > 32767 THEN tot& = tot& - 65536!
    Baseline! = Baseline! + (tot& / 204.8)
NEXT I
Avgbaseline! = Baseline! / 10  'Avgd baseline is
LOCATE 12, 1
'mean of 1st 10 pts.
PRINT "The averaged baseline is ", Avgbaseline!, "V"
PRINT
LPRINT "The averaged baseline is ", Avgbaseline!, "V"
LPRINT
PeakSigVal! = MaxV! - Avgbaseline!
PRINT "Peak Signal Value is ", PeakSigVal!, "V"
PRINT
LPRINT "Peak Signal Value is ", PeakSigVal!, "V"
LPRINT
RETURN
'*****************************************************************************

Plot.and.Print:

GOSUB Set.Parameters
GOSUB CLintensity

RETURN
'*****************************************************************************
Subroutines for Plotting:

Set Parameters:

CLS
   LOCATE 10, 1
   INPUT "Low Time Value: ", Time.Lo
   Time.Lo = 0
   INPUT "High Time Value: ", Time.Hi
   Time.Hi = 6000  '6000 msec data acquisition time.
   Time.Range = Time.Hi - Time.Lo
   Time.Range = Time.Range * 3 / 50
RETURN

'***************************************************************

CLIntensity:

Plot CL intensity (V) as a function of time.
LOCATE 24, 1
PRINT SPACE$(70);
LOCATE 24, 1
SCREEN 2   'supports CGA, EGA, VGA, and MCGA
SCREEN 2   'screen mode for Hercules
CLS
HiPot! = 10   'Highest potential: 10 V;
LowPot! = 0   'lowest: 0 V.
FOR I = 1 TO TOPNUM
   tot& = 256! * H(I) + L(I)
   IF tot& > 32767 THEN tot& = tot& - 65536!
   D!(I) = tot& / 204.8
   IF D!(I) > 10 THEN D!(I) = 10
   IF D!(I) < -.5 THEN D!(I) = -.5
   IF D!(I) >= HiPot! THEN HiPot! = D!(I)
   IF D!(I) <= LowPot! THEN LowPot! = D!(I)
NEXT
   IF LowPot! > 0 THEN LowPot! = 0

'Set up plot:

WINDOW (-40, -2)-(Time.Range * 1!, HiPot!)
LINE (0, LowPot!)-(Time.Range, HiPot!), , B
FOR X.Pos = 0 TO Time.Range STEP Time.Range / 20
   LINE (X.Pos, LoPot!)-(X.Pos, LoPot! + .035 * HiPot!)
NEXT X.Pos
LOCATE 22, 6: PRINT Time.Lo;
LOCATE 22, 75: PRINT Time.Hi;
LOCATE 22, 35: PRINT "Time (msec)";
LOCATE 21, 2: PRINT USING "+#.###": LowPot!;
LOCATE 1, 2: PRINT USING "+##.##"; HiPot!
LOCATE 10, 1: PRINT "Voltage";
PSET (0, D!(1))                'For graphics screen.
FOR I = 1 TO TOPNUM
LINE -(I, D!(I))
NEXT

Peak.Integration:

LOCATE 23, 1: PRINT SPACE$(75): PRINT SPACE$(75);
LOCATE 23, 1
'INP "Enter time ms = start of CL peak:", Begin.Peak.t
Begin.Peak.t = 0
Delt.Time.A = Begin.Peak.t - Time.Lo
X.Index.A = Delt.Time.A / Time.Range * TOPNUM
LOCATE 23, 1: PRINT SPACE$(75)
LOCATE 23, 1
'INPUT "Enter time (ms) = end of CL peak:", End.Peak.t
End.Peak.t = 6000
Delt.Time.B = End.Peak.t - Time.Lo
Peak.Area! = 0
FOR I = X.Index.A TO X.Index.B
     Peak.Area! = Peak.Area! + D!(I)
NEXT
LOCATE 23, 1: PRINT SPACE$(75)
LOCATE 23, 10: PRINT "Total Area is "; Peak.Area!
LPRINT "Total Area is ", Peak.Area!
LPRINT
Cor.Peak.Area! = Peak.Area! - (TOPNUM * Avgbaseline!)
LOCATE 24, 10: PRINT "Peak Area is", Cor.Peak.Area!
LPRINT "Peak Area is ", Cor.Peak.Area!
LPRINT : LPRINT : LPRINT : LPRINT : LPRINT
RETURN