ON-LINE FERMENTATION MONITORING VIA RECOMBINANT FIREFLY LUCIFERASE

by

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ABSTRACT

A novel method the for on-line monitoring of bacterial fermentations was devised and implemented in batch cultivations of *Escherichia coli*. The bacteria were first modified by transfection with a plasmid containing the recombinant gene for firefly (*Photinus pyralis*) luciferase. The plasmid led to constant activity levels of luciferase in cells grown on a glycerol minimal medium. Luciferase catalyzes the oxidative decarboxylation of luciferin to oxyluciferin. The reaction consumes ATP and emits photons stoichiometrically. Intact bacteria expressing luciferase emitted light when luciferin was supplied in the growth medium. A lack of ATP prevented non-viable cells from emitting light. Applying this method did not effect the growth rate or productivity of the bacteria.

The monitoring method was demonstrated in a 4.5 liter benchtop bioreactor. An optical fiber carried the light signal to a computer-monitored detector. The light signal generated by the viable cells was shown to indicate the intracellular concentration of ATP in the growing cells. During exponential growth, that concentration initially was constant at about 3 mM, but then decreased to about 0.5 mM, the reported value for K_M. The light emission obeyed Michaelis-Menton kinetics with respect to both luciferin and ATP. When the ATP concentration was constant, the light signal provided a direct measurement of the number of viable cells in the bioreactor. Neither non-viable cells nor the extracellular portion of the culture broth emitted any light. Signals were detected from fewer than 10^6 viable cells per milliliter and from the maximum density of 14 g/L DCW.

The medium pH was adjusted to 6.0 to facilitate luciferin diffusion into the cells. Luciferin that was added to the bioreactor was consumed by luciferase. An equation was derived to correct the light signal for this changing substrate concentration. It required knowledge of the light detection efficiency, which was on the order of 10^{-11} photons detected per photon emitted. Experimental evidence proved that inner filter effects were not significant, even at the highest cell densities. When it was above 15%, the dissolved oxygen level did not effect light emission. This method could be a valuable tool for laboratory investigations into energy metabolism.

Thesis Advisor: Daniel I. C. Wang
Title: Institute Professor

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1. INTRODUCTION

1.1 BACKGROUND

Modern bioprocesses have descended from food- and beverage- producing fermentations which date back to antiquity. These early processes were probably random events, brought about by the inadvertent exposure of foodstuffs to airborne organisms. The ensuing changes in the food presumably were appreciated, and attempts were made to re-create them. More controlled processes were developed much later, including strain maintenance and propagation for bread, brewing and dairy cultures as well as environmental controls to improve product consistency. Early this century, fermentations began to be used for making bulk chemicals and fuels. In the 1940s, bioprocess production of pharmaceuticals began with the development of a process for producing penicillin. Bioprocesses based on microbial fermentations are now producing goods worth billions of dollars annually.

The relatively recent development of techniques for gene manipulation is in large part responsible for the success of this industry. In the 1960's and 1970's, as the understanding of genetic manipulation grew, it became possible to use familiar, well-characterized organisms to produce peptides and proteins discovered in other species. Once a gene was isolated, it could often be expressed in virtually any cell line. For instance, human insulin could be made in yeast, or human growth hormone produced by *Escherichia coli*.

Nearly as important to the success of the nascent biotechnology industry as genetic engineering was the advent of mammalian cell culture for the production of therapeutic proteins. Many of the human proteins of interest to the medical community have complex tertiary and quaternary structures that cannot be properly duplicated in bacterial or yeast cells. Attempts to do so lead to improper protein folding, improper disulfide bond formation and/or inadequate glycosylation. Expressing the appropriate gene in mammalian cells instead of yeast or bacteria, however, will usually result in production and secretion of the fully formed, properly glycosylated product. Proteins made in mammalian cell cultures are sold for many billions of dollars annually.

As with all injected or ingested drugs, pharmaceuticals produces via bioprocesses must be subject to quality control and purity regulations. The complex nature of the protein and especially glycoprotein products makes them virtually impossible to characterize completely; so alternative means are used to assure quality. The United States Food and Drug Administration (FDA) requires rigid maintenance of process parameters, including feeds, inoculum, process variables, and cell content, to assure consistency between batches. The FDA approves a new biologically produced drug based not only on the drug's effectiveness, but also upon the process used to make it. This approach attempts to ensure that the product is always identical to that which was used during the evaluation period.

One absolute requirement common to both fermentation and cell culture bioprocesses is living cells. These cells use their natural metabolic pathways to convert the relatively simple and abundant feeds into the highly complex, valuable products encoded in their DNA. But determining the number of viable cells in a bioreactor is a difficult task because they can be very difficult to distinguish from their non-viable counterparts. One rarely utilized distinction between viable and non-viable cells is the production and storage of energy in the form of adenosine 5'-triphosphate, or ATP, in the former but not the latter.

Firefly luciferase is an interesting enzyme which uses ATP as a substrate. Luciferase comes from the light organs in the tails of the North American firefly, *Photinus pyralis*. It catalyzes a reaction requiring among the substrates and emitting yellow-green light as one of the products. The number of photons released is directly proportional to the amount of ATP consumed. Light in this color range is easily measurable by electronic equipment; so luciferase activity can readily be quantified electronically. Thus, firefly luciferase provides an excellent means for quantifying ATP in solutions. So expression of the luciferase gene could lead to a means of monitoring intracellular ATP levels, since the light produced will leave the cell and can be measured externally.

1.2 MOTIVATION

It is clear that accurate monitoring of bioprocesses is vital to the biotechnology industry. Also, there is currently no method for rapidly distinguishing viable, growing cells from non-viable cells in culture. An on-

line method to monitor the viable cell content of a bioreactor could be very helpful in the monitoring process. Especially in mammalian cell culture, the fraction of cells which are viable is known to fluctuate during the course of a batch culture. Bacterial cultures tend to have viability fractions very near to unity, but they can still serve as simple model systems for the development of new techniques for culture viability measurements. On-line knowledge of the fractional viability of a culture may permit the use of more robust control strategies which maintain high viabilities, thereby increasing productivity and reducing the occurrences of poor batches.

The extracellular environment of a bioreactor can be well-monitored and accurately modeled. The intracellular space is less amenable to external observation; although decades of research have improved our understanding of the inner workings of the cell. Simplified metabolic models can describe the internal workings of these cells on a mathematical basis. The structures and functions of countless enzymes, macromolecules and multimolecular assemblies have been elucidated. But while a great deal is known about the internal metabolism and biochemistry of cells, there is a lot that is still unknown. This is especially true in regards to the maintenance and regulation of various internal parameters. Additional knowledge in this area could be extremely beneficial in attempts to increase the level and/or duration of cellular productivity. A monitor which provides a rapid assessment of the intracellular energy availability could prove extremely beneficial to investigations of the metabolism of cultured cells. Such a monitor would also be useful in investigating the energetics of subcellular organelles in mammalian cells. Since energy is required for protein production and secretion, it may eventually lead to control strategies which improve bioprocess productivity.

1.3 RESEARCH OBJECTIVES

The goals of this research project fall into two categories. The first pertains to viable cell determination and the second to intracellular ATP quantification. In both cases, expression of the firefly luciferase gene by the cultured cells is the key step in the approach. *Escherichia coli* fermentations will be used as the model system. These cells will provide a relatively simple, well understood platform from which to analyze and

understand the new techniques. Future research can utilize these findings to adapt the processes to mammalian cell cultures.

First, this project seeks to introduce and evaluate a novel approach to viable cell determination. Because the most significant way in which viable cells differ from non-viable cells is in their activity or lack thereof, this approach will attempt to distinguish them on the basis of intracellular energy stores in the form of ATP. Cells which lack ATP can have no chemical energy available for metabolic activity; so they must be considered non-viable. Those which do have ATP available not only can metabolize feeds and make products but also can make light via the ATP-requiring luciferase reaction. This project seeks to demonstrate the utility of luciferase expression to the on-line determination of viable cell density in active cultures by demonstrating the correlation between light production and culture viability.

As mentioned above, ATP stores are vital to cellular function. It follows that fluctuations in the ATP pool will effect culture behavior. So understanding the behavior of cells in response to changes in ATP availability would be beneficial to all processes which rely upon viable cells. Such knowledge must begin with measurements of the intracellular ATP pool. The second objective of this thesis is to provide a means to rapidly and accurately quantify the intracellular ATP pool in a culture of growing cells. These measurements will be a significant improvement over more tedious techniques which require external manipulations and extractions prior to assays for ATP.

1.4 THESIS ORGANIZATION

This thesis is divided into six major chapters. The first chapter introduced the topic by a brief discussion of bioprocess engineering and an explanation of the reasons for pursuing this research project, as well as its specific aims. The second chapter provides additional background information which may be of relevance to this project. It covers both fundamental concepts and recent advances in biomass and ATP quantification, the genetics and enzymatics of firefly luciferase, and light collection and measurement. The third chapter details all of the equipment and techniques used over the course of this research project. Special attention is paid to

the construction of the luminometer, or light-measuring device, that was used.

Results and discussion of the experiments are presented in the next two chapters. Chapter 4 covers the cell viability aspects of the project. The usefulness and limitations of firefly luciferase expression as a tool for quantifying viable cell densities are discussed, and results of experiments in this area are presented. Chapter 5 presents and discusses the results of experiments designed to evaluate the ability of expressed firefly luciferase to quantify the intracellular ATP pool. The final chapter summarizes the findings of this thesis and presents some conclusions. Additional sections that follow Chapter 6 include brief recommendations for the continuation of this research project, a list of cited references and an appendix containing a listing and explanation of the original computer code used in this research.

2. LITERATURE REVIEW

2.1 MONITORING BIOMASS IN FERMENTATIONS

Fermentors utilize live cells to convert feed compounds to extremely complex products. Required feeds are a carbon source, a nitrogen source and some salts or trace elements. The products are usually polypeptides or proteins. In mammalian cell culture, certain hormones, vitamins and amino acids are also required, and more complex glycoproteins can be produced. In each case, the bioreactor must be monitored to evaluate the progress of the fermentation and to control the bioreactor. Many basic monitoring devices are well-established and will not be discussed here, including pH and dissolved oxygen (DO) probes and thermocouples. Biomass monitoring and optical methods will be covered in some detail.

2.1.1 Traditional Off-Line Analyses

Most commonly used means of quantifying biomass are performed off-line. That is, a sample of reactor broth must be removed and analyzed in a manner not connected to the computer that monitors the bioreactor. The cell content of the reactor is measured in terms of either cell mass or cell number (Gerhardt 1994). Mass measurement techniques provide dry cell weights (DCW) and wet cell weights (WCW). Total cell numbers can be evaluated by absorbance, by particle counters (Coulter Counter®), or, for anchorage-dependent mammalian cells, by nuclei counts. Viable cell numbers can be determined by either colony formation assay for bacteria or by dye-exclusion hemocytometry for mammalian cells.

For both DCW and WCW, the procedure requires a known volume of broth that is assumed to accurately represent what is in the reactor. The sample must be treated to isolate the cells from the liquid medium, generally by centrifugation. WCW is simply the mass of the pellet normalized by the sample volume. The pellet may be subjected to an additional wash step to ensure removal of all medium components. DCW continues from the WCW process, drying the washed pellet by heating until all water, including intracellular water, is removed. DCW is the mass of the dried pellet normalized by the sample volume. DCW and WCW tend to have significant standard errors, especially for bench-scale fermentations, because the sample volumes are small. This means that the masses being measured

are very small. In the early stages of a bacterial fermentation, the DCW of a 5 ml sample can be less than one milligram. Also, DCW measurements are slow. The time required to dry the samples is on the order of a day.

Total cell counts are most easily obtained by particle counts or by absorbance (OD). For either method, the sample is simply diluted into the linear range of the instrument with an appropriate blank solution, and the Coulter Counter® or spectrophotometer provides the output. According to the Beer-Lambert Law, OD is directly proportional to the number of particles (i.e.: cells) absorbing at the chosen wavelength. The Coulter Counter counts particles directly. It monitors the resistance between two electrodes, one of which is placed in the sample and the other is inside an electrolyte-filled glass tube. The electric current carried by the electrolyte must flow through a small orifice in the glass tube. A slight vacuum slowly pulls the sample through the same orifice. When cells pass through the orifice, they momentarily decrease the area filled by electrolytes, thereby increasing the resistance. The orifice diameter must be chosen to be roughly one order of magnitude greater than the cell diameter. Rough size determinations can be made by measuring the magnitude of the resistance change; this is proportional to the cross-sectional area of the cell in the orifice.

Nuclei counts require more procedural steps by the researcher. The sample must first be treated to release and stain the nuclei, usually with crystal violet (Freshney 1987). The nuclei are then counted using a microscope and calibrated slides. Nuclei counts are preferable to cell counts for anchorage-dependent cells both because mammalian cells growing on any curved surfaces are virtually impossible to count visually and because most methods used to release whole cells damage some of them. So deliberately lysing the cells and releasing nuclei gives a more complete count, although it does not permit distinction of viable cells.

Counting viable cells requires some means of distinguishing them from the non-viable cells. With mammalian cells, the most common method is testing the plasma membrane permeability. A dye that does not pass through intact plasma membranes, usually trypan blue, is added to sample of cell suspension. Any cells which take up the dye are considered non-viable. The dyed sample is spread on calibrated slides and manually counted as viewed through a microscope. Bacterial viability, on the other

hand, is usually defined by the ability to grow and reproduce into colonies. Cell samples are diluted and a known volume is spread on solid nutrient medium. After sufficient time has passed, the resulting colonies are counted. Each represents one colony-forming unit (cfu), which is assumed to have been a single bacterium in the original sample. Both of these techniques are subject to errors in dilution and counting, and they are time consuming and labor intensive. Neither can be adapted to on-line monitoring.

2.1.2 Other Off-Line Analyses

Several newer techniques have been developed to determine the mass or number of cells in a bioreactor. There are also some novel techniques for determining viability, including two off-line methods. The first uses the electro-optical method to determine bacterial viability (Lapysh et al. 1989). For this method, sample of fermentation broth must be washed and suspended in an electrolyte solution of a fixed conductance at a pre-set cell concentration and a known OD. The cuvette is then placed in an orienting electrical field. The frequency of the electrical field is varied in a controlled manner while the OD is continuously measured. The change in OD from the pre-set value is recorded, and the fractional viability is simply the ratio of the change in OD of the sample to a the change in OD of the standard, which is measured for a suspension of 100% viable cells. This measurement is valid in the frequency range $7.5 \leq \lg(\text{freq.}) \leq 7.8$, where damaged bacteria make little or no contribution to the change in OD. The process is much faster than the colony-forming-unit method, as it requires only 20 minutes per sample as compared to 15 hours.

The other technique is for determining the viable cell content from a plant cell bioreactor (Sowa and Towill 1991). This method relies on Fourier transform infra-red spectroscopy to quantify intracellular aqueous CO_2 in the sample. This compound was discovered to be proportional to the viable cell number, and was absent from cells killed by freeze-thaw cycling. Thus, the magnitude of the CO_2 peak is proportional to the number of viable cells in the sample. Internal reflectance sampling of the cells is used to avoid damaging them; so if they are treated aseptically, the samples may be returned to the bioreactor. Adaptation of this technique to on-line application may be difficult because internal reflectance sampling requires the

cells to be close-packed around the internal reflectance element (IRE). It would be very difficult to design an on-line or *in situ* device that will have proper packing geometry.

2.1.3 Direct On-Line Analyses

Some progress has been made in the direct, on-line measurement of the cell content of bioreactors. Among the first developments in this area was a fiber-optic device to measure optical density (Hancher, Thacker et al. 1974). This turbidimeter relied on retroreflectance, rather than transmission, to measure the turbidity. Retroreflectance spectroscopy captures and measures the light reflected by the sample directly back at the source (180° scatter). An optical fiber carried the incident light to a flow cell and the reflected light to the detector. A bubble trap prevented air bubbles from interfering with measurements. The light source was calibrated to a known intensity, and the intensity of the reflected light was measured. The ratio of these intensities was directly proportional to the number of cells in the reactor. The device provided a linear response to 50 g/L WCW.

Lee refined this technique to make a probe which was directly insertable into a bioreactor (Lee 1981). He measured the OD by transmission, with the light supplied by a light emitting diode (LED) and detected by a photodiode. Optical fibers carried the light into the reactor and back to the photodiode. The ends of the fibers faced each other at a fixed distance. The ends were well-polished to minimized fouling, which would lead to falsely high readings. They also used a pulsed light source and a matching electronic filter on the photodiode signal. This allowed the probe to accurately measure OD even in the presence of ambient light of the same wavelength. The signal was linear up to 6 g/L DCW and accurate to over 15 g/L. Unfortunately, the probe was not steam-sterilizable. Nor could it be used in aerated fermentors, because the bubbles could not be distinguished from cells. This technique has since been improved, in large part by the availability of small, inexpensive lasers. Reliable, steam-sterilizable, laser turbidity probes are now available from many commercial sources.

A different group devised a technique to measure total cell mass by acoustic resonance densitometry (ARD) (Kilburn et al. 1989). With a commercially available ARD device, the oscillation period, t, of the sample was

measured. The reciprocal of the sample density is linearly related to t^2, with the slope and intercept determined by calibration. A flow cell and bubble trap were used to deliver the sample to the ARD instrument and to remove the gas phase prior to measurement. Manually operated valves could send the medium through filters to remove the cells and permit readings of cell-free medium. Biomass density was the difference between the densities of the cell-containing and the cell-free samples. This method was tested for both bacteria and mammalian cells. It was shown to be very accurate, provided the biomass density was above a certain minimum (at least 10^6 cells/ml for mammalian cells).

Another group attempted to use a bacterial luciferase to make luminescent *Escherichia coli* for an on-line detection system (Huang et al. 1993). They transfected the *E. coli* with the genes *luxA-E* of *Vibrio fischeri* (see Section 2.2.1 below). They then monitored the luminescence from a batch fermentation of the transfected *E. coli*. The luciferin was being steadily supplied within the cells by the *luxC-E* gene products; so the enzyme and $FMNH_2$ were the limiting reagents. They found that the light production was very low through most of the fermentation, but rose to a maximum at the same time as the maximum rate of viable cell production was reached. The light output was also extremely sensitive to DO levels; so they had to maintain the DO above 60% air saturation. Through extensive calibration and statistical analysis, a method was devised to calculate the cell density as a function of light and elapsed time. The calculation method is based on the instantaneous growth rate; so the viable cell number must be known at some time to find the value at any other time.

2.1.4 Indirect On-Line Analyses

Other methods for estimating the viable biomass or cell number in an operating bioreactor are frequently used. These are indirect techniques. A different parameter is measured, and the biomass is estimated based on that value. The last example above could be considered an indirect measurement technique because it estimates cell number based on a growth rate calculated from observed luminescence.

One indirect biomass estimation method is based on the oxygen uptake rate (OUR) and/or CO_2 evolution rate (CER). Most bacterial fermentations monitor the off-gas composition and have defined feed gas

compositions and flow rates. The difference in oxygen levels between the feed and off-gas gives the OUR for the reactor and the difference in CO_2 levels gives CER. If it is assumed that the per-cell oxygen demand and/or CO_2 productivity is constant for viable cells, then the number of viable cells in the bioreactor can be easily calculated from the OUR or CER. A nearly identical method can be applied to glucose consumption. On-line glucose sensors can provide measurements of the glucose level in the medium, and glucose feed rates can be recorded. So the glucose uptake rate (GUR) can be calculated. The assumption of constant specific glucose demand leads to an easy calculation of the viable cell content of the reactor.

The other commonly used indirect measure of viable biomass is based on NADH and NADPH. These molecules, which are jointly referred to as NAD(P)H, are not secreted from cells, and are only released upon cell lysis. In aerated media, they are rapidly oxidized to the $NAD(P)^+$ form. It has been shown that NAD(P)H is be a good indicator of cellular activity in some cultures (Li et al. 1991). Cellular activity by definition only occurs in viable cells; so a probe for NAD(P)H gives an indirect measure of viable cells. Such probes, based on the work discussed below, are commercially available. They have been improved to be steam-sterilizable and insertable into the bioreactor.

2.1.5 Other Optical Sensors

Rapid detection of NAD(P)H is best achieved by fluorometry. NAD(P)H fluoresces strongly at an emission peak of 460 nm when excited with light at 340 nm. The oxidized form, $NAD(P)^+$, does not fluoresce. Harrison and Chance were the first to take advantage of this property for an *in situ* probe for total culture NAD(P)H (Harrison and Chance 1970). They directed the excitation beam through the glass wall of the bioreactor and measured the emission intensity in the same manner. Enzymatic assays on samples confirmed that they were accurately recording total culture NAD(P)H. This fluorometric technique was further refined by the use of multiple excitation and emission wavelengths to quantify additional fluorophores that are common in bioreactor cultures (Li et al. 1991). The four species measured were tryptophan, pyroxidine, riboflavin and NAD(P)H. The emission spectra for these fluorophores overlap, but by exciting with one wavelength at a time and comparing the emission spectra,

these overlaps were resolved to give accurate concentrations of each of the fluorophores.

Several other optical biosensors are based on fluorescence coupled to enzymatic reactions. The general idea is to immobilize the enzyme(s) in a compartment which is separated from the fluorophore by a gas-permeable membrane. The whole assembly is at the end of an optical fiber for fluorescence measurements. The gaseous product or substrate of the enzymatic reaction crosses the membrane and quenches or stimulates fluorescence (Arnold 1990). For example, O_2 quenches the fluorescence of many dyes; so DO can be measured directly by fluorescence quenching (Junker 1988). If such a probe for DO is coupled with immobilized glucose oxidase, the device could indirectly measure glucose levels from direct measurements of the O_2 consumption rate. Similar work has been done with NH_3 and CO_2 as the gaseous species. These may alter the fluorescence properties by causing pH shifts and/or than quenching. Among the devices based on this concept that have been reported are ones that measure lactate, xanthine, ethanol, H_2O_2, urea, and glutamate (Arnold 1990).

A different family of fiber-optic biosensors are based on luminescence, rather than fluorescence. For these biosensors, there is no need for excitation light; so the fiber only carries light to the detector from the reaction site. The best examples of this approach use firefly or bacterial luciferases for the detection of ATP or NAD(P)H (Blum et al. 1988; Blum et al. 1989). NADH detection was accomplished by immobilizing bacterial oxidoreductase, which catalyzes the oxidation of NAD(P)H by FMN to give $NAD(P)^+$ and $FMNH_2$ in addition to bacterial luciferase. ATP and $FMNH_2$ are substrates for the firefly and bacterial luciferase reactions, respectively (see below). Use of a flow cell permits a steady supply of buffer and reagent (luciferin) and prevents interference by ambient light. Light intensity from these probes gives a direct measurement of concentration. Another luminescent reaction studied used a supply of luminol and was catalyzed by horseradish oxidase. The concentration of the other substrate, H_2O_2, was found directly from the light intensity. The limit of detection was around 0.3 nM for ATP and NAD(P)H and around 20 nM for H_2O_2, The probe range covered 3-4 orders of magnitude for each species. The reactions can be coupled to permit indirect measurement of other species (i.e.: alcohol dehydrogenase plus the bacterial luciferase system measures

ethanol) (Blum et al. 1989). Another study combined the two luciferase systems in a single probe, but it resulted in a reduced range for NAD(P)H and did not suggest any means for distinguishing the very similar spectra generated by ATP and NAD(P)H (Gautier et al. 1990).

2.2 FIREFLY LUCIFERASE

There are over 1800 known species of bioluminescent organisms (Ziegler and Baldwin 1981). As a group they cover a large portion of the visible spectrum, but each emits light at a specific wavelength. In each case, the light is produced by a reaction catalyzed by an enzyme known as a "luciferase". The primary substrate of a luciferase is "luciferin". The luciferase reaction leads to a product molecule with at least one electron in an excited state. Light is produced when the electron goes to the ground state. Both luciferins and luciferases vary among the bioluminescent species. In addition to a luciferin, luciferases may require two other substrates. One, often O_2, reacts with the luciferin to form the product molecule, and the other provides the driving force for the reaction. Thus bioluminescence is a drain on the internal energy supply of the organisms. It is unknown why bioluminescence exists, although in fireflies it appears to be involved in mate selection (Shimomura 1982).

2.2.1 Bacterial Luciferases

Many species of marine bacteria are bioluminescent. The enzymes responsible for this activity have been characterized for many species and cloned for at least one, *Vibrio fischeri*. The *V. fischeri* luciferase/luciferin system is a good model for bacterial bioluminescence (Shimomura 1982). The luciferase is a heterodimer consisting of α and β subunits which each have a molecular weight of around 40 kDa. The luciferin for bacterial luciferin is a long-chain aldehyde, which is oxidized to a fatty acid of the same length. $FMNH_2$ provides the oxidative force, and O_2 provides the extra oxygen atom (see Reaction 2-1).

$$RCHO + FMNH_2 + O_2 \longrightarrow RCOOH + FMN + H_2O + h\nu \qquad (2\text{-}1)$$

When the luminescent system for *V. fischeri* was cloned, seven genes were found and named *luxA-E, luxR* and *luxI*. The first two code for the luciferase subunits, and the last two code for regulatory elements on the *lux* operon (Engebrecht and Silverman 1984). The remaining three

genes encode enzymes involved in recycling the fatty acid product to the aldehyde substrate for the luminescent reaction. Thus, this system is quite robust because the enzymes necessary to make the luciferin are all in the same operon as the luciferase genes are.

The robust nature of the *lux* operon has led to some interesting uses. One such use is as a cellular reporter on the effectiveness of biocides and virucides (Jassim et al. 1990). The operon is inserted on a plasmid and expressed at high levels in the cells of interest. Effective biocides will prevent light production, whereas effective virucides will prevent active viruses from killing the host and preventing light production. Another interesting use of the operon is for sensitive detection of pollutants such as naphthalene in soil samples (King et al. 1990). The *lux* operon was inserted into naphthalene-metabolizing bacteria and placed under the same genetic control as the *nahG* protein, which is part of the naphthalene degradation pathway. In a naphthalene-containing environment, the bacteria emit light in rough proportion to the amount of naphthalene.

It could seem that the *lux* operon would be very useful to this research, especially for viable cell detection. However, the regulatory portion of the operon leads to autoinduction and overexpression of all the *lux* genes at or above a certain cell density. Also, the aldehyde luciferins can be toxic to mammalian cells at fairly low concentrations. In addition, expression of the heterodimeric luciferase would be very difficult in mammalian cells. Because this research is intended to continue in mammalian cell culture, the last two problems are quite significant. The latter problem has been solved. A chimeric protein which shows normal luciferase activity in both prokaryotes and eukaryotes was created by fusing the two subunits of *Vibrio* luciferase. (Boylan et al. 1989).

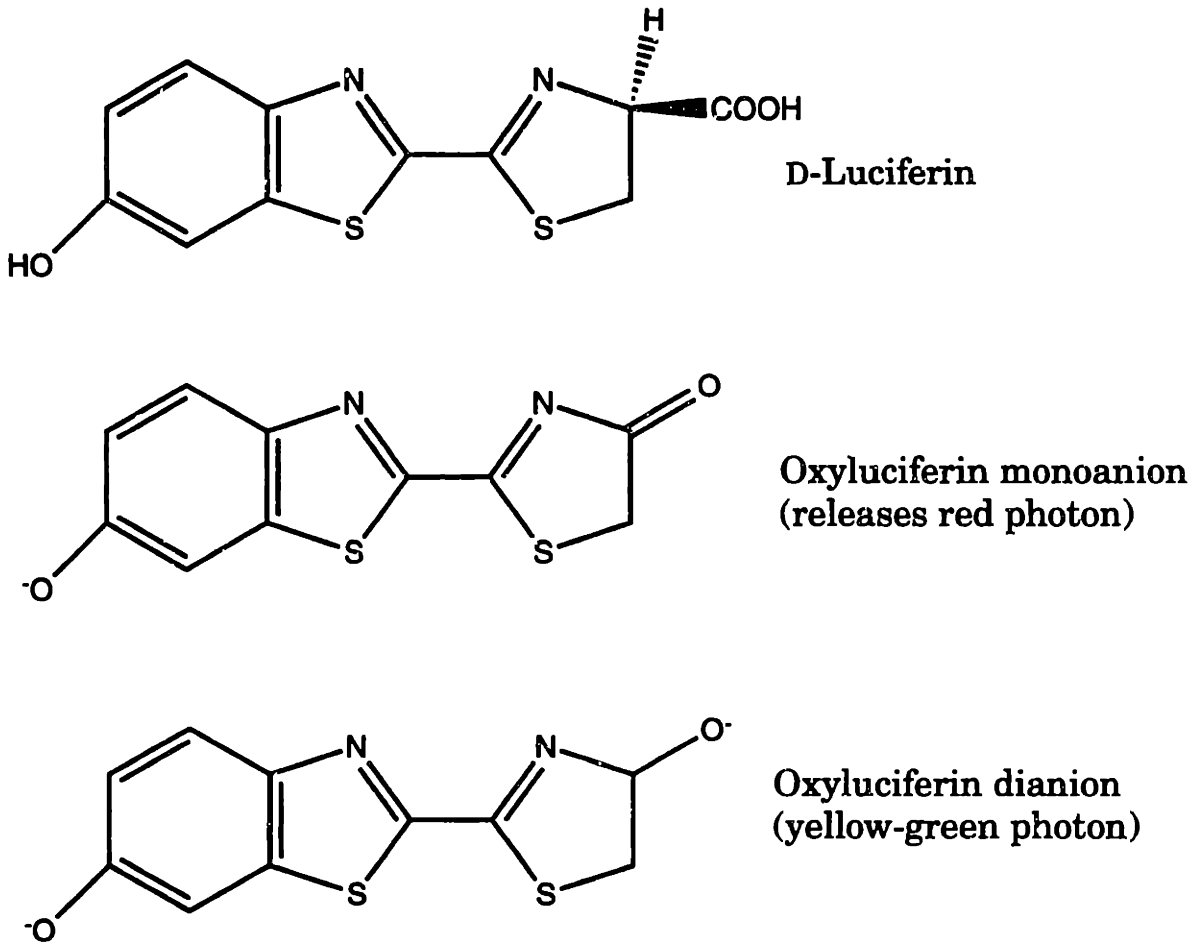

Figure 2-1: Structures of Luciferase Substrate and Product
The excited electron is in a resonance structure around the carbonyl carbon of oxyluciferin. The double ring on the left side is the benzothiazole portion. Note that the right side of luciferin is very like cysteine.

2.2.2 Structure and Reaction

Firefly (*Photinus pyralis*) luciferase (EC 1.13.12.7) is a single polypeptide which, according to its cDNA sequence, has a molecular weight of 62 kDa (De Wet et al. 1985). Non-reducing gels run before the DNA was cloned indicated that is had an apparent molecular weight of around 50 kDa and functions as a 100 kDa homodimer (McElroy and DeLuca 1985). A large number of bioluminescent insect species have very similar enzymes with identical substrates and products. For the remainder of this thesis, "luciferase" will refer to firefly luciferase, and "luciferin" will refer to firefly luciferin.

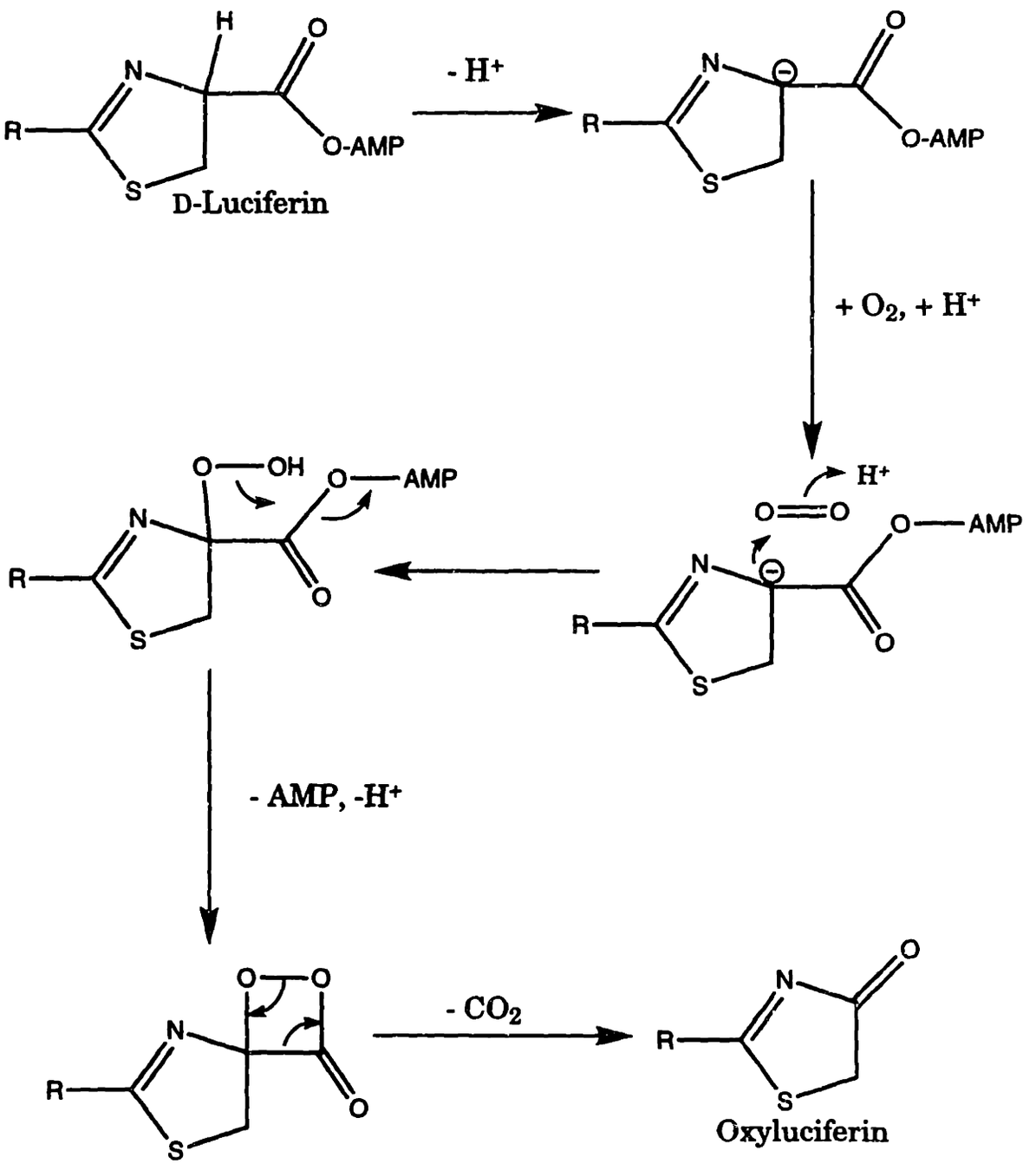

Figure 2-2: Mechanism of the Reaction Catalyzed by Luciferase
The dioxitanone intermediate is the structure at bottom left.

Firefly luciferin (LH_2) is a stereo-specific, multi-ring molecule with the structure shown in Figure 2-1. Its biosynthetic pathway is unknown, but an organic synthesis for its production was devised in the 1960's (White et al. 1965). Luciferase first catalyzes the reaction of LH_2 with ATP to form the activated luciferin adenylate (LH_2AMP), which then reacts with oxygen. The magnesium salt of ATP is the actual form of the substrate. The magnesium ion is bound to the charged oxygen molecules on the two outermost phosphate groups (Lee et al. 1970). If available, LH_2AMP can also serve as a substrate without requiring additional ATP. The reaction with oxygen proceeds through a dioxitanone intermediate (Shimomura 1982) (see Figure 2-2). The product is an oxyluciferin (L=O) molecule (see Figure 2-1) with an electron in an excited state. A photon of yellow-green light is normally released when the electron goes to the ground state. This occurs while the L=O is still held by the enzyme. AMP, pyrophosphate, and carbon dioxide are by-products.

At the optimum reaction pH of 7.8, the light produced by luciferase bioluminescence has a maximum at a wavelength (λ_{max}) of 562 nm. The emission spectrum has a half-height peak width of 70 nm. The quantum efficiency of the reaction is 88%, meaning that for every 100 molecules of luciferin reacted, 88 photons are released. As the pH becomes more acidic, a second peak appears at 610 nm. Below pH 6.5, this peak is the dominant one. This red shift is due to photon release from the monoionic form of oxyluciferin. The quantum efficiency of red light production is only 20% (McElroy and DeLuca 1985).

Little is known about the active site of luciferase. Removing the twelve C-terminal amino acids reduced luciferase activity by two orders of magnitude. So the C-terminus is near, but not essential, to the active site (Sala-Newby et al. 1990). It has been shown that the adenylation of luciferin is accompanied by a conformational change in the enzyme (Thompson et al. 1991). In this conformation, the hydroxyl proton on the benzothiazole portion of LH_2 remains bound even at high pH, indicating that the active site is very hydrophobic. Another known feature of the active site is that a free sulfhydryl group from an unpaired cysteine is required for luciferase activity.

Each of the luciferase substrates has its own binding site. Once ATP is bound, luciferin binding is enhanced. The bicyclic backbone of luciferin

is the structure that is held in the binding site. The benzothiazole nitrogen has been shown to be especially important for binding (Denburg et al. 1969). The ATP binding site is highly specific for the magnesium salt of ATP. The only other nucleotide that was found to bind there was 3-iso-ATP (Lee et al. 1970). ATP ion without magnesium acts as a competitive inhibitor against MgATP; so an excess of Mg^{++} is beneficial. Other divalent cations also lead to luciferase activity, but not as well as Mg^{++}. Luciferin only has a minimal effect on ATP binding.

Luciferase is also sensitive to the presence of detergents. With the exception of the anionic ones, which are inhibitory, most detergents stimulate luciferase activity. However, cationic and zwitterionic detergents permanently inactivate the enzyme. Non-ionic detergents tend to stimulate activity without inactivating the enzyme (Simpson and Hammond 1991). However, it is important to note that neither polypropylene glycol nor Triton X-100 has an effect on luciferase activity. These sensitivities indicate that, *in vivo*, luciferase is probably membrane-associated. This theory is supported by Ugarova, who found that, in solutions, luciferase associated with liposomes when they were available (Ugarova 1989).

Luciferase has been successfully expressed in a wide variety of cell types, including mammalian, plant, fungal and bacterial (Subramani and DeLuca 1988). However, luciferase is not very stable *in vivo*. In mammalian cells, it has a half-life around 3 hr, and in *Escherichia coli*, the half-life is only 45 min. The mRNA shows normal stability; so it must be the enzyme that is degraded. When a strongly competitive inhibitor to luciferin is bound by luciferase, the accompanying conformational change protects the enzyme from proteolytic degradation (Thompson et al. 1991). In many of the expression systems, attempts were made to assay for luciferase activity *in vivo*. Luciferin was added externally to the cultures. The only reported case of partial luciferin toxicity was in plant cell culture at concentrations above 400 μM (Ow et al. 1986). Other researchers reported that luciferin was non-toxic.

2.2.3 Reaction Kinetics

In fireflies luciferase catalyzes the bioluminescent reaction in a manner characterized as "flash kinetics." Within half of a second from the initial mixing of the enzyme and substrate, the maximum level of light

production is reached. This level declines rapidly in an exponential fashion to an equilibrium level many orders of magnitude below the maximum (McElroy and DeLuca 1985). However, if one or more of the substrates are limiting, the rate of the decline decreases. If the limiting substrate is one to two orders of magnitude below the K_M, there will not be any decline at all.

The reaction catalyzed by luciferase can be summarized as in 2-2 thru 2-5:

$$E + LH_2 + MgATP \longrightarrow E{\cdot}LH_2AMP + PP_i + H_2O \qquad (2\text{-}2)$$

$$E{\cdot}LH_2AMP + O_2 \longrightarrow E{\cdot}L{=}O^* + AMP + CO_2 \qquad (2\text{-}3)$$

$$E{\cdot}L{=}O^* \longrightarrow E{\cdot}L{=}O + h\nu \qquad (2\text{-}4)$$

$$E{\cdot}L{=}O \longrightarrow E + L{=}O \qquad (2\text{-}5)$$

The asterisk indicates an excited electron. Note that the photon is released while the oxyluciferin is still bound by the enzyme. This is why enzyme structure affects the color of the photon (see Section 2.2.6). Reactions 2-2 and 2-5 are both rate-limiting steps when the substrates are in excess (Schram et al. 1981). When either ATP or LH_2 is limiting, there is enough enzyme to overcome the slow turnover rate represented by reaction 2-5, and so only reaction 2-2 is rate-limiting.

Interestingly, pyrophosphate (PP_i) not only is a product of the luciferase reaction, but it also affects the reaction rate. At concentrations as low as 10^{-7} M, PP_i accelerates reaction 2-5, increasing the turnover rate for the enzyme and decreasing the flash effect (Ahmad and Schram 1981). Many commercial ATP and luciferase assay kits provide steady light output by means of an appropriate amount of PP_i in the reaction mixture. However, at concentrations above 10^{-5} M, PP_i inhibits the activation step (reaction 2-2). Since reaction 2-2 is a rate-limiting step, it is very important to keep [PP_i] below 10^{-5} M in the reaction mixture.

The K_M values for each of luciferin and ATP vary with pH. They have a minimum when the pH is below 6.3 and a maximum when it is above 8.0 (Denburg et al. 1969; Lee et al. 1970). In the region between these pHs, the K_M's change in a roughly linear fashion with pH. This similarity in the pH dependencies of the two K_M values suggests that there is a con-

formational change associated with changing pH. The K_M values for luciferin range from 1 to 20 μM while those for ATP range from 60 to 400 μM.

A Russian researcher has done extensive kinetic analyses on a closely related luciferase (EC 1.2.3.13) from an Asian firefly (Ugarova 1989). She found two sites which bind ATP as an allosteric activator. The activator binding sites have lower binding constants than does the reactive site; so they are filled first. She also analyzed the kinetics with one additional step for the activation of the enzyme-LH_2AMP complex. In her analysis, this activation is the rate-limiting step. The K_M values for ATP varied from 400 to 800 μM, depending upon whether or not the allosteric ATP binding sites were filled.

2.2.4 Detection *In Vivo*

When luciferase cDNA is expressed in mammalian cells, the C-terminus of the enzyme marks it for transport to the peroxisomes (De Wet et al. 1987; Keller et al. 1987). It is also found in peroxisomes in firefly tails. When expressed in bacteria, the enzyme is scattered throughout the cytoplasm, although there is some evidence that it tends to associate with the inner surface of the plasma membrane. Targeting the luciferase to the cytoplasm in eukaryotic cells may increase *in vivo* activity by halving the number of membranes luciferin must cross from the medium. An attempt to remove the twelve C-terminal residues resulted in greatly decreased activity (Sala-Newby et al. 1990). But other attempts to eliminate peroxisomal targeting by removing fewer residues have succeeded (Gould and Subramani 1988).

It is also possible to modify the N-terminus by deletion of a few residues or by fusion with another protein (Subramani and DeLuca 1988). The size of the fusion partner is important, as very large ones appear to eliminate the luciferase activity by steric hindrance. But a short peptide, such as the KDEL endoplasmic reticulum retention sequence should leave normal luciferase function. This would permit the use of luciferase to study the endoplasmic reticulum, or some other organelle if an appropriate signal is known and fused to luciferase.

Most assays for luciferase expression require rupturing the cell membranes and extracting the enzymes for analysis. This is due to the carboxylic acid moiety of luciferin. At normal culture pH, the molecule is

anionic and will not cross the plasma membrane. So some groups have investigated methods of getting luciferin inside viable cells. For bacteria, it was found that simply shifting the pH of the medium was sufficient for luciferin to pass into the cells (Wood and DeLuca 1987). The magnitude of the shift had to be chosen to avoid harming the cells while still allowing a significant portion of the luciferin in solution to be in the free-acid form. A buffer of pH 6 seemed to suffice. In mammalian cells, however, such a pH shift would cause necrosis under otherwise normal conditions (Freshney 1987). In a buffer of pH 5.2 containing 15% DMSO, luciferin entered CV-1 cells without killing them, although their growth was arrested. Even less harmful is a DMSO buffer at pH 6.9, which is only slightly lower than the normal pH of growth media. Another possible means of getting luciferin into mammalian cells is low concentrations (≤1 μM) of nigericin, an antibiotic which permeabilizes the plasma membrane, but does not kill them (Gould and Subramani 1988).

Modifying luciferin itself would be a better and less harmful way to get it into mammalian cells without harming them. The carboxylic acid cannot be eliminated because it is the reactive center, and the LH_2AMP ester is too large and too polar to cross the membrane. But other, smaller esters might cross the membrane and, once inside, hydrolyze to re-form luciferin. Craig et al. synthesized five luciferin esters and showed that each of them did, to some extent, pass through the plasma membrane into the cytoplasm (Craig et al. 1991). They also showed that endogenous esterases hydrolyzed these esters in the cytoplasm, creating active luciferin. The most reactive of the esters was luciferin ethyl ester (LH_2OEt), which was also the one which crossed the membrane most readily.

Unfortunately, LH_2OEt is difficult and costly to make because its synthesis is a fairly complex process, and the yields for the described techniques are very low. An easily synthesized ester developed by Yang (Yang and Thomason 1993). Its synthesis is an easy, one-tube process with yields on LH_2 of over 90%. The ester is luciferin 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane, or luciferin DMNPE ester. It has been shown to cross the plasma membrane and be hydrolyzed by endogenous esterases. It can also be photolyzed to release luciferin rapidly or if no esterases are present. The precursor reagent is available in a kit from Molecular Probes of Eugene, OR.

A different reason to modify of luciferin is to provide a means of covalently linking it to another molecule as a marker. This marker could serve as a substrate for luciferase and its location visualized by film or another photometric detection technique. Farace et al. have had some success in this area by adding a methyl group to the benzothiazole portion of luciferase, creating 4-methyl-D-luciferin (Farace et al. 1990). This molecule reacted with luciferase with kinetics and spectra similar to unmodified LH_2. Unfortunately, it was only about one-tenth as active as the unmodified substrate.

2.2.5 Genetics and Applications

The luciferase gene was cloned and patented by researchers from the University of California at San Diego in 1985 (De Wet et al. 1985). The form they patented and made available is the intronless cDNA because the genomic DNA was inactive in bacteria and mammalian cells (De Wet et al. 1987). The inactivity appears to be due to the length of the six introns, each of which is much shorter than those typically found in mammalian genes. The cDNA can be expressed to make active luciferase in a wide variety of cell types. The gene is about 1800 base pair (bp) long, coding for 550 amino acids. There is also a short (13 residue) open reading frame (ORF) immediately to the 5' end of the luciferase-coding portion. This ORF has been deleted in several DNA constructs to eliminate any unanticipated effects it may have on luciferase translation.

The same research group constructed many plasmids for luciferase expression. They created four forms of the luciferase cDNA: the complete cDNA (L), L without the firefly polyadenylation signal (L-A), L-A without the 5' ORF (L-AΔ5'), and L without the ORF (LΔ5'). These were each inserted into six different vectors for transient expression in mammalian cells. The vectors are all based on parts of pBR322 and pSV2. One of these constructs is pRSVL, which has the L cDNA expressed from the Rous Sarcoma Virus Long Terminal Repeat (RSV LTR). The RSV LTR has been shown to be a constitutive promoter in prokaryotes (Mermer et al. 1983). They also made at least one vector for stable expression in mammalian cells, pSV2L/SV2Neo (De Wet et al. 1987). This vector contains the gene for neomycin resistance, which renders mammalian cells immune to G418

toxicity. In pSV2L/SV2Neo, L is expressed from the SV40 Early promoter, which also functions as a constitutive promoter in prokaryotes.

The most common use for these plasmids is analyzing promoter and enhancer activity with luciferase cDNA as a reporter. Before luciferase was cloned, chloramphenicol acetyl transferase (CAT) and β-galactosidase were the reporters most frequently used. Reporter genes are placed under the control of the promoter to be studied and the product expression is assayed. Luciferase assays are easy and rapid and do not require radioactive or toxic compounds. The assay for it is linear over more than five decades of concentration; so it is ideal as a reporter gene (Subramani and DeLuca 1988). Nordeen made promoter studies easier by constructing a shuttle vector with several unique restriction endonuclease sites at the 5' end of the luciferase cDNA (Nordeen 1988). This makes inserting promoter sequences in the proper direction a very straightforward process.

The most common use for luciferase is for assaying ATP. This is probably the most accurate means of and sensitive method for assaying ATP in solutions. ATP concentrations as low as 80 fM (8×10^{-18} mole or 8 amole per100 μl) are detectable with a sensitive luminometer and proper assay conditions (Simpson et al. 1990). Purified luciferase, with luciferin, magnesium and buffers are added to a sample containing ATP, and the light level corresponds to the ATP level. A variation on this is determination of bacterial content of samples, especially food samples. Before assaying for ATP, the sample is treated in such a way as to lyse any bacterial cells that may be there, releasing their ATP to be assayed (Stanley 1989). This is only valid if no other ATP is present.

Perhaps the most interesting published application of luciferase cDNA is for the screening of tuberculosis infections for antibiotic resistance. The infectious agent in tuberculosis, *Mycobacterium tuberculosis*, grows very slowly. Its doubling time is around 24 hr; so conventional plating assays to screen for antibiotic resistance take between 2 and 18 weeks. Two groups reduced this time to under 48 hr using luciferase (Cooksey et al. 1993; Jacobs et al. 1993). Both begin by exposing sputum samples to antibiotics, as in the conventional assay. The Jacobs group have constructed a mycobacteriophage, phAE40, which contains luciferase expressed from a strong promoter. They add luciferin to the sputum samples and infect them with the virus. This leads to light production in viable, drug resis-

tant, samples, but those which are susceptible to the antibiotic make no light. The Cooksey group uses a plasmid rather than a phage. The plasmid is inserted by heat-shock, and luciferase is only expressed in drug resistant cells. The samples are lysed by sonication, freeing the luciferase for assay to determine the effectiveness of the antibiotics.

2.2.6 Other Luciferase Emission Spectra

Other modifications to luciferase have changed the spectrum of the light output from the reaction. These efforts were suggested by the isolation and cloning of four luciferases with different emission spectra from a single species of Jamaican click beetle, *Pyrophorus plagiophthalamus* (Wood et al. 1989). Each beetle emits light of one color from its head and a different color from its tail. There are also many color variants between beetles. Sequence analysis of the cloned DNA showed that the sequences are mostly homologous; so a few amino acid changes led to the color variation. *E. coli* expressing the cDNA emitted light ranging from green to orange (λ_{max} from 546 to 593 nm) when supplied with luciferin.

Rearrangement hybrid cDNAs from the same species enabled the researchers to determine exactly which amino acid substitutions were responsible for each color change (Wood 1990). The color shifts were found to be additive in terms of the energy or frequency of the light peak. That is, substituting two groups shifted the frequency of the light output by the sum of the frequency shifts of each individual substitution. Another group introduced point mutations in Genji (*Luciola cruciata*) firefly luciferase by random mutagenesis (Kajiyama and Nakano 1991). They found five single-residue mutants with significantly altered color output ranging from 558 to 612 nm. In some of these mutants, the pH-dependent red shift had been eliminated as well.

Other red shifts can be caused or eliminated by certain modifications of either the luciferin or the ATP structure. While most modified forms of LH_2 or ATP will not react at all, a few will. One that does is 6-amino-luciferin, which emits red light even at high pHs. Another is 3-iso-ATP, which leads to emission of yellow-green light at lower pHs. Both of these reagents reduce the enzyme activity significantly (McElroy and DeLuca 1985).

2.3 ADENOSINE TRIPHOSPHATE OVERVIEW

Adenosine 5'-Triphosphate (ATP) is quite possible the single most important intermediary metabolite in all living organisms. The adenine nucleotides, which also include the di- and mono-phosphates (ADP and AMP, respectively), serve as the primary intracellular chemical energy storage and transfer system. The phospodiester bonds linking the second and third phosphates to AMP are high energy bonds. Countless enzymes couple exothermic reactions to ATP production and endothermic ones to ATP hydrolysis (Zubay 1983). This provides a means for the cell to transfer energy between otherwise unconnected reactions.

In addition to energy storage and transfer, ATP is usually the phosphate donor for phosphorylation. Phosphorylation serves to activate metabolites for future catabolic reactions and to prepare molecules for transport across the plasma membrane. It is also important for switching certain enzymes between active and inactive forms. This function is vital for control of many regulatory pathways such as the blood clotting cascade in humans. The enzymes necessary for clotting are always present in the blood, but they need to be activated by addition or removal of a phosphate group. This type of activation cascade leads to much shorter response times than genetic control would permit.

Another important contribution of ATP to cellular function is as a precursor to RNA and DNA. These macromolecules are built from nucleotides and deoxynucleotides; so the source of all adenosine bases in ribonucleic acids is ATP. ATP is also involved in many cellular responses to extracellular stimuli. It is converted to cyclic AMP (cAMP) for intracellular signaling purposes in several receptor-mediated processes.

2.3.1 ATP and Protein Production and Secretion

ATP has a great deal of significance to recombinant protein production and secretion, primarily as an energy source. First there is the metabolic energy required to produce amino acids for protein assembly. Recombinant peptides can account for over 20% of the total protein in a cell; so this can be a very significant drain. Another ATP drain comes in the ribosomes, where the attachment of each new residue to the nascent peptide change hydrolyzes two GTP molecules. The GTPs are restored by enzymes which convert GDP to GTP at the cost of an ATP. The cost of

elongation does not include the additional ATP required to initiate transcription and translation.

In eukaryotic cells, secreted proteins start as nascent peptide chains in the endoplasmic reticulum (ER). There they undergo the necessary manipulations to fold into the proper tertiary structure and, if necessary, associate into proper quaternary forms. Certain ER-resident proteins called chaperone proteins assist in this process. One of these, heavy chain binding protein (BiP), functions by binding and releasing hydrophobic areas on nascent chains (Darnell et al. 1990). BiP binds these regions quickly, but it must hydrolyze an ATP molecule to release them. Release is necessary for proper folding and eventual secretion. BiP may hydrolyze many molecules of ATP for each molecule of secreted protein.

In addition, ATP is necessary for the secretion of proteins. In prokaryotes, the chaperone protein which recognizes the secretion signal binds and transports the peptide to the plasma membrane. There is secreted by an active transport mechanism requiring ATP hydrolysis. In eukaryotes, secreted proteins are transported by vesicles from the ER to and through the Golgi bodies. From there, they move into secretory vesicles, which can either go directly to the plasma membrane (constitutive secretion) or wait for a signal (regulated secretion). All of this vesicle formation and transport requires energy that is supplied via ATP.

2.3.2 Extraction and Measurement

Because of its importance to so many cellular functions, ATP has been the subject of many studies. For most of these, it was necessary to assay the ATP in the samples being studied. In many cases, it was also necessary to extract the ATP from intact cells in order to assay it. The techniques devised for these processes are discussed below.

Prior to the development of the firefly luciferase assay for ATP, it was usually measured by a combination of purification and quantitation steps. One commonly used method was separation of nucleotides by two-dimensional thin-layer chromatography (Cashel et al. 1969). The isolated nucleotides were then quantified by fluorescence or by radioactive decay, in the case of radiolabeled samples (Bagnara and Finch 1972). Another, more complicated technique involved coupled enzymatic reactions which

led to stoichiometric changes in another, more easily assayed species (Estabrook et al. 1967).

The discovery and characterization of firefly luciferase eliminated the need for these more complicated procedures. ATP could be assayed quickly and without prior purification or radioactive labeling. Other adenine nucleotides could be assayed by enzymatically converting them to ATP and then assaying the ATP (Chapman et al. 1971). If desired, other nucleoside triphosphates can also be enzymatically converted to ATP and then assayed by firefly luciferase. But a simpler way to assay a wide variety of nucleotides is by ^{32}P nuclear magnetic resonance (NMR). This technique requires expensive equipment, but allows accurate quantitation of many nucleotides in one step (Cashel et al. 1969).

The extraction process is more involved. One study compared ten different techniques for extracting adenine nucleotides from bacteria (Lundin and Thore 1975). These methods use acids, bases, solvents, and/or heat to rupture the plasma membrane and release the nucleotides. The stronger acids and bases also deactivate any proteins, as can prolonged heating. The advantage of this deproteination is that it removes all of the many enzymes which can effect the ATP concentration. The number of available techniques leads to different research groups advocating different methods as the most effective.

The two most commonly used techniques for intracellular nucleotide extraction are the trichloroacetic acid (TCA) and the perchloric acid ($HClO_4$) techniques. In each case, the sample is mixed with a quantity of acid on ice. After an incubation period, the acid is neutralized and removed. $HClO_4$ works best between 0.5 and 0.75 M (Dhople and Hanks 1973), while for TCA, final concentrations around 0.25 M suffice (Stanley 1989). $HClO_4$ is neutralized by adding KOH, which leads to $KClO_4$ formation. $KClO_4$ precipitates and can easily be removed by centrifugation along with the cell debris. The KOH may be in solution with a buffering compound to help control the final pH (Bagnara and Finch 1972). TCA can be neutralized with any base, but it must be removed from the samples by repeated extractions with ether. Lundin and Thore found that TCA extracts mono- and di-phosphates from bacteria better than $HClO_4$ does, but the two methods are equally efficient at extracting ATP. They also showed that ATP in the extracted samples is stable indefinitely if kept at or below

0°C. They also report that inhibits the luciferase reaction, but it does so in a consistent manner. So $HClO_4$ extraction leads to accurate ATP quantification provided the standards are treated the in the same manner as the samples are.

Others advocate different extraction procedures. One such study is that of Dhople and Hanks (Dhople and Hanks 1973). They extracted ATP from three different organisms using their own technique. They placed the samples in 23% (v/v) chloroform in a boiling water bath for 10 min. The chloroform was removed by applying a vacuum for another minute. This method extracted approximately 8% more ATP than was obtained using a variation of the $HClO_4$ technique. They also showed that additional extractions using their technique did not yield any more ATP, suggesting that it leads to complete extraction in a single step. They did not address the question of sample stability.

2.3.3 Intracellular Concentrations

Several groups have researched the behavior of ATP in normal and stressful environments. An early study was that of Franzen and Binkley in which they examined the effect of growth rate on nucleotides in *E. coli* (Franzen and Binkley 1961). The *E. coli* were grown on various media leading to various growth rates. They found that per-cell nucleotide quantities increased with increasing growth rates. This corresponded with increasing per-cell dry weight, which suggests that the more rapidly growing cells are simply larger, and that intracellular nucleotide concentrations are under tight controls.

A more directed study reinforced the perception of tightly controlled intracellular adenosine nucleotide concentrations (Chapman et al. 1971). They extracted the nucleotides both with $HClO_4$ and with heated ethanol and obtained identical results with each technique. They were exploring the value of the "energy charge" of *E. coli*, which is an indication of the availability of energy in the adenosine phosphates (ANPs). The energy charge is the mole fraction, with regard to total ANPs, of ATP plus one-half of the mole fraction of ADP. In equation form, with *EC* representing the energy charge and ATP, ADP and AMP representing the moles of each:

$$EC = \frac{(\mathrm{ATP} + \tfrac{1}{2}\mathrm{ADP})}{(\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP})} \qquad (2\text{-}1)$$

They found that the energy charge was maintained at a very constant value in *E. coli*, barring a major change in culture conditions. When the energy charge was between 0.5 and 0.8, the culture was in stationary phase. When the energy charge rose higher, the cells grew, and when it dropped lower the cells died. They also compiled data from many other studies in other organisms and cell lines to show that the same approximate values were valid for all organisms. The value for *E. coli* growing exponentially varies between 0.90 and 0.94, depending on the medium. The rigid maintenance of the energy charge even under the stress of changes in the extracellular environment indicates the importance of careful adenosine balance to cellular function.

A group led by Cole investigated the time course of intracellular ATP concentrations in *E. coli* growing on various media with and without aeration (Cole et al. 1967). They found that the per-cell ATP concentration could rise, fall, remain constant or even cycle up and down, depending upon the growth medium. In the case of aerobic growth on a glycerol minimal medium, they found a gradual increase through the early portion of exponential growth, followed by a more pronounced decrease to well below the initial concentration. They also showed that the ATP levels respond rapidly to lack of oxygen. When the aeration was stopped, the ATP level dropped to below 10% of its prior value in less than two minutes.

2.4 OPTICAL DEVICES AND MEASUREMENTS

A complete review of optical devices is beyond the scope of this thesis; so what follows is background information and a summary of basic theory regarding light measuring devices and fiber-optics. There is also a more detailed discussion of inner filter effects. For further information regarding optical theory, an excellent source is the book, Photometry and Radiometry for Engineers, by Allen Stimson (John Wiley and Sons, New York, 1974). Other, more compact, sources of information are in the catalogs of the Oriel Corp. (Stratford, CT) and Hamamatsu Corp. (Bridgewater, NJ) and the Photomultiplier Handbook, published and distributed by Burle Industries, Inc. (Lancaster PA), a manufacturer of photomultipliers.

2.4.1 Commercial Instruments

A wide variety of instruments are capable of detecting and measuring the light produced by luminescent reactions in small samples (Van

Dyke 1985; Jago et al. 1989). The most sensitive are called luminometers; they can detect as little as 0.01 pg of luciferase in an excess of luciferin and ATP (De Wet et al. 1987). In terms of CV-1 (monkey kidney) cells expressing the luciferase gene from the plasmid pSV2L/SV2Neo, these instruments are capable of detecting the luciferase extracted from fewer than five cells. That is far more sensitive than is necessary for most applications. However, the commercial luminometers cannot measure light input arriving from an external source via fiber optics. Nor can they be used in an automated, on-line fashion, although some of them support data transfer via RS-232 connections on command.

Scintillation counters can also be used to detect luciferase, although they are less sensitive than luminometers. This is possible only if the photon coincidence circuits can be turned off, which is an available option on most new models. Scintillation counters with this capability can detect samples containing as little as 1.0 pg of luciferase. The scintillation counters also are not designed to be used on-line or with fiber-optic input.

While many fluorometers can be adapted to on-line use, and many can accept inputs from fiber-optic sources, they are usually not suitable for sensitive luminescence measurements. The reason for this lies in the differing nature of luminescence and fluorescence. For the former, the energy required for light emission is supplied from within the luminescing molecule, usually by a chemical reaction. For fluorescence, the energy is supplied from without in the form of the excitation light. So fluorometers are generally designed to measure greater light intensities than are luminometers, because they supply a bright (high-energy) light source for excitation. This means that most fluorometers are not capable of detecting small quantities of luminescent material.

2.4.2 Light Sensors

Within luminometers, scintillation counters and fluorometers, there are smaller components which actually detect and measure light intensity. This can be accomplished both with film and with electrical devices. The film assay is the simplest form of light detection. A piece of film sensitive at the appropriate wavelengths is exposed to the light source for a fixed time period. It is then developed, and the resulting negative can be scanned by a densitometer to determine the degree of exposure. Film assay

sensitivity is easily adjusted by varying the time of exposure from as short as seconds to as long as weeks. This is commonly done with autoradiographs of gels which were run using radiolabeled material. When small samples can be optically isolated from one another, this technique can be used to test many samples simultaneously. One such use is testing clones for luciferase activity in 96-well plates.

For more rapid, on-line quantification of light intensity, electrical or electronic devices are used. The oldest and most sensitive of these is the photomultiplier tube (PMT). PMTs are vacuum tubes containing a photocathode which releases electrons when impacted by photons; several dynodes arranged in a cascade to amplify the electron flux; and an anode to receive the electrons. The anode current can be measured and is directly proportional to the photon flux at the photocathode. Very large (≥1000V) electric potentials must be supplied to the photocathode and the dynodes to help dislodge the electrons. The built-in amplification cascade of dynodes makes PMTs sensitive enough to provide a clear signal in response to a single photon hitting the photocathode.

A solid-state device known as a photodiode is the rough equivalent of the photomultiplier. When photons strike the photodiode surface, they dislodge electrons and move them into "holes" in a positively doped region. This leads to a detectable current. Another solid-state light detector is called a charge-coupled device (CCD). The CCD is a sandwich of two layers. When photons strike the outer, photosensitive layer, they bump electrons to the inner layer, where they aggregate in nodes. The nodes are evenly spaced around the device, and each can maintain its charge independently. The charges are read and cleared periodically by a process known as charge coupling (Hooper et al. 1990). This arrangement provides a two-dimensional image of the light impact on the CCD.

Because PMTs are so sensitive, they are very subject to interference. Radio frequencies and magnetic fields can dislodge electrons from charged dynodes, leading to so-called black noise, or anode current in the absence of light. This problem is offset by the ability to count photons, as opposed to the light intensity measurements provided by CCDs and photodiodes. The latter, however, can constructed so they are more sensitive than PMTs at longer wavelengths. Sensitivity to various wavelengths is a function of the materials used for the photocathode coating. The practical range for PMTs

is from 200 to 900 nm, and that for photodiodes is from 400 to 1100 nm. Most PMT and photodiode manufacturers provide sensitivity spectra for their products in order to assist end-users in choosing an appropriate device.

The signals from these devices can be detected and amplified, if necessary, by appropriate electronic devices. Photodiodes generally supply currents that are readily be measured by solid-state circuits. CCDs, as mentioned above, are monitored by special charge-coupling readers. PMT output can either be monitored by an ammeter or oscilloscope in the case of high light intensity, or by a photon counter when there is less light. Photon counters detect the individual current pulses associated with each photon's impact on the photocathode. They discriminate any overlapping pulses and convert them to short, separated, square-wave pulses. Many electronic devices can accurately and rapidly measure the square waves, especially if industry standards such as TTL are followed. Some such readers are built into cards that are designed to be inserted in desktop computers. for examples of two such cards, see the section on Light Detection in the Materials and Methods chapter.

2.4.3 Light Gathering (Lenses and Optical Fibers)

While PMTs can quantify light which reaches the photocathode by any means, they are most useful when the light from a source or sample can be focused there. The focusing can be done by a system of lenses or one of optical fibers and lenses. The basic science of lenses and fibers and the parameters which describe them are outlined below. Some of the terms introduced here may be better understood with the help of Figure 2-3.

Lenses work by refracting the light that passes through them. If the incident light rays are effectively parallel, such as those from a distant light source, the rays are focused to a single point. This point is known as the focal point of the lens and its distance from the center of the lens is called the focal length (f). Conversely, light originating at the focal point leaves the lens as parallel rays. This process is called collimation. The focal length is a function of both the curvature of the lens and the material from which it is made. The power of the lens, θ, is the reciprocal of the focal length. They are related to the refractive index, n, of the lens material and

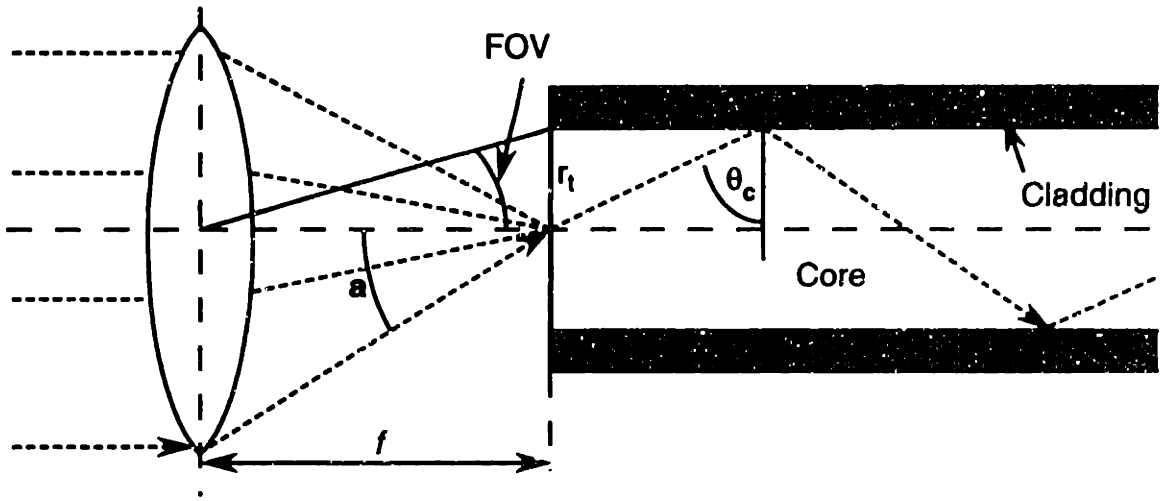

Figure 2-3: Important Parameters for Lenses and Optical Fibers Focal length (f) is a property of the lens. Critical angle (θ_c) and acceptance angle (**a**) are properties of the fiber. Field of view (FOV) is a function of f and the target radius (r_t), which, in this case, is the fiber radius.

the two radii of curvature, r_1 and r_2 by the following formula (r_2 is negative for bi-convex lenses such as that shown in Figure 2-3):

$$\theta \equiv 1/f = (n-1)(1/r_1 - 1/r_2) \qquad (2\text{-}2)$$

Lenses also have a characteristic number known as the lens speed, or F/# (written as F/2 for a speed of two). Speed is an indicator of the throughput of the lens. Lower F/#'s indicate faster lenses and higher throughputs. The throughput is inversely proportional to the square of the F/#. The lens speed is calculated by dividing f by the lens diameter, d. F/#'s are very useful for matching lenses in systems for manipulating images. Another useful property of a lens is the field of view (FOV). FOV is the maximum angle, relative to the lens axis, from which light can originate and still hit a defined area, such as the end of an optical fiber, on the other side of the lens. Based on the radius of the target area, r_t, the FOV is determined by:

$$\text{FOV} = \tan^{-1}\left(r_t/f\right) \qquad (2\text{-}3)$$

All of the above definitions and relationships are based on the light source being either at the focal point of the lens or infinitely far away. Practically speaking, infinitely far means a distance of at least 25 times the focal length. As the distance to the source (L_s) decreases, the effective

focal length (L_f) increases. This relation is defined by the Newtonian Lens Formula, as shown in Equation 2-4.

$$L_s L_f = f^2 \tag{2-4}$$

In addition to the effect of the distance to the source, other factors called lens aberrations lead to non-ideal lens performance. Even a perfectly ground lens will have these problems, which cause the spot at the focal point to actually be what is known as a blur circle. Lens aberrations are beyond the scope of this thesis. It is sufficient to note that they prevent lenses from performing perfectly even in ideal situations.

Optical fibers can often be used to minimize the need for lenses to transmit light from a source to a target because of their ability to transmit light well even when curved. They are made of a core of a flexible, transparent fiber surrounded by cladding of a different material. The core must have a higher refractive index than does the cladding. Fiber transmission is based on "total internal reflectance." When a ray of light strikes the boundary between the core and the cladding at an angle greater than the critical angle, θ_c, it is reflected back into the core. The angles are measured relative to a line normal to the surface. The critical angle is defined by the refractive indices of the core and cladding (n_1 and n_2, respectively):

$$\theta_c = \sin^{-1}\left(n_1 / n_2\right) \tag{2-5}$$

In order for light to enter the fiber and remain in it, the light must originate from within the acceptance cone. The cone is defined by an acceptance angle, a, measured from the fiber axis. The acceptance angle is related to the numerical aperture (NA) and the F/# of the fiber. They are all defined by the refractive indices of the core and cladding as well as that of the external medium from (n_0), by Equation 2-6 (for air, n_0 = 1.0).

$$1/2(\text{F/\#}) = \text{NA} = \sin a = \sqrt{n_1^2 - n_2^2} / n_0 \tag{2-6}$$

The F/# can be used to match the fiber with an appropriate focusing or collimating lens at the input or output, respectively.

These relationships and definitions are also valid for fiber bundles. Fiber bundles are used to provide a larger cross-sectional area and therefore capacity without losing flexibility. They consist of many individual fi-

bers close-packed in cladding and are often surrounded by an opaque material for containment. The fraction of the cross-sectional area of the core of a bundle that is actually fiber is called the packing fraction, P. P is exactly 1.0 for single fibers, and gets as low as 0.8 for some fiber bundles.

In real fibers, the internal reflectance is not actually 100%. Because of flaws in the fiber surface or in the border with the cladding, or because of photon-photon interactions inside the fiber, some light escapes. This is quantified by a number called the reflection loss, R, which is less than 0.05 for most commercially available fibers. There are also light losses associated with absorption by the core material, defined by the absorption coefficient, $\alpha(\lambda)$. These factors are interrelated, and the overall fiber transmittance, T, is a function of them all according to Equation 2-7.

$$T = P(1-R)^2 \exp(-\alpha(\lambda)L) \qquad (2\text{-}7)$$

The length, L, is the actual path length of the light ray being followed. This is longest for rays entering at the acceptance angle because they must reflect off the walls most frequently, and shortest for those entering directly on the axis. It is impractical to calculate T for each ray of light; so in practice the average path length is used instead. It is important to note that T is an exponential function of wavelength, λ; so a fiber must be chosen that absorbs little at the important wavelengths. It should also be noted that refractive indices are a function of λ. For instance, the refractive index of crown glass ranges from 1.531 at 400 nm to 1.511 at 800 nm. This difference is why prisms diffract light into spectra. It also has some effect on lens focusing ability. For fiber transmittance calculations, it is lumped into the reflection loss parameter.

2.4.4 Inner Filter Effects

Inner filter effects (IFE) are caused by the absorbance or scattering of luminescence or fluorescence by other species in the sample. IFE leads to falsely low readings of light intensity at the detector. In the case of fluorescence, it can affect either excitation or emission light or both. In the case of *in situ* culture measurements, many compounds may absorb light, but virtually all of the scatter will be caused by the cells or their organelles (Srinivas and Mutharasan 1987).

Many groups have examined IFE in fluorescence measurements (Ratzlaff et al. 1984; Eisinger and Flores 1985; Srinivas and Mutharasan 1987; Puchalski et al. 1991). They have developed analytical expressions for the correction required for IFE in many fluorometer geometries. The absorbing species can be the same as the fluorescent species or they can be unrelated. Some researchers have even developed sensors based on the IFE (He et al. 1993; Leisley et al. 1994). The absorbance information can be derived from theory or measured experimentally. However, all of the studies which calculate the absorbance from theory begin with the Beer-Lambert law:

$$\log\left(I_0/I\right) \equiv -\log(T) = OD = \varepsilon c l \tag{2-8}$$

Where T is the transmission, I is the intensity of the light after it passes through the sample, I_0 is the original light intensity, l is the distance throughout the sample, c is the concentration of the absorbing species in the sample and ε is the molar extinction coefficient, which is a function of the absorbing species and the wavelength. The same method used in these studies can be used for luminescence as well. This was done by Yappert and Ingle, resulting in the following correction (Yappert and Ingle 1989). I is the measured light intensity and I_{act} is the actual light intensity.

$$I_{act} = I\left(\frac{\ln T}{T-1}\right) = I\left(\frac{2.3\mathrm{OD}}{1-10^{-\mathrm{OD}}}\right) \tag{2-9}$$

The Beer-Lambert law is valid in almost all situations when the absorbing species is very small relative to the wavelength. However, it begins to break down with larger particles at significant concentrations (practically speaking, at OD > 1 when l = 1 cm). Under these conditions, scattering effects are too strong to be lumped with absorption, as they are in Equation 2-8 (Tam and Zardecki 1982). In these cases, a better descriptor is:

$$-\log(T) = \mathrm{OD} = \varepsilon l \log(c) \tag{2-10}$$

This does not effect the Yappert correction factor, because they assumed that the sample was uniformly concentrated in their derivation of Equation 2-9. However, OD or T measurements must be taken in the

proper regime to be used in the correction factor. Otherwise, they must be converted to the high density form before being used.

For a more detailed evaluation of scattering effects, one must incorporate an in-depth analysis of radiation diffusion and the Maxwell equations. The resulting equations for corrections become far too cumbersome to be used without considerable computer power. Thus, empirical, experimentally determined corrections are recommended (Groenhuis et al. 1983a; Groenhuis et al. 1983b). Experimentally determined IFE corrections have also been reported for fluorescence measurements (Subbarao and MacDonald 1993).

3. MATERIALS AND METHODS

3.1 BACTERIAL STRAINS AND PLASMIDS

The organism used in this study was *Escherichia coli*. In the initial studies, DH5α cells from Bethesda Research Laboratories (Bethesda, MD) were used. Subsequently, the major portion of the research was performed using a strain from the American Type Culture Collection (Rockville, MD), *Escherichia coli* accession #15224. This strain's genotype is *lac* $i^{-}z^{+}y^{+}$, making it a constitutive producer of β-galactosidase (β-gal). β-gal was considered as the product when addressing various issues related to the effects of luciferase expression on strain productivity.

Two different plasmids were used to transfect these strains with the luciferase gene. The first, pRSVL, was provided by Prof. Donald Helinski, and the second, pSV2L/SVNeo, by Prof. Suresh Subramani, both from the Department of Biology at the University of California at San Diego (De Wet et al. 1987). Both plasmids contain pBR232 segments coding a bacterial origin of replication as well as ampicillin resistance. They both contained the cDNA for luciferase under the control of a mammalian viral promoter which acts constitutively in prokaryotes. In the case of pRSVL, the promoter is from the Rous Sarcoma Virus Long Terminal Repeat, while in pSV2L/SVNeo it is the SV40 Early promoter. In addition, pSV2L/SVNeo contains the gene for neomycin resistance, also expressed from the SV40 Early promoter.

The host strain was transfected by electroporation. A colony was grown in100 ml of Luria Broth (LB) to an optical density (OD) of 0.86. The cells were centrifuged and resuspended in 40 ml of 5% glycerol in 20 mM HEPES pH 7.2, then recentrifuged and resuspended in 5 ml of the same solution. This was further centrifuged and resuspended in 1 ml of ice-cold 10% glycerol in 10 mM HEPES 7.2 and then split into 5 × 200 µl aliquots and frozen overnight at -70°C. An aliquot was thawed as needed, and centrifuged and resuspended twice with the 10% glycerol solution. Then 40 ng of plasmid DNA was added, and the mixture was placed in an electroporation cuvette.

Electroporation was performed using a Gene Pulser by BioRad (Hercules, CA) with the settings as follows: 2.5 kV, 25 µF and 200Ω. The mixture was immediately added to 800 µl of LB and left to sit at 30°C for

Table 3-1: Defined Media Compositions (Quantities in g/L)

	Bioreactor		Shake Flasks	
Operating pH	7.0	6.0	7.0	6.0
$(NH_4)_2SO_4$	7.00	7.00	7.0	7.0
$(NH_4)_2HC_6H_5O_7$	0.50	0.50	0.5	0.5
$NaH_2PO_4 \cdot 7H_2O$	8.39	3.65	17.0	5.7
K_2HPO_4	1.11	3.35	3.0	9.0
Glycerol	22.20	22.20	15.0	15.0
Trace Salts (ml/L)	10.0	10.0	10.0	10.0
$MgSO_4$ (ml/L)	5.0	5.0	5.0	5.0

Table 3-2: Trace Salts Solution Composition

Component	g/L
$CaCl_2 \cdot 2H_2O$	0.50
$FeCl_3$	10.00
$ZnSO_4 \cdot 7H_2O$	0.18
$CuCl_2 \cdot 5H_2O$	0.16
$MnSO_4 \cdot H_2O$	0.12
$CoCl_2 \cdot 6H_2O$	0.18
$Na_2 \cdot EDTA$	20.10

an hour. Then 200 µl was spread on each of four LB-agar plates containing ampicillin. Twelve large colonies were selected at random for further study. One substrain was chosen that showed luciferase activity and had a growth rate similar to the host strain. It was stored for long-term stability as follows.

A colony was grown in LB medium to an OD of about 1.0. Glycerol was added to the culture to yield a final glycerol concentration of 30% (w/v). The resulting culture was placed in 1 ml aliquots in cryogenic vials. The vials were then rapidly frozen in liquid nitrogen and stored at -70°C to provide consistent inocula. Approximately 80 vials were stored for each plasmid.

3.2 GROWTH MEDIA

Various media were used over the course of this study. The choice of medium was determined by the circumstances of each experiment. Culture dishes for plating cells were prepared using 1.5% agar in LB. These were used primarily for viable cell counts (see below) and for short-term storage of strains. They were also vital for the selection of successfully transfected bacteria. For selection purposes, ampicillin was added to a final concentration of 50 μg/ml.

In all cases for growth studies in liquid medium, the first stage of growth was in 5 ml of LB in culture tubes. Either a single colony or a portion, usually 200 μl, of a frozen aliquot was used to inoculate the tube. If less than a complete, 1 ml aliquot was used, the remainder was discarded. Occasionally, larger volumes of LB were used in shake flask experiments, but most of the time a defined medium was used. For selection in liquid media, ampicillin was added as above (50 μg/ml).

The defined media used were all closely related and are listed in Table 3-1. The major difference was in the amounts and the phosphate buffer concentrations. Glycerol was chosen as the carbon source for two reasons. One is because glucose is known to affect expression levels from many promoters. Because the promoters used in this study are not well characterized in bacteria, it is uncertain if they respond to variations in glucose concentration. To avoid the problem, glucose is not used as the carbon source. The other reason is to minimize acetate formation, because acetate is known to lower expression levels of genes on plasmids (Jenson and Carlsen 1990).

For experiments requiring light production during cultivation, the acidity of the medium was maintained at pH 6 while in other situations, the pH was neutral (7.0). For fermentations in the bioreactor, less total phosphate was needed because the bioreactor has the capacity to control and maintain the pH through acid and base additions. For light production in the bioreactor, luciferin was added to the medium immediately before inoculation to a final concentration of 15 μM. The solutions of trace salts and of magnesium sulfate were prepared and autoclaved separately, then added to the bulk medium before inoculation.

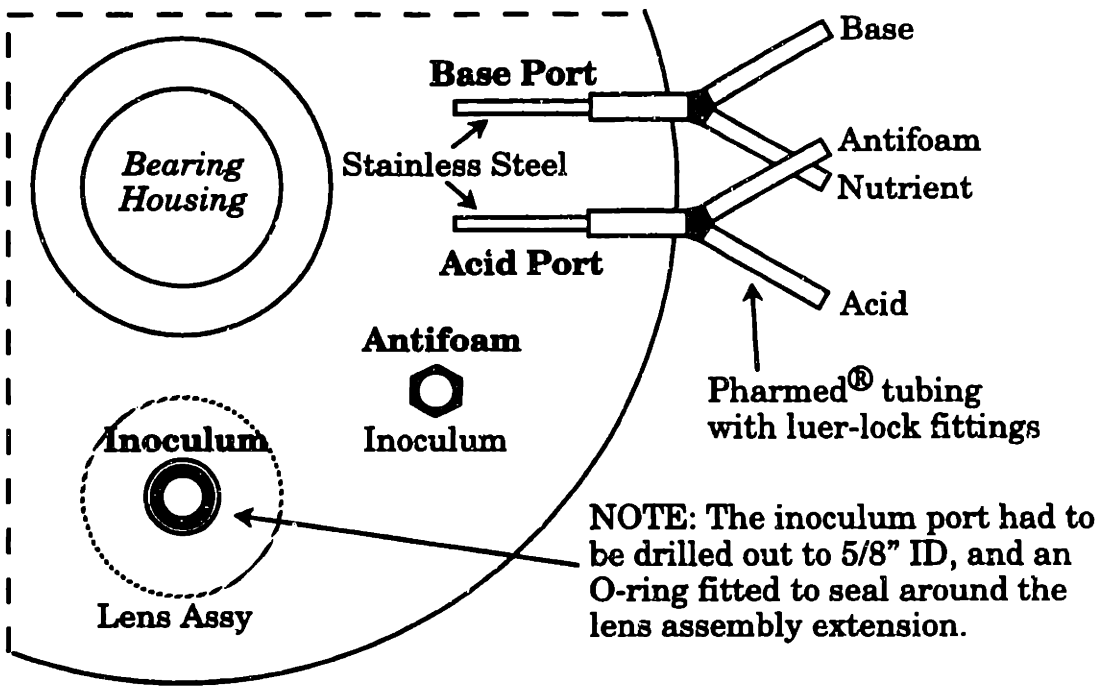

Figure 3-1: Modified Quadrant of the Bioreactor Headplate. Original designation of ports are in **boldface**. The bearing housing and the rest of the headplate are unchanged from the BioFlo II specifications.

3.3 FERMENTATIONS

Small-scale fermentations were performed using shake flasks, as were seed cultures for the bioreactor. All shake flasks were baffled and from Bellco Biotechnology, Inc. (Vineland, NJ). The 250 ml flasks contained 25-40 ml of culture broth, and the 500 ml flasks contained 50-80 ml medium. Seed cultures were 80 ml; 50 ml of which was used for the bioreactor inoculum. Flask cultures were grown in an orbital shaker-incubator (Queue Systems, Parkersburg, WV) at 37°C and 220 rpm. LB tubes were grown in the same shaker-incubator.

3.3.1 Bioreactor Operation

Bench-scale fermentations were performed in a New Brunswick Scientific Co., Inc. (Edison, NJ) BioFlo II bioreactor fitted with 7.5 L vessel (5 L working volume). The initial culture volume was always 4.5 L. The fermentation vessel headplate had been modified to allow the lens assembly to fit in through the inoculum port (see Luminometer Components and Connections below). The side ports were unavailable because a light-tight aluminum shield was placed around the vessel during fermentations to

prevent ambient light from overwhelming the light measurements. This requirement and the headplate change resulted in several adjustments to the standard methods of inoculation and feed connections.

The antifoam feed port of the fermentor was used as the inoculum port. It was fitted with 8 mm red rubber septa with a PTFE coating on one side (VWR Scientific, Boston, MA, Catalog #66011-180). Two septa were used at a time, and both were placed with the PTFE on the top side. The septa were replaced after each fermentation. The trace salts, $MgSO_4$, ampicillin and luciferin were also injected through this port. Solutions and seed cultures were added using sterile syringes fitted with hypodermic needles. Only small-diameter needles (up to 16 gauge) could be used without damaging the septa. Inocula were 50 ml to a 4.5 liter fermentation.

The headplate is also equipped with two small-diameter, stainless steel feed ports used for acid and base feeds. To provide sufficient feed ports without access to either the antifoam port or the side ports, two lines had to be connected to each of these (see Figure 3-1). This was accomplished by connecting a "Y" splitter made of PharMed® tubing (Cole-Parmer, Niles, IL) above each port. Each branch ended in a Luer-type connector. This allowed the bioreactor vessel to be autoclaved independently of the feed bottles. The Luer connectors provided a means of rapid, aseptic connection for the feed lines. The nutrient feed used the same port as the base feed, and antifoam was fed through the same port as the acid. The base used was 4N NaOH; the acid was 1N HCl; the carbon source feed was water, defined medium, or 50% glycerol; and the antifoam was 20% (w/v) poly-propylene glycol (m.w. 2000) in ethanol.

The BioFlo II has built-in controls for temperature, pH, antifoam and dissolved oxygen (DO). Base, acid, antifoam and nutrient are delivered by built-in peristaltic pumps from freshly filled and sterilized feed bottles. The DO control is achieved by manipulating the agitation speed of the impellers. The air flow rate is fixed by an external needle valve. During the initial stages of this research, all of the built-in controls of the BioFlo II were utilized. Air was supplied from a cylinder. In order to eliminate the large changes in agitation rates, which ranged from 200 to 1000 rpm, the monitoring computer was used to control the DO in later staged of the research.

This required the addition of a nitrogen tank, two mass flowmeters (model 5850E, Brooks Instruments, Buena Park, CA) and a flow controller (Brooks model 5878). The air and nitrogen flow rates were controlled separately by the mass flowmeters, and the computer provided a setpoint for each via a voltage sent by an analog-to-digital (A/D) board (Omega Engineering, Inc., Stamford, CT. Model DAS-16). The setpoints were calculated using a PID algorithm and on-line DO data. The air and nitrogen streams each then passed through a hydration chamber before they were combined and fed to the sparger. The needle valve was kept fully open. The impeller agitation rate was set manually and remained unchanged over the course of the fermentation. The computer software allowed the user to set and change the DO setpoint as well as the total gas flow rate. For more details on the control/monitoring software, see Computer Boards and Software.

The same computer that controlled the DO also monitored and recorded several parameters during the fermentations. However, in the first few fermentations, only light production was recorded via computer. The light levels were monitored by a multichannel scaler board (model ACE-MCS by EG&G Ortec, Oak Ridge, TN) (see Computer Boards and Software). After the A/D board was installed, some or all of the following were monitored: broth temperature, pH and DO; air and nitrogen flow rates; mass of the base feed bottle from a digital scale (Ohaus Brainweight 5000); and the composition of the off-gas, as measured by a Perkin-Elmer (Pomona, CA) MGA 1200 mass spectrometer. The off-gas analysis measured nitrogen, oxygen, carbon dioxide, and water vapor. The contents of the gas feed tanks were analyzed before and after each fermentation with the MGA to check the MGA calibration and instrument drift as well as the feed gas composition. A prior user had soldered two wires onto a board in the Ohaus scale to provide a voltage-based signal. This modification allowed the A/D board to interpret the scale readings.

3.3.2 Biomass Quantitation

Biomass was measured off-line by the following methods: optical density (OD), colony counts, dry cell weights (DCW), and Coulter Counter®. The OD measured the absorbance of a sample of broth at 630 nm. The OD corresponded to the number of particles or cells in the solution, provided no other species absorb at that wavelength. Dilutions were

performed to ensure that the values were always in the linear range of the instrument and of the Beer-Lambert law, which is valid under an OD value of 1.0. The OD was shown to be linearly related to each of the other three measurements.

Colony counts provided an estimate of the viable cell number in the broth. A sample containing the cells was diluted serially in LB broth. Each dilution was between 10- and 500-fold. Micropipetters were used to keep total volumes at or near 1 ml. The OD was used to for an initial estimate of cell concentration in the broth, based on a conversion factor of around 10^8 cells/ml/OD. The size of the final dilution step was chosen to result in a cell concentration of around 250 cells/ml. This cell density was optimal because it provided a sufficient number of colonies on a plate for statistical significance and at the same time avoided overlapping colonies. It was found that about 50 colonies per plate was sufficient for reproducible counts.

To obtain the viable cell count, 200 µl of the dilute broth was spread on an agar plate and left under the hood for up to two hours. The plate was then placed in the incubator overnight, and colonies counted in the morning. If the plate was properly prepared, each colony grew from a single colony-forming unit (cfu) that was in the 200 µl placed on the plate. Each cfu was assumed to be one viable cell. Factoring in the dilutions gave the viable cell density for the broth. Ampicillin-containing agar could be used for plating to determine plasmid-containing cfu densities.

Dry cell weight is a common measure of the total mass of cells in the broth. Five ml of a culture sample was centrifuged at 4°C in a pre-weighed glass tube. The supernatant was discarded or used for different assays. The pellet was resuspended in MilliQ water and centrifuged again, and the supernatant was discarded. The tube was dried for at least 24 hr in a gravitic oven at 110°C. Finally, the tube was removed, cooled, and weighed. The DCW of the 5 ml sample was the difference in mass between the final mass and that of the preweighed tube. This generally ranged from 0.5 to 65 mg. These small differences in mass introduce significant error into this method. DCW was calculated for the broth by conversion to g/L.

The Coulter Counter® (Coulter Electronics, Inc., Hialeah, FL) is a device which counts particles in solution by electrical conductance across an orifice (see Section 2.1.1). For this study, the orifice diameter was

30 μm. Between 0.5 and 20 μl of sample was suspended in 20 ml of Isotone® which had been filtered with a 0.22 μm filter. The electrolyte was a 4.0% (w/v) NaCl solution. A Coulter Channelyzer® was used to provide size distributions of the particles. Data from the Channelyzer® was dumped directly to a computer for storage and later analysis. The number density of cells in the broth was calculated from the Coulter Counter® data.

3.3.3 Intracellular Enzyme Extraction

Additional off-line assays were required to measure other parameters. These measurements fell into two categories: intracellular and extracellular. The intracellular compounds of interest in this study were ATP, β-gal, and luciferase. The two enzymes were extracted by sonication, and the ATP by the perchloric acid method (see below).

Sonication utilizes ultrasonic vibrations to break open the cell wall and membrane. Samples were prepared for sonication by centrifugation followed by resuspension in ice-cold sonication buffer so that the OD of the new suspension was 1. The sonication buffer was 1% Triton X-100, 25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 4 mM EDTA (pH 8.0) and 1 mM dithiothreitol (DTT). Three ml of the cells in sonication buffer was placed in a 12 × 75 mm culture tube. The tube was positioned and clamped so that the end of the sonicator microtip was 3-4 mm above the bottom of the tube and no part of the tip touched the tube. A beaker containing ice in water was placed around the tube so that most of the tube was submerged. The ice water minimized overheating and subsequent denaturing of the sample. The sample was sonicated for six minutes total time, 40 seconds on and 20 seconds off, with a VirSonic sonicator by The Virtis Co. (Gardiner, NY) set at power level 5 (maximum for microtips). The sonicated samples were stored at -20°C until assayed.

3.3.4 Perchloric Acid Extraction

ATP was extracted from cells by the perchloric acid method because it is rapid, provides complete and consistent extraction, and deproteinates the sample (Lundin and Thore 1975). Deproteination and rapidity are vital to reduce the enzymatic degradation of the ATP. For further discussion of nucleotide extraction from cells, see the literature review. The samples were subjected to the following procedure:

Prior to drawing the sample from the reactor, 200 µl of ice-cold 3.0 N $HClO_4$ was added to a labeled microfuge tube placed on ice. Eight hundred µl of sample was added to the cold $HClO_4$ as rapidly as possible. The solution was vortexed and kept on ice for 10 minutes and then 200 µl of 3.0 N KOH in 0.3 M HEPES (original pH 7.8) was added to the tube, still on ice. This neutralized the solution to stabilize the ATP and precipitated $KClO_4$. The HEPES helped to maintain a consistent final pH in the case of inexact neutralization, and the final HEPES concentration matched that of the ATP assay buffer. After the precipitate formed and settled, the supernatant was removed and stored at -20°C until assayed. Extraction efficiency declined significantly for samples more dense than 0.8 OD units. For these cases, the sample was diluted with a quantity of filtered, spent medium from at least two hours into the fermentation. This was necessary because the spent medium affected the ATP assay differently than did the fresh medium. The diluent was added to the $HClO_4$ before the sample was drawn.

A stopwatch showed that there was about 7-8 seconds between when the sample was removed from the reactor and when it was added to the $HClO_4$. To study the effect of extraction time on the ATP level, some samples were deliberately extracted more slowly. For this study, four tubes were prepared for each extraction. The first was used as quickly as possible (7-8 seconds). For the others, the sample was held outside the bioreactor for the following times: 15, 23 and 35 seconds. They were subsequently in the same manner as those above.

3.4 LIGHT MEASUREMENTS

Luminometers are devices which measure light production, or luminescence, of samples. Several commercial models are available (Van Dyke 1985). However, all are designed to for samples that can fit in a cuvette, and few can be monitored by computer. In addition, none can accept light input from an optical fiber. This study required both computer connectivity and fiber-optic input. This necessitated the construction of a more flexible, modular luminometer.

3.4.1 Luminometer Components and Connections

The primary components of the luminometer are the following: a light detector, housing for the detector, sample chamber, optical fiber,

power supplies and computer boards. There are also many other components, including connectors, wiring, shutters and lenses. A description of the major parts and connections of this luminometer follows. Placement of other components is also described.

The light detector is a photomultiplier tube (PMT) by Hamamatsu (Bridgewater, NJ), model R1527PHA. This model is shielded against magnetic interference. The PMT was chosen over a solid-state device because it is more sensitive over a greater range of incident wavelengths. A drawback is that PMTs can be damaged by exposure to too much light. This includes normal room lighting; so a light-tight housing is required. The R1527 model is a low-noise PMT and does not require a cooled housing. It is mounted inside a radio frequency shielded housing with no cooling made by Products for Research (Danvers, MA), model 1405RF. The housing comes with a socket pre-wired for the PMT by the manufacturer, who does this based on the specific model of PMT used. This housing model also has a shutter, which helps to protect the PMT.

The PMT requires a high voltage (-1000 VDC) to generate a current from photons. This is supplied by an Oriel Corp. (Stratford, CT) power supply, model 70705. Output from the PMT goes to a photon counter by Hamamatsu (model C3866). This connection must be kept as short as possible; a six-inch section of 50Ω coaxial cable with BNC connectors is used. The photon counter requires low voltage power (+ and - 5 VDC) for its amplification circuitry. A DC tracking power supply (model LPS-151, Leader Instruments Corp., Hauppauge, NY) provides this voltage with the necessary current (up to 300 mAmp). Output from the photon counter is TTL-compatible and is carried by another 50Ω coaxial cable. The far end of the cable should be terminated with 50Ω resistor in parallel with a 0.1 μF capacitor (termination is built in on one of the computer cards used – see below). For a diagram of these connections, see Figure 3-2.

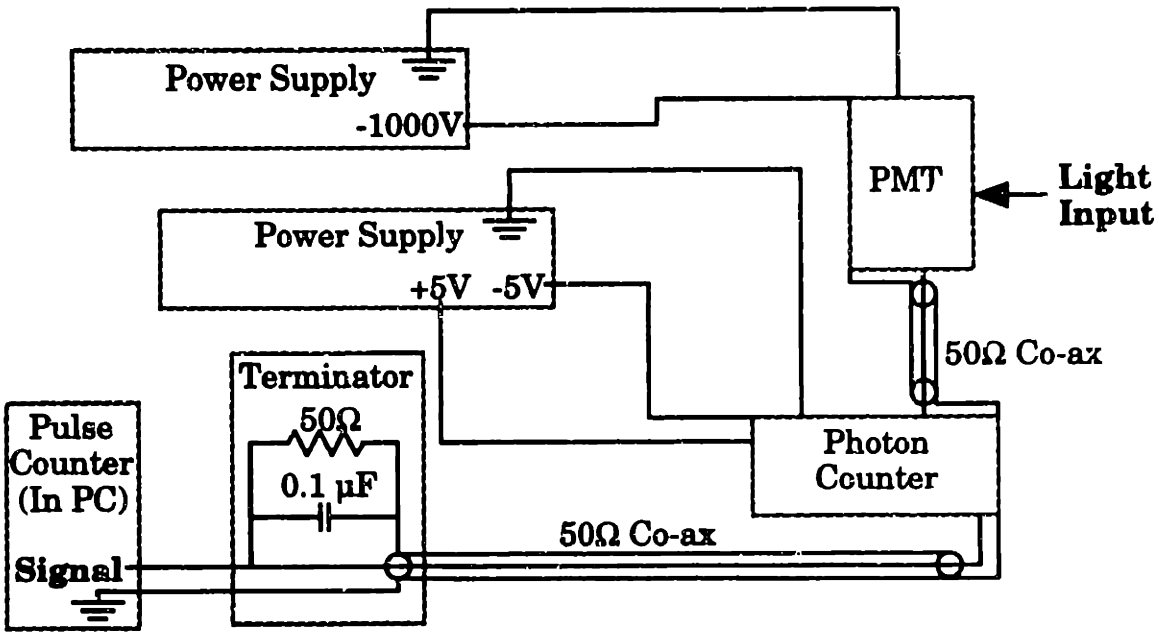

Figure 3-2: Electrical Connections for the Modular Luminometer. Solid lines are electrical connections and dashed lines represent pieces of equipment except the terminator, which actually uses a 47Ω resistor. The outer conductor of the coaxial (Co-ax) cables always carries the ground.

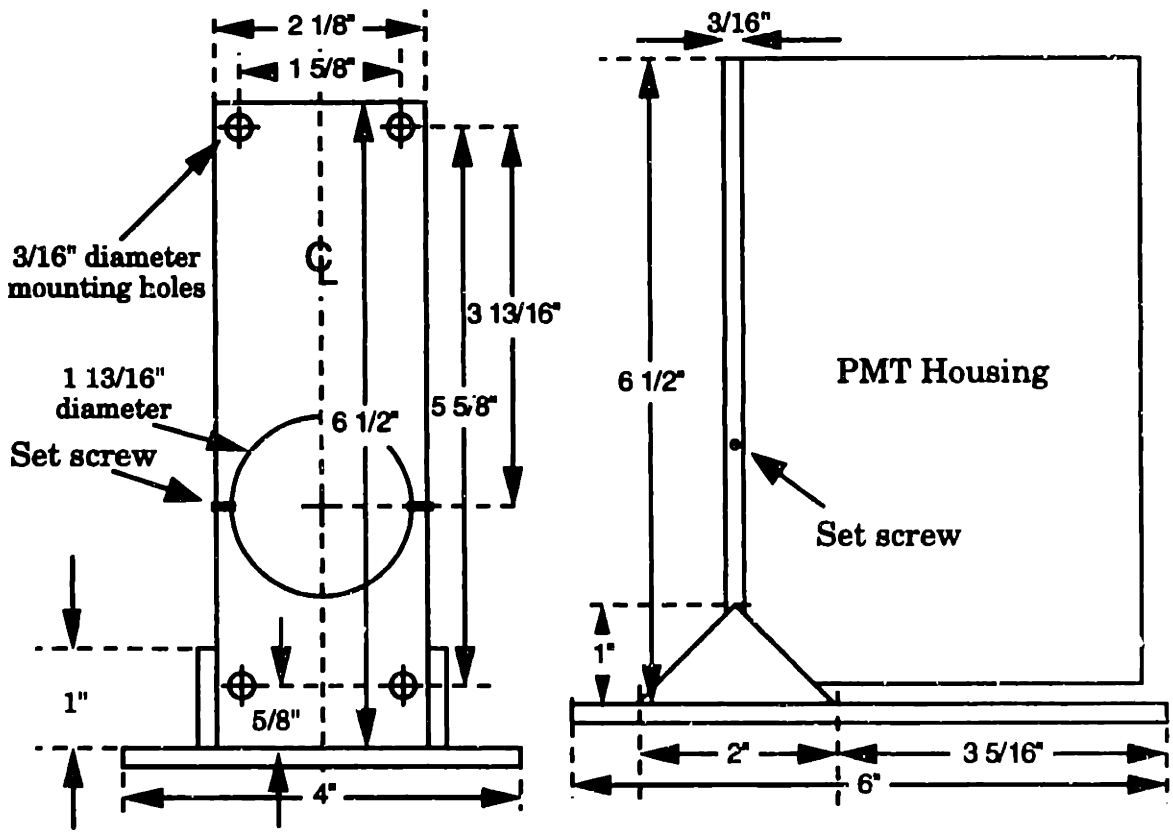

Figure 3-3: Stand for Photomultiplier Tube Housing. Front view on left and side view on right. Dimensions in inches.

An aluminum stand was constructed for the PMT housing (see Figure 3-3). Shaped like an inverted "T," it has holes in the vertical piece so that the housing can be bolted to the stand. In addition, it has a 1.75" hole centered on the shutter opening. This hole can accept either the cuvette sample chamber male connector or the optical fiber adapter. If desired, a filter housing can be inserted into the hole and the light source connected to the filter. A black O-ring on the housing seals the connection against light leakage. Two set screws, one on either side of the hole, serve to hold the connector in place. This piece allows easy switching between fiber-optic and cuvette sample modes.

The optical fiber, sample chamber and associated lenses and holders are all from Oriel Corp. The sample chamber (Oriel #30750) is used for measurements of cuvette-sized samples with only minor modifications. The only change is the removal of the handle for the lid to allow reagent injection through the hole. Black electrical tape is used to seal all openings to the chamber to minimize background light noise. A red rubber septum is taped over the hole to prevent light leakage around the injection site. When the sample chamber is in use, the housing shutter must be closed before the chamber lid is raised.

The optical fiber is a three foot long glass fiber bundle (Oriel #77525). Bundles transmit more light and are more flexible than single fibers of the same diameter. The glass bundle was chosen because it can transmit light which enters the bundle at the greatest angle (up to 34°); so more light can be gathered by the glass bundle. At the PMT end of the fiber, light from the bundle is focused with a collimating beam probe (Oriel #77645) into a tight beam. The probe is focused visually by the appearance of the projection of a bright source through the fiber bundle. The collimating beam probe is fixed in a 1.5" flange mount (Oriel #77803) to allow connection to the PMT housing. The entire fiber assembly, including all but the 6" of fiber bundle that goes in the lens assembly (see below) is wrapped in black electrical tape to prevent light leakage.

As mentioned above, a lens assembly passes through the modified inoculum port in the bioreactor headplate. The lens assembly is modified from the Oriel part #77799 by removal of the shutter and extension of the fiber-holding portion to a length of 6" (see Figure 3-4). This permits the vertical position of the lens in the reactor vessel to be adjusted. A me-

chanical stop in the assembly sets the fiber at the focal point of the glass, F/# 0.85 lens supplied with the assembly. The immersed side of the assembly has been cut as short as possible without harming the lens. A 2" Pyrex watchglass is glued to the end as an aseptic, gas-tight barrier. The lens was also glued in place, but this is not recommended for future constructions. The lens and the watchglass were treated with Prosil before placement to prevent fogging. The reactor is autoclaved with the lens assembly in place, but not the fiber. After the fiber is inserted, black electrical tape is used to seal the connection against light leakage. The lens assembly was kept immersed for most of the fermentations in this study.

3.4.2 Computer Boards and Software

As mentioned above (Bioreactor Operation), two different computer boards can be used to monitor the signal from the PMT. The first is a multi-channel scaler (MCS) by EG&G Ortec (ACE MCS). The MCS counts the TTL pulses from the photon counter for the user-set dwell time and stores the result in a channel. It then performs the same operation for the next channel, etc. There are a total of 4096 32-bit channels. The dwell time can be set as low as 1 μs. The MCS has built in termination on the "Data In" connection.

The MCS comes with a graphical software interface. It provides a rough graph of the channel contents as they change. Zooming and scaling capabilities allow precise examination of the data. Data storage formats are defined in the manual; so an APL program (SINGLE) was written on an Apple Macintosh to translate the data into a spreadsheet-compatible format for analysis, when necessary (see Appendix for code). When used to monitor the reactor, the MCS is used in a continuous mode. In this mode, each time it fills the last channel, it stores the data to the hard disk, clears itself and starts again until it detects a user-requested stop.

However, the MCS cannot be used to monitor the analog data which come from the BioFlo II, the MGA 1200, the flowmeters or the scale. Nor can it run simultaneously with another card in the same computer gathering the analog data. So the Omega A/D card is necessary to gather the data during a fermentation. The MCS is preferred for use for assays

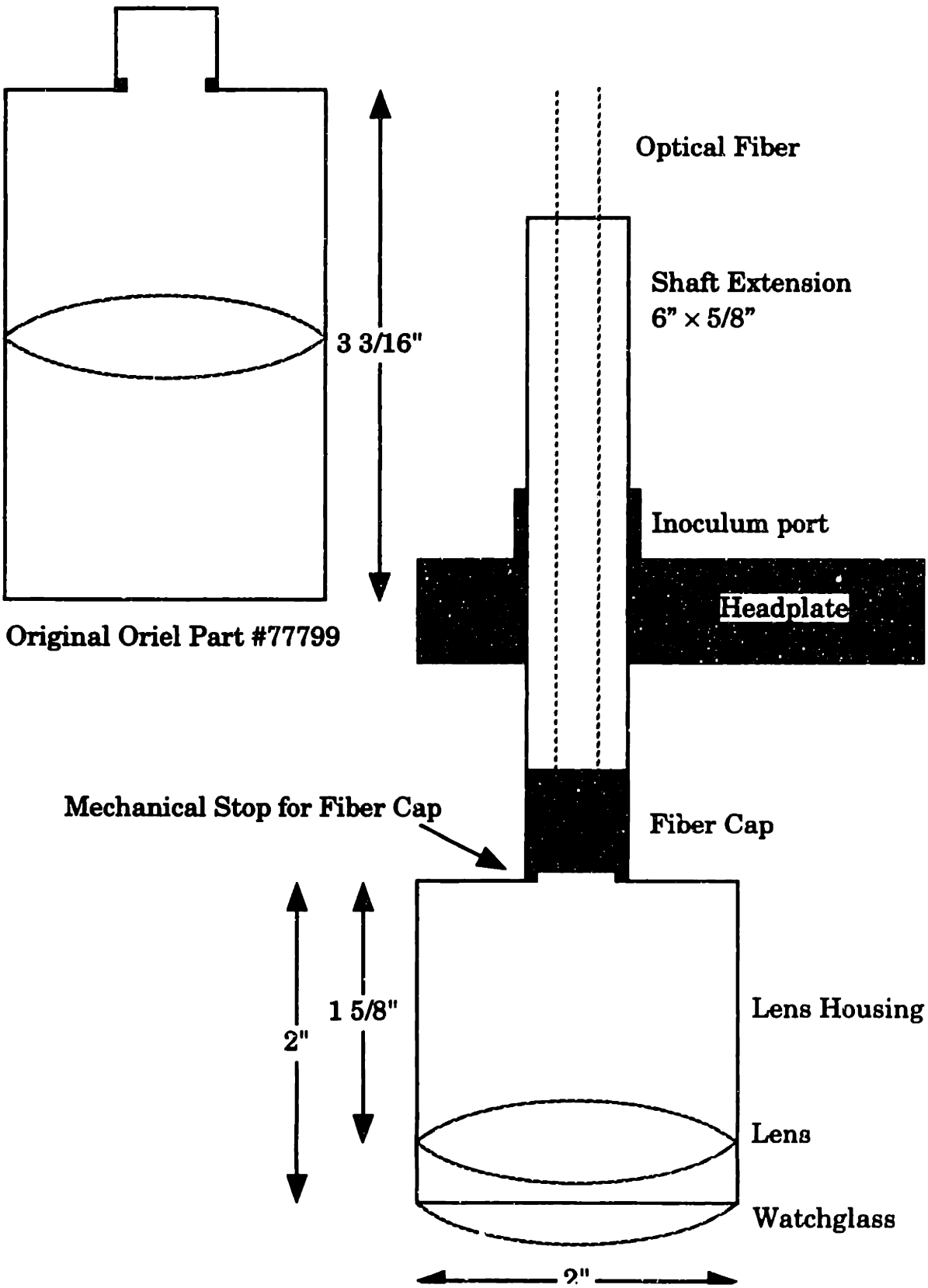

Figure 3-4: Lens Assembly Schematic.
At the top left is the Oriel part (#77799) without the shutter, and the right is the modified form as used in this research.

and other light detection when other data do not need monitoring because it has better time resolution and a graphical interface. The dwell time is usually set to 0.1 s, which provides excellent detail and still allows one pass to last almost seven minutes.

The A/D card converts up to 16 analog signals to 12-bit data (0-4095). Hardware switches set the upper and lower limits of these signals. The lower limit can be zero or the negative of the upper limit, which can be 1, 5, 10 or 20V DC. It also can send up to 2 independent 5 VDC signals and send and receive up to 4 digital, 5V signals. In addition, it has 3 digital pulse counters and a built-in 100 MHz clock chip. Two of the counters combine with the clock to convert the 100 MHz signal to a square wave as low as 1 Hz. This signal can then be used to trigger reading of the analog signals and/or to gate the third pulse counter for reading external pulse (i.e.: photon counter) data.

The A/D board is set to receive signals between 0 and 10 VDC to accommodate all of the equipment used in this research. Each piece of equipment is connected to the A/D board by small-gauge wiring and an appropriate plug (i.e.: DB25 or DB9) at the equipment end. Pin-outs are provided by the equipment manufacturers. The computer end connects to a screw-down box (Omega model STA-16), which is connected to the board by a ribbon cable. All of the signal grounds must connect to the same computer ground. A data file (CALIB.DAS) keeps track of what channel reads what data; the upper limit of the voltage and what that voltage corresponds to; and which channels carry the DO and flowmeter data. A compiled QuickBasic program (CALIB.EXE) facilitates changes to CALIB.DAS.

3.4.3 Procedures and Techniques

In the gathering of the data, the major goal is to avoid damaging the PMT by exposure to ambient light when the high voltage power supply is on. This is typically > 10^9 photons per second (ph/s), which is above the capacity of the luminometer without filters. Therefore the shutter is kept closed until the system, either the sample chamber or the reactor and fiber, is properly sealed. With proper sealing, the light leakage into the sample chamber is between 50 and 100 ph/s, and the reactor/fiber assembly lets in between 60 and 300 ph/s. Black tape is used over most connections;

it often makes a 10- to 100-fold or greater difference. For example, even the untaped, gasketed port where the foam probe penetrates the headplate can leak as much as 200 ph/s. The photon counter power supply should be turned on before the PMT power supply is, and turned off after it.

When luminescent bacteria in the bioreactor are the light source, the photon counter is set to +10 mode and its output is connected to the A/D board. During fermentations, a compiled QuickBasic program, CONTROL.EXE, manages the system. This program asks for confirmation of the CALIB.DAS data before proceeding to execute the run. The user confirms the computer time and date stamp and then supplies the total gas flow rates, the O_2 fraction in the "air" tank, the DO setpoint and a one-line run description. The program then begins controlling the DO by mixing the air and nitrogen flows, maintaining the total gas flow rate as requested. When the user is satisfied that DO is in the proper range, the program collects 3.5 minutes of light data to compute the leakage or background light level. It then counts down on-screen and beeps to signal when inoculation should occur.

After the inoculation time, the program simply monitors the inputs and controls the DO. It reads the analog signals and counts pulses once every three seconds. Every minute, it averages the 20 data points, discarding the highest and lowest as noise, converts the result to actual data (i.e.: % O_2 or pH) using the information in CALIB.DAS, and stores it to the hard disk. It also checks the keyboard frequently for user requests to change total gas flow or DO setpoints or to stop the program. Upon detecting a stop request, it closes the main data file and writes all of the detailed light data (every 3 seconds) to the disk. Both data files have a header containing user-provided information about the run as well as inoculation time and column headers for the data. The data files are written in a format easily read by Apple Macintosh spreadsheet and graphing programs.

When the sample chamber is used, the photon counter output is connected to the MCS card and the photon counter is set to +1 mode. The cuvette to be analyzed is placed most of the way into the chamber, and the lid pushes it into place as it is closed. The total contents of the cuvette should be at least 0.5 ml to ensure a good line-of-light to the PMT. The MCS program (MCS.EXE) brings up a graphic interface. After the dwell time is set, usually to 0.1 second, a keystroke begins light data collection.

When the last channel is full, the data can be stored and/or analyzed. Pan and zoom features allow the user to read the contents of each channel, if desired. Custom settings, such as dwell time, are stored a separately

When changing between light sources, it is extremely important to remember to change the photon counter connections and settings. Neither board can interpret the data from the photon counter if the "+" switch is in the wrong position. The "+1" position provides greater accuracy, but the A/D card is too slow to notice the TTL pulses. The "+10" position lengthens the pulses at the expense of one significant figure of detail. After reconnecting the cables, it is a good idea to check that they are not coiled, because that could cause induction. Nor, for minimum noise, should the power supply lines pass close to the signal cables. Also, do not bend the fiber bundle too tightly, because it may cause some of the fibers in the bundle to break, reducing throughput.

3.5 LIGHT-BASED ASSAYS

Each of the three components of the luciferase-catalyzed reaction, ATP, luciferin and luciferase, can be assayed enzymatically in a system which supplies a controlled quantity of the other two. Luciferase and ATP were purchased from Sigma Chemical Co. (St. Louis, MO), and luciferin was purchased as both the free acid and sodium salt from either Analytical Luminescence Laboratory (San Diego, CA) or Molecular Probes, Inc. (Eugene, OR).

These closely associated assays all use the luminometer with the sample chamber and the MCS board. In each assay, the sample, some buffer and possibly some additional reagent are mixed in a cuvette and placed in the sample chamber. The chamber is then sealed and the shutter opened. After it is clear from the display that there is a steady background light level, the remaining reagent mixture is injected to start the reaction.

Light production is recorded by the computer as a function of time. A typical assay result is shown in Figure 3-5. The value which corresponds most closely to the limiting concentration is the peak height. The background light level is subtracted from the measured peak height and

the difference divided by the dwell time to give the actual activity in counts per second (cps). The linear range of the assay and the formula for conversion of activities to concentrations is established by testing of standards. Note that the time required to perform the assays, including loading and changing the cuvettes, is around 65 seconds. This means that 6-7 assays can be performed before the MCS is full, assuming the dwell time is 0.1 second. After the MCS is full, peak and background values are recorded, the data file is stored, and the MCS is cleared to start again.

3.5.1 Luciferase

The luciferase assay is unchanged from that reported by Brasier et al. (Brasier et al. 1989). Their assay is designed for luciferase extracted from mammalian cells using the sonication buffer for this thesis. For this assay, 100 µl of sonicated sample are mixed in a cuvette with 360 µl of the ATP reagent cocktail. After the cuvette is placed in the sample chamber and the background level determined, 200 µl of the luciferin reagent cocktail is injected to start the reaction. For the reagent cocktail compositions, see Table 3-3. These reagents and samples are stored at -20°C when not in use. The linear range of the luciferase assay spans several decades of concentration. The lower limit is the limit of detectability, somewhere below 1 µg/L, and the upper limit is over 5 mg/L.

3.5.2 ATP

The ATP assay uses luciferase and has been reported frequently (McCapra 1987; Gould and Subramani 1988; Cole et al. 1967; Dhople and Hanks 1973; Garewal et al. 1986). The reagent concentrations used in this study are derived from the commercially available assay sold by Molecular Probes and are shown in the first column of Table 3-3. There is no ATP in the reagent mixture; it only contains luciferin. One hundred microliters of sample is mixed with 200 µl of 50 mM HEPES pH 7.8 in a cuvette. The cuvette is placed in the sample chamber and, when the background level is established, the reaction is started by the injection of 200 µl of reagent cocktail. The linear range of the assay is from about 0.1 µM, the lower limit of detection, to over 14 µM. Standards are checked each day the assay is run to account for possible degradation of reagents by the freeze-thaw cycle. Reagents and the reagent cocktail are stored at -20°C when not in use.

Table 3-3: Composition of Reagent Cocktails for Light Assays

ATP/Luciferin Assay		Luciferase Assay			
		ATP Cocktail		LH_2 Cocktail	
Luciferase	3 µg/ml	HEPES 7.8	25 mM	HEPES 7.8	25 mM
HEPES 7.8	50 mM	$MgSO_4$	15 mM	$MgSO_4$	15 mM
$MgSO_4$	10.0 mM	EDTA 8.0	4 mM	EDTA 8.0	4 mM
Luciferin	0.3 mM	$Na{\cdot}PO_4$ 7.8	15 mM	DTT	2 mM
OR		DTT	1mM	Luciferin	0.2 mM
ATP	10.0 mM	ATP	2 mM		

Standards are treated in the same manner as samples, including perchloric acid treatment and freezing and thawing episodes, if necessary.

3.5.3 Luciferin

The luciferin assay is derived directly from the ATP assay. Any residual luciferin in the ATP assay samples has insignificant influence on the assay due to the excess available in the reagent mixture, and the same is true of ATP in the luciferin assay. The cocktail is identical to that of the ATP assay, except that the 0.3 mM luciferin is replaced by 10 mM ATP (see Table 3-3). The assay procedure is also the same: 100 µl of sample mixed with 200 µl of 50 mM HEPES 7.8, then the reaction started by injection of 200 µl of reagent cocktail. The linear range is nearly as great as that of the ATP assay, from 0.1 µM to 8 µM. Again, standards are run each day that the assay is performed and are treated the same as are the samples.

3.5.4 Intact Cells

For various experiments, it was desirable to measure the light output of intact cells grown in shake flasks. These include early experiments to confirm that viable cells make light, comparison of luciferase activity of various clones, and instantaneous K_M investigations. The latter will be described separately below. The others followed the same general procedure as above. The sample is placed in the sample chamber, a background light level is established, and the reagent, dilute luciferin, is added to start the reaction. The sample was generally intact bacteria in medium or buffer. It was often diluted or mixed with other solutions before being tested.

The instantaneous K_M (K_{Mi}) offers a means to check the cell membrane permeability, because K_M can be considered an indicator of the dissociation constant for the substrate and enzyme. The K_{Mi} is measured as described below with intact cells, thus accounting for the resistance imparted by the cell membrane. It is "instantaneous" because the peak light production is measured, as opposed to the equilibrium production after several minutes.

Cells were grown in defined media in shake flasks. The media were at various pH's. The pH changes were made by changing the relative amounts of Na_2HPO_4 and KH_2PO_4 added while keeping the total [PO_4] constant. Immediately prior to the first light measurement, a sample was taken and the pH and OD were measured. Then several samples were taken in rapid succession and their light output was measured. Each sample was injected with the same volume of luciferin solution, but the luciferin concentrations varied. Up to 14 samples, or two passes on the MCS, were treated this way, with the time recorded for each. Immediately after the final sample, the pH and OD were checked again.

The initial and final ODs were used to calculate the ODs for each sample. The light production of each was then normalized based on the OD. The normalized light production was treated as the reaction velocity, and both a Lineweaver-Burke and an Eadie-Hofstee plot was used to determine the K_{Mi}. Finally, the K_{Mi} could be related to the pH, which did not change in the brief time required for these experiments.

3.6 OTHER ASSAYS

While this research was primarily concerned with the three light-based assay results, other compounds were monitored. Chief among these were the oxygen and carbon dioxide compositions of the feed and off-gas. These compositions were provided by the MGA 1200 mentioned in Bioreactor Operation above and were used to calculate O_2 uptake rates (OUR) and CO_2 evolution rates (CER). Other important compounds assayed regularly were β-gal, as a representative product, and certain medium components.

3.6.1 β-Galactosidase

The β-galactosidase assay is based on the one described in Sambrook et al. (Sambrook et al. 1989). This colorimetric assay measures the quan-

tity of ortho-nitrophenyl β-D-galactopyranoside (ONPG) digested by the enzyme over a fixed time period. The samples were first sonicated as described above (Intracellular Enzyme Extraction). Frozen samples were thawed and kept on ice during the assay procedure.

The assay procedure is as follows. Prepare fresh assay buffer by mixing 100 µl each of 3.36 M β-mercaptoethanol and 30 mM $MgCl_2$ with 2.3 ml of 0.1 M sodium phosphate buffer, pH 7.3. Add 100 µl of sample and place in a 37°C water bath. To start the reaction, 400 µl of warm (37°C) 0.51% ONPG in $Na{\cdot}PO_4$ buffer, pH 7.3 was added to the buffered sample. Temperature was maintained at 37°C for the duration of the reaction, usually 10 minutes. One ml of 1.0 M Na_2CO_3 was added to stop the reaction. The final absorbance at 420 nm (OD_{420}) was measured and recorded.

The activity of the sample, A, in units/ml, is given by the formula below.

$$A = \frac{40 \times OD_{420}}{3.5\,\mathrm{min}^{-1} \times t} \tag{3-1}$$

where t is the incubation time of the reagent mixture. A control sample using buffer in place of sample should give zero activity to make certain that no contamination exists.

3.6.2 Medium Components

The only medium component analyzed, besides luciferin, was the carbon source, glycerol. Samples from the fermentation were analyzed for the metabolic byproducts which are secreted into the medium, and these include lactate, pyruvate and acetate. The samples were assayed by high performance liquid chromatography (HPLC). An HPLC (WISP 701B and pump model 501, Waters Chromatography, Division of Millipore Corp., Milford, MA) fitted with an Aminex HPX-87H column from BioRad (Hercules, CA) was used for the analysis. Compounds were detected in the effluent by a differential refractometer (Waters R401) and data gathered by a Hewlett-Packard integrator (model 3390A).

The samples were prepared by filtration through a 0.22 µm filter. They were diluted to keep the glycerol content under 6 g/l, usually 4:1. An internal standard of 5 g/l glucose was added, as was 0.5 g/l NaN_3 as a preservative. The column was preconditioned to NaN_3. The elution buffer was

6.0 μM H_2SO_4. The internal standard permitted slight changes in injection volume to be accounted for.

As mentioned above (Growth Media), little acetate was expected because glycerol was used as the carbon source. None was found in the HPLC analysis. To double check this, samples from one fermentation were analyzed for acetate using a commercial assay kit from Boehringer Mannheim (Mannheim, Germany). The kit confirmed the HPLC results.

4. VIABLE CELL QUANTIFICATION

The first part of this research project was devoted to developing a novel method for the rapid, on-line determination of viable cell density. This was carried out in fermentations of *Escherichia coli* as a simple model of the more complex mammalian cell cultures, where such a tool may be more useful. This method utilizes intracellular expression of firefly luciferase to provide an easily measured light signal. Because firefly luciferase requires ATP as a substrate and non-viable cells cannot produce ATP, only viable cells will be able to produce light.

The results presented in this chapter will show that transfection of the parent *E. coli* strains resulted in expression of active luciferase. Also, when luciferin was added to the medium, only the viable cells were producing light. Typical on-line light data gathered using the equipment described in Section 3.4 are also presented. Factors which had a direct effect on the light output are analyzed, including agitation rate, inner filter effects and luciferin depletion by the luciferase-catalyzed reaction. A simple model corrects the light output during cell growth to account for the decreasing concentration of luciferin during each batch cultivation.

4.1 LUCIFERASE EXPRESSION AND ACTIVITY

Luciferase activity in intact cells was determined off-line by suspending the cells in an isotonic citrate buffer at pH 6 in a cuvette. Once the cuvette was in the sample chamber of the luminometer, luciferin was injected to start the assay. The luciferase activity should be directly proportional to the maximum light output from the sample. For intact cells, the maximum occurs within 2-3 seconds of luciferin addition. Samples were also lysed by sonication and the lysate was assayed for luciferase as described in Section 3.5.1. Luciferase activity was seen both in intact cells and in the lysates. The extracellular portion of the medium was tested for luciferase activity only after the cells had been removed by filtration by the same assay that was used for the lysates. Passage through a 0.22 μm filter ensured that no cells remained in the medium samples. After this treatment, the samples were unable to produce light even when ATP and luciferin were both added. To demonstrate that the medium does not deactivate luciferase through this treatment, luciferase was then added to the samples, and light was produced.

In order to generate bacterial cell lines with luciferase activity, each of the *Escherichia coli* strains was transfected with pRSVL, and the ATCC #15224 strain was also transfected with pSV2L/SVNeo. In each case, the plasmid-containing bacteria were shown to express active luciferase, but the untransfected host cells had no discernible luciferase activity. Each line had a different relative level of activity. DH5α cells transfected with pRSVL exhibited the highest luciferase activity, and ATCC #15224 transfected with pSV2L/SVNeo showed the lowest. ATCC #15224 transfected with pRSVL exhibited luciferase activity between the other two cell lines. The high expression levels in the DH5α line were expected, because the parent DH5α strain was designed for expression of plasmid DNA. The difference in activity between the lines derived from the different plasmids inserted into strain 15224 suggests that the RSV LTR is a stronger promoter in bacteria than is the SV40 Early promoter.

There was some concern that the insertion of these plasmids would the maintenance burden to the cells and thus decrease their productivity. To determine if this was the case, the parent and modified cells were characterized. No significant difference in either the growth rate or the β-galactosidase production rate was found between the parent and transfected cell lines growing in the presence or absence of luciferin. This is shown in Figure 4-1 A and B. In each part of this figure, the solid lines and open circles represent data for the parent ATCC #15224 strain and the dashed lines and squares represent the transfected cell line. In graph A, on top, the growth rates are compared and in graph B, on the bottom, the β-galactosidase productivity is compared. The higher slopes of the lines for the recombinant strains indicate increased growth and productivity rates. But the difference is less than 5% in each case. Furthermore, there was no detectable difference in the oxygen demand of the transfected and untransfected ATCC #15224 cells. Because the DH5α cells were not cultured in the bioreactor (see below), no quantitative oxygen uptake or carbon dioxide evolution rate analysis was performed.

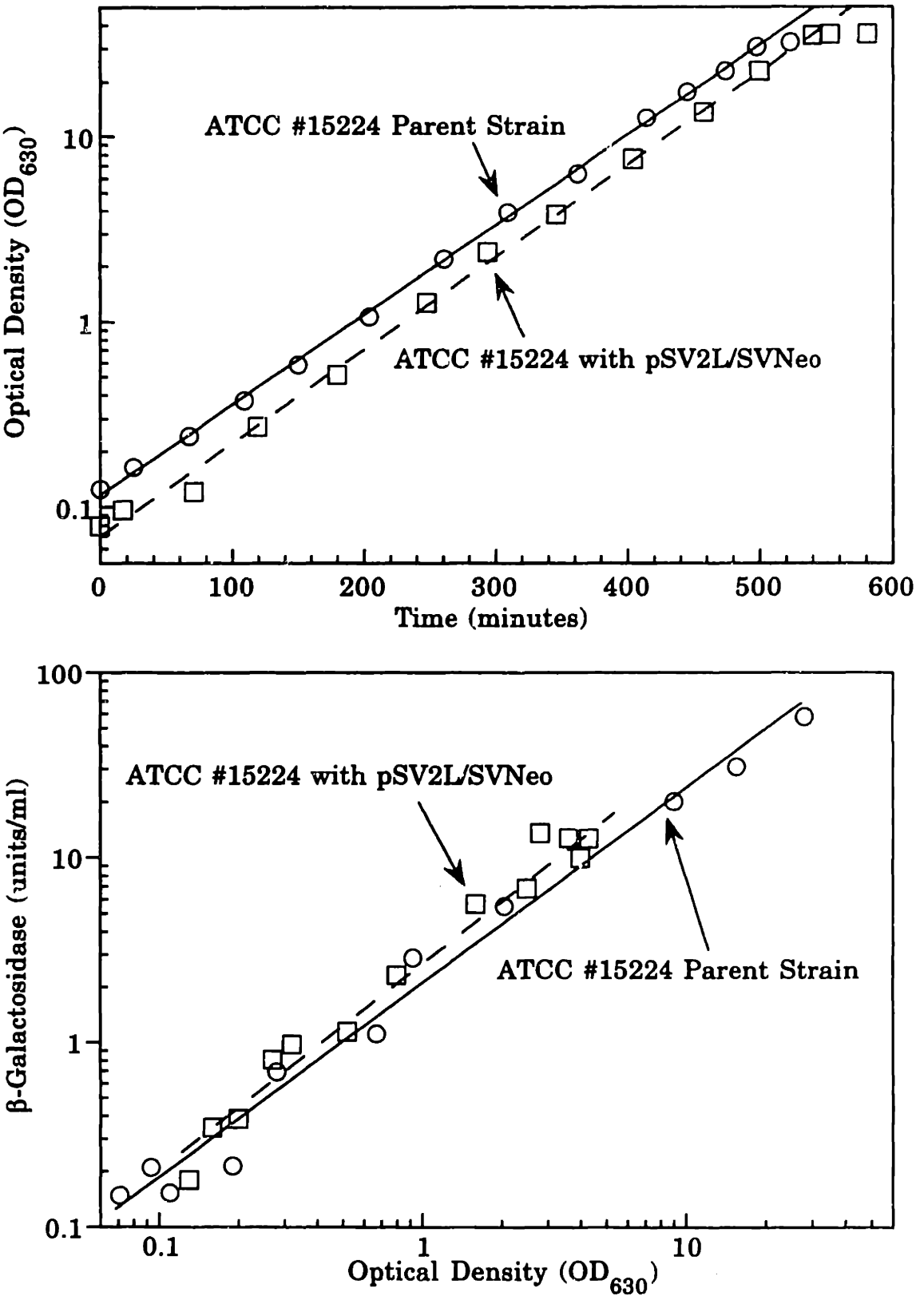

Figure 4-1: Effect of Transfection on Growth and Productivity
Figure 4-1A, on the top, compares the growth rates and Figure 4-1B, on the bottom, compares the productivity of parent and recombinant cell lines.

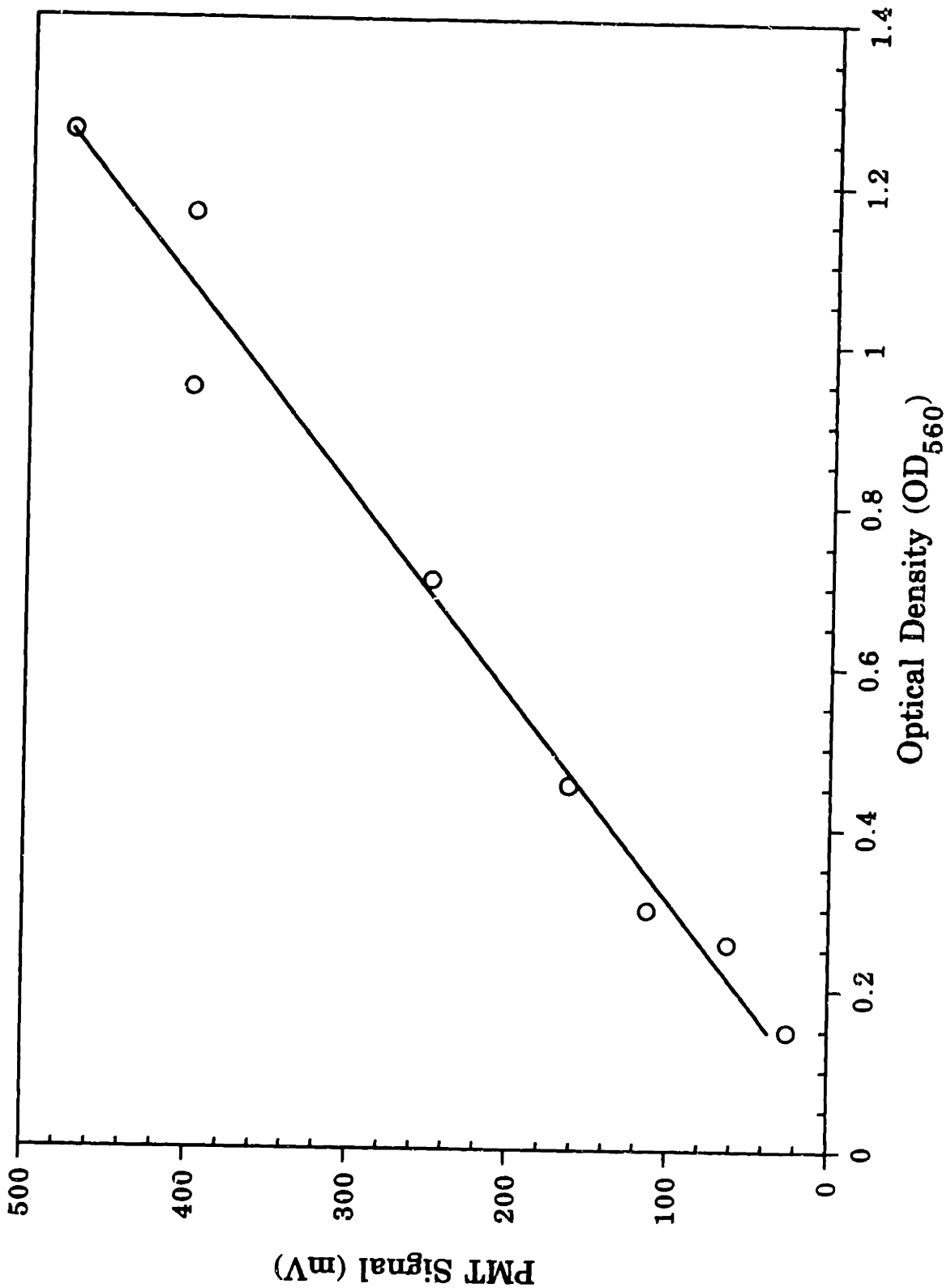

Figure 4-2: Initial Proof of Luciferase Activity in Intact Bacteria Sample are DH5α transfected with pRSVL and growing on LB medium.

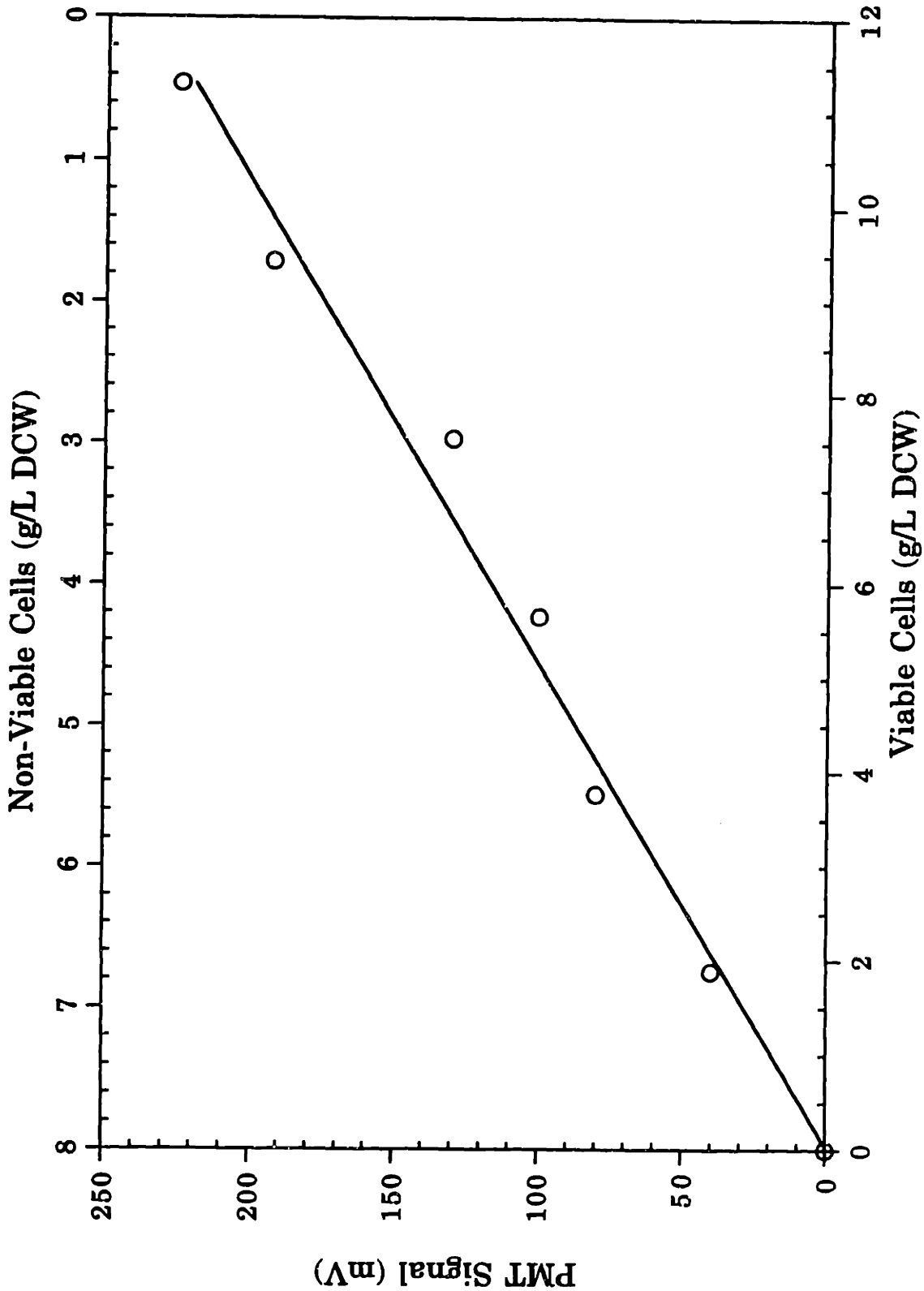

Figure 4-3: Light Emission from Mixtures of Live and Dead Cells
Samples are mixtures of intact viable and non-viable *Escherichia coli*.

4.2 LIGHT EMISSION FROM VIABLE CELLS

Once the intact, transfected cells had been shown to emit light in the presence of luciferin (LH_2), it remained to be shown that only the viable cells were the source of the light emission. Cultures of DH5α cells expressing luciferase from the plasmid pRSVL were grown in shake flasks on LB medium. After the OD was measured for each sample, it was placed in the luminometer sample chamber and luciferin was added by injection. The luminometer described in Section 3.4 was not fully assembled; so the signal was an amplified voltage that was measured with an oscilloscope. The amount of light emitted was proportional to the OD, provided the [LH_2] was kept constant (see Figure 4-2).

Additional shake flask experiments were performed to eliminate the possibility that dead cells could produce light during these measurements. Bacteria were grown in LB to in shake flasks with periodic addition of fresh medium. At about 9 g/L DCW, the broth from the flask was split into two flasks. One portion was returned to the shaker to continue cell growth, and the second was heated to 56°C for 60 minutes. The duration and temperature were chosen to ensure that fewer than one in 10^{14} cells remained viable without denaturing the proteins (Wang et al. 1979). OD measurements indicated that the final cell density of the heated portion was 8 g/L and that of the growing portion was 12 g/L DCW.

Light measurements were taken on mixtures of samples from the two batches. Each mixture had the same total volume, but the content ranged from 0 to 8 g/L of dead cells from the heated broth and 0 to 12 g/L of the unheated cells. The resulting linear relationship between the light production and the unheated cell density in these samples (see Figure 4-3) indicates that the non-viable cells do not effect light production. This experiment was repeated using streptomycin at 80 μg/ml instead of heat to kill the cells, and similar results were obtained. Thus, neither dead cells nor culture medium can serve a light source. This is due to lack of ATP in the non-viable cells and to the lack of ATP and luciferase in the medium.

4.3 ON-LINE DETECTION OF LIGHT

Once the linear relationship between light production and viable cells was confirmed, the feasibility of light production as a means for *in situ* monitoring of batch fermentations remained to be established. For

this purpose, the fermentor and light detection equipment described in Section 3.4 were assembled. Because the DH5α strain does not grow well on minimal media, the ATCC #15224 *E. coli* were used for all of the experiments in the bioreactor. Unless otherwise noted, a known quantity of luciferin was added to the medium prior to inoculation. The on-line monitoring computer measures the background light level over a four minute span before inoculation, and thereafter it is automatically subtracted from all light readings. So all of the light data presented in this thesis are those above the background level.

4.3.1 Typical Bioreactor Light Output

A number of different but interesting phenomena occurred in each and every run. The light data from two typical, bioluminescent fermentations are shown in Figure 4-4. The top graph is from the ATCC #15224 *E. coli* transfected with pRSVL and the bottom is from those transfected with pSV2L/SVNeo. In both graphs, the open circles and the left axis are the optical density data, and the solid line and the right axis are light emission data. As mentioned above, higher light production resulted from the cells transfected with pRSVL. The small blip in the light data in the top graph at approximately 650 minutes was caused by a temporary failure of the agitation motor controller.

Light levels that were well above the background level were seen immediately after inoculation. At the end of exponential growth, the light output rapidly decreased by a factor of about 2.5 in a period of only three minutes. This phenomenon always occurred approximately 15 to 20 minutes prior to the onset of cell lysis as determined by the demand for acid feed and cessation of base feed for maintenance of the broth pH. The optical density measurements shown in Figure 4-4 accurately reflect the viable cell densities as determined by colony forming unit assays. So this cannot be explained by a concurrent change in the number of viable cells. This rapid change appears to be triggered by carbon source limitations or possibly by a change in the compound serving as the carbon source.

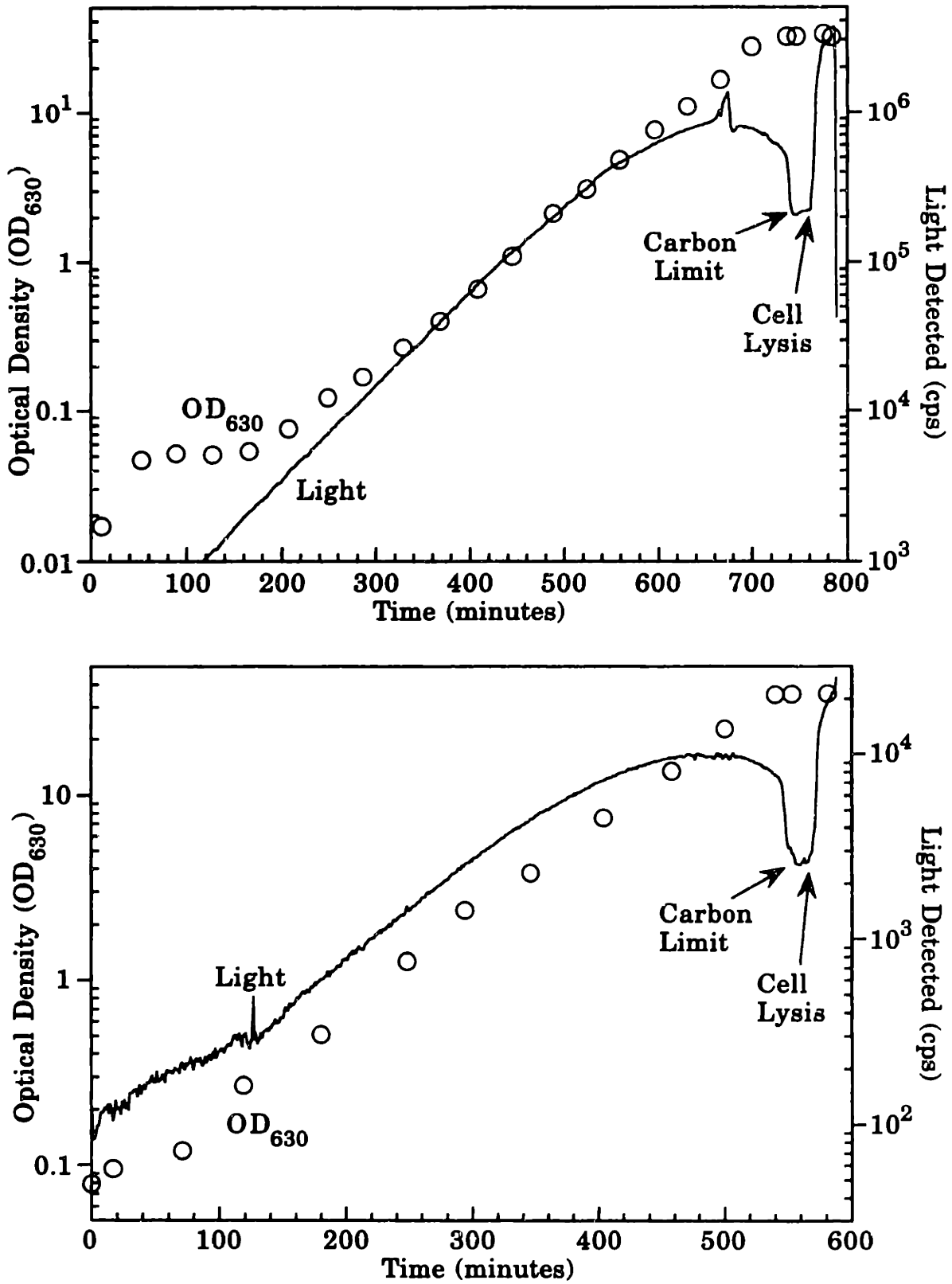

Figure 4-4: Light and OD Data from Typical Fermentations
Open circles are ODs (left axis) and solid lines are light (right axis).

Upon lysis, the light production rapidly rose over tenfold as compared to the lowest value caused by carbon source limitations. It seemed likely that this increase was due to luciferase activity outside of the cells. There the light would not have to pass through the plasma membrane to be detected, and this would give the appearance of increased light production. This possibility was tested by first analyzing the sterile filtrates of samples taken after the onset of lysis. These did not emit light. However, the unfiltered samples removed from the reactor at the same time continued to do so. Thus, the extracellular space can be ruled out as the source of the excess light production. The reason for these changes in light output are not yet clear, but they seem to be intracellular in nature. This suggests that this method may be quite useful for monitoring the metabolic state of a culture as well as its viability.

4.3.2 Effect of Lens Position

The lens assembly, which passes through the modified bioreactor head plate, was constructed with a six-inch long neck to enable its vertical position in the vessel to be varied (see Section 3.4.1). Bioluminescent fermentations were performed both with the lens immersed in the broth and with it kept one to two inches above the surface. The light levels detected showed minimal differences caused by the lens position. The data appeared less noisy with the lens immersed in the medium; so this was the preferred configuration. However, small leaks in the seal around the watchglass could lead to a significantly decreased detection efficiency if cells entered the lens assembly and absorbed or scattered light.

Fermentations run with the lens above the medium required stringent antifoam control to minimize light scatter in the foam layer. Another drawback to this configuration was that, at any given cell density, the amount of light detected decreased as the agitation rate increased, as shown in Figure 4-5. The second set of data for 400 rpm (open circles) was obtained during a different batch cultivation and is included to demonstrate run-to-run consistency. This decrease was probably caused by the changes in the morphology of the air-liquid interface in the reactor vessel. The deeper, conical surface seen at higher agitation rates exaggerated the light scattering at the interface. It also decreased the effectiveness of the antifoam controller because the foam probe is located near the vessel wall.

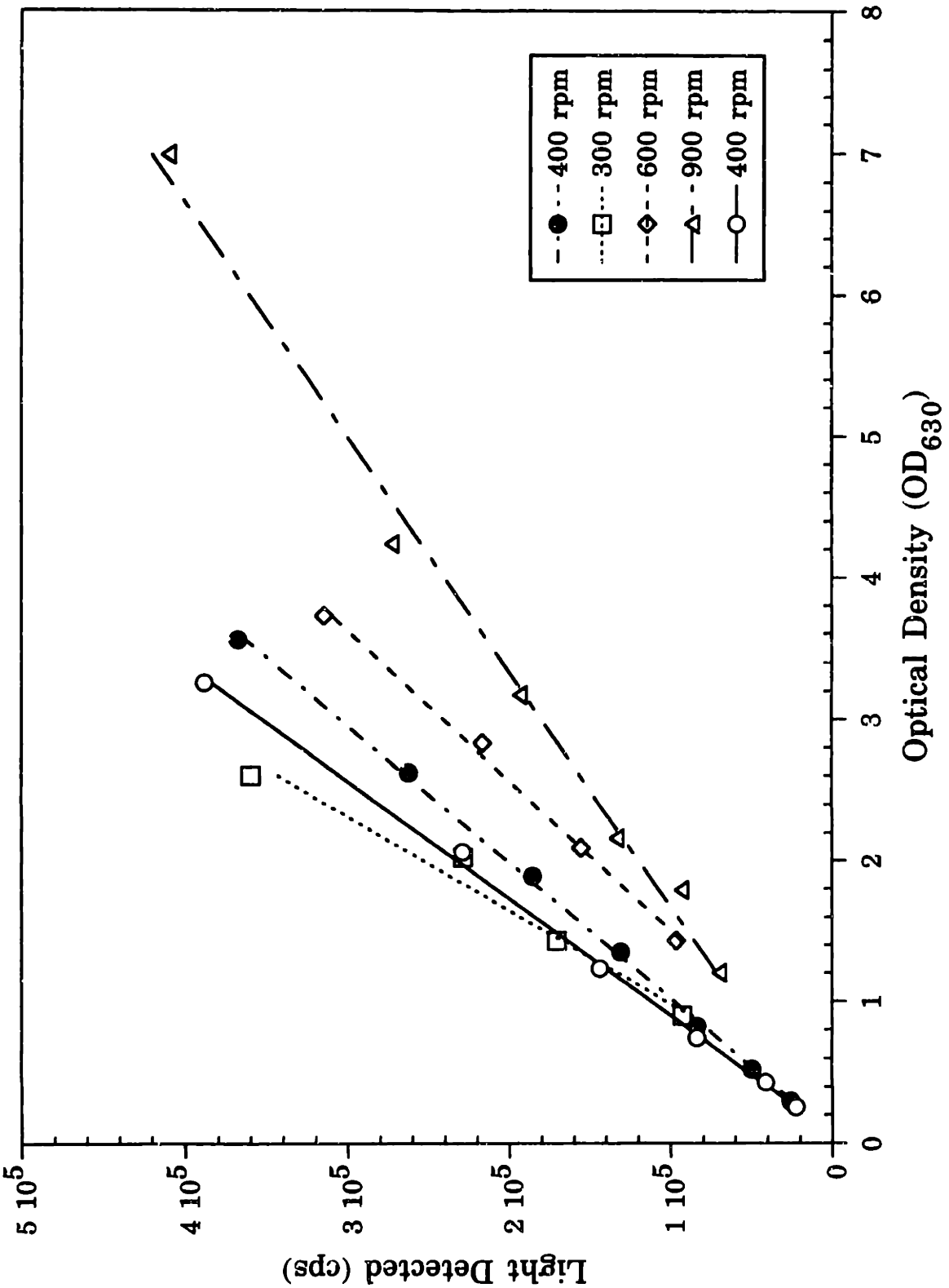

Figure 4-5: Effect of Agitation Rate on Light Detection

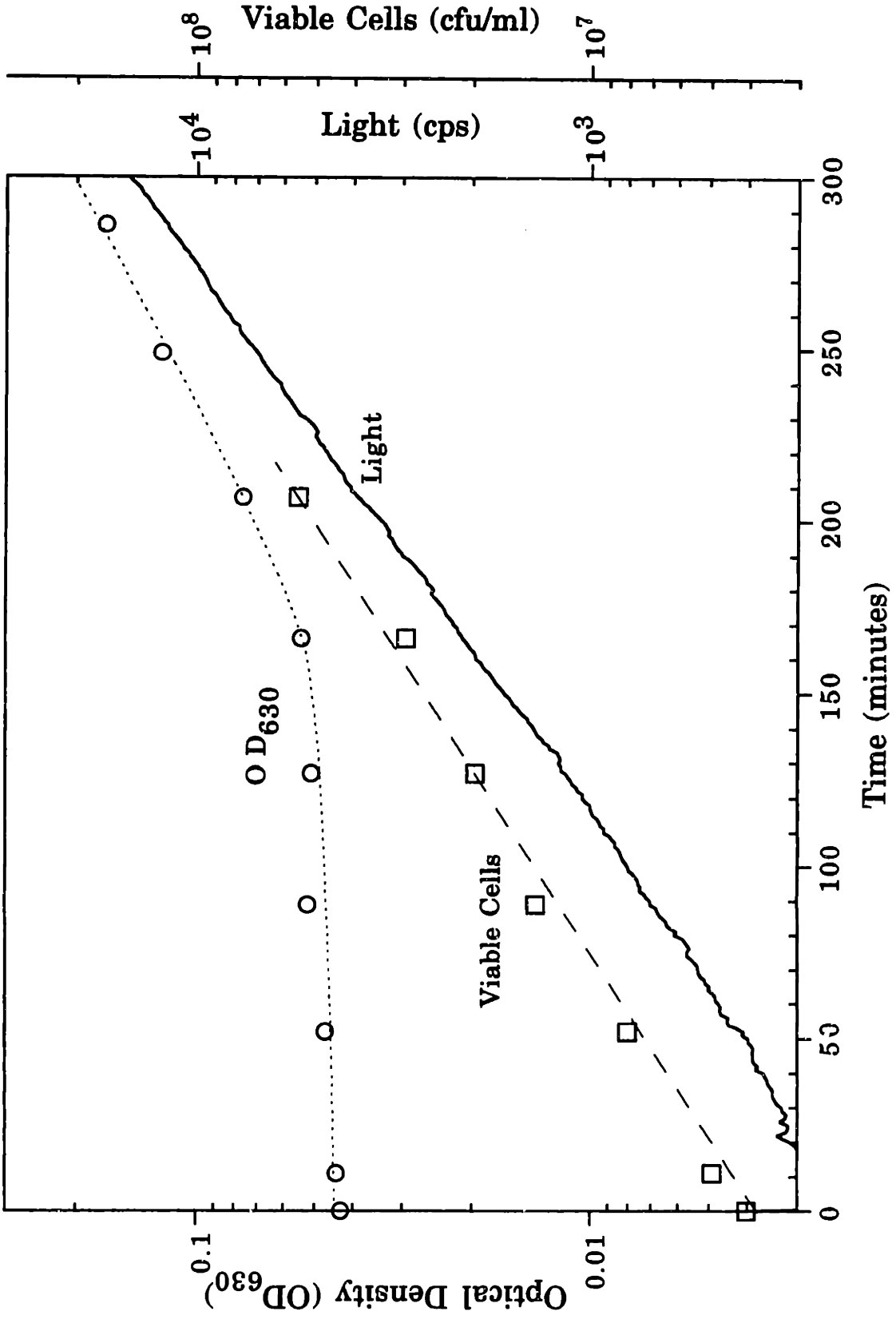

Figure 4-6: Light Data Tracking Viable Cells During Lag Phase

Since the foam-air interface was not conical, a significant layer of foam could build up in the center of the headspace without contacting the probe.

4.3.3 Lag Phase Viable Cell Detection

The most dramatic demonstration of the ability of luciferase to distinguish viable from non-viable cells occurred during the period known as the lag phase at the beginning of each fermentation, as shown in Figure 4-6.

During this period, light production increased exponentially even though the optical density was not increasing. Furthermore, the transition to exponential growth was not detectable by examination of the light data alone. Lag phase was only apparent when the optical density or other total cell data were examined. Colony forming unit assays of samples taken during the lag phase confirmed that the viable cell number was increasing exponentially and was tracked very accurately by the light data (see Figure 4-6). This shows that, at low cell densities with ample nutrients, light emission provides an accurate measure of the number of viable cells in the bioreactor.

This is also an indication that the so-called lag phase is actually part of the exponential growth phase, but only a small portion of the initial inoculum was viable. The remainder of the inoculum was either inactive or dead. A comparison of colony counts on LB plates with and without ampicillin showed that over 98% of the colony forming units in the inoculum contained the plasmid. This means that all of the inoculum was still resistant to ampicillin, and so it was not killed by the fresh ampicillin in the bioreactor. The exact reason why a significant portion of the initial inoculum does not grow is still unknown.

4.3.4 Non-linear Response at Higher OD

It is apparent from Figure 4-4 that the light production is not a linear function with the number of viable cells as exponential growth progresses. The deviation from linearity generally begins when the OD of the broth is greater than five absorbance units. As the cell density continues to increase, the light detected per viable cell decreases. There are many possible explanations for this behavior. Two that will be addressed in this chapter are the inner filter effect and consumption of luciferin by the lu-

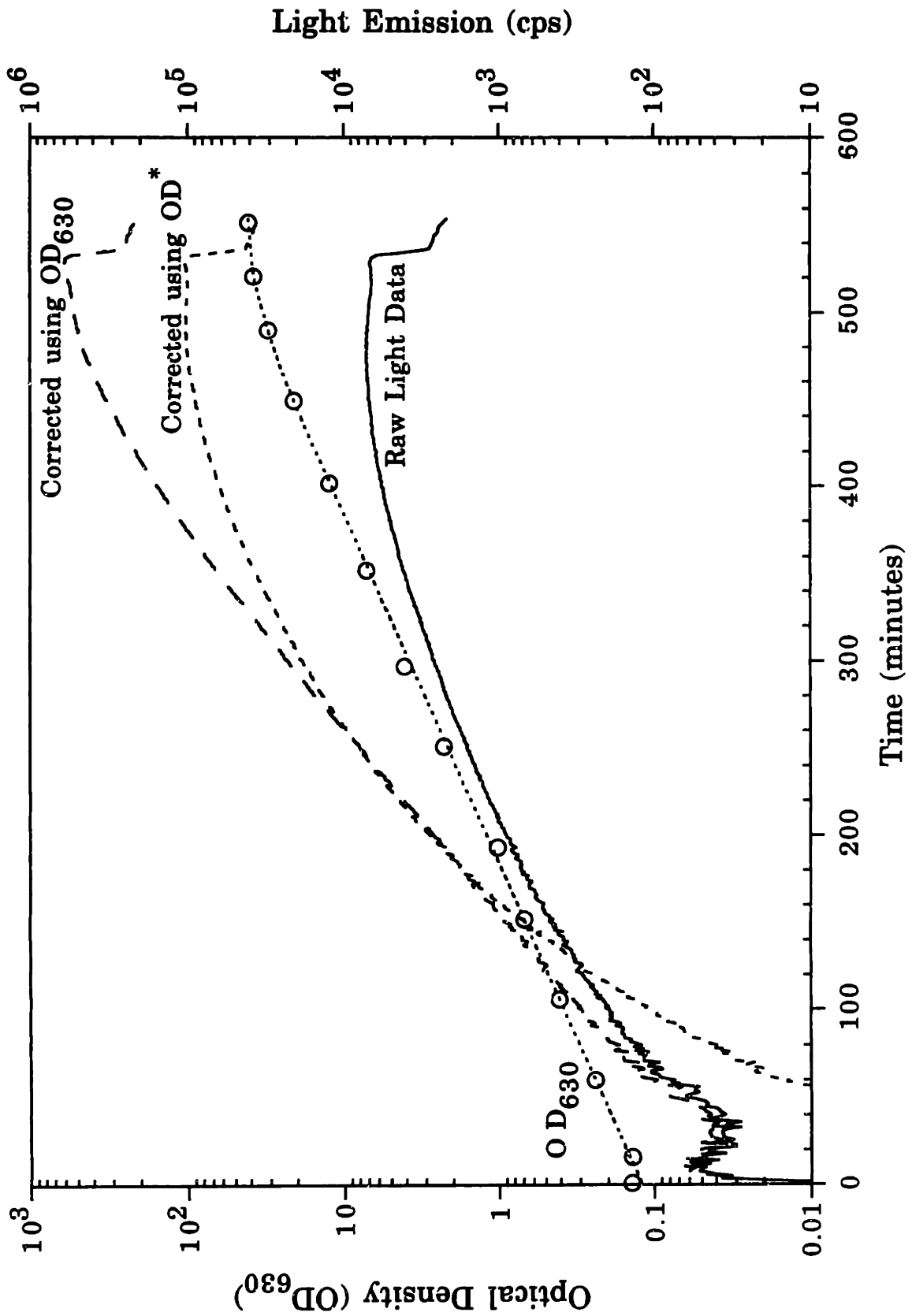

Figure 4-7: Inner Filter Corrections Using Yappert's Theory

ciferase reaction. Other possibilities, including further investigations into the inner filter effect, will be covered in Chapter 5.

As was discussed in Section 2.4.4, the inner filter effect is the absorption or scattering of the light emitted from a sample by some other species within that sample. In the case of culture bioluminescence, the inner filter effect is the result of light scatter caused by the cells. Because the OD of the broth is known quantitatively, it is possible to correct for the inner filter effect using the Yappert correction, as given in Equation 2-9. This OD was measured in samples that were diluted into the regime where the Beer-Lambert law is valid. At higher cell densities, the alternate optical density (OD^*) given in Equation 2-10 and shown below can be used.

$$OD^* = \varepsilon' l \log c = \varepsilon' l \log\left(\frac{OD}{\varepsilon l}\right) \tag{4-1}$$

The corrected light outputs calculated using the Yappert correction factor as computed with both of these optical density definitions are shown in Figure 4-7. It is obvious that the correction using the measured OD is far too great. It apparently over-corrects because the Beer Lambert law does not account for the significance of multiple scattering effects at the higher densities. The other correction is in the wrong direction at low densities, where it is not expected to perform well. At higher densities, it does not change the relation between the light and OD data, except for a change in the magnitude of the difference, which is increased. From this analysis it is concluded that there must be another source of the decreasing per-cell light output. This will be discussed further in Chapter 5.

4.4 CORRECTING FOR LUCIFERIN CONSUMPTION

As presented previously, the light data from the bioreactor (see Figure 4-4) are not linear with the OD or the viable cell number late in the exponential growth phase. This decrease in per-cell light emission is not explainable by the inner filter effect. Another likely cause could be the decrease in luciferin due to its consumption from the medium. As the bioluminescent fermentations proceed, luciferin is continually converted by luciferase to oxyluciferin, and the decreased substrate concentrations may account for the decrease in light production. Data which support this

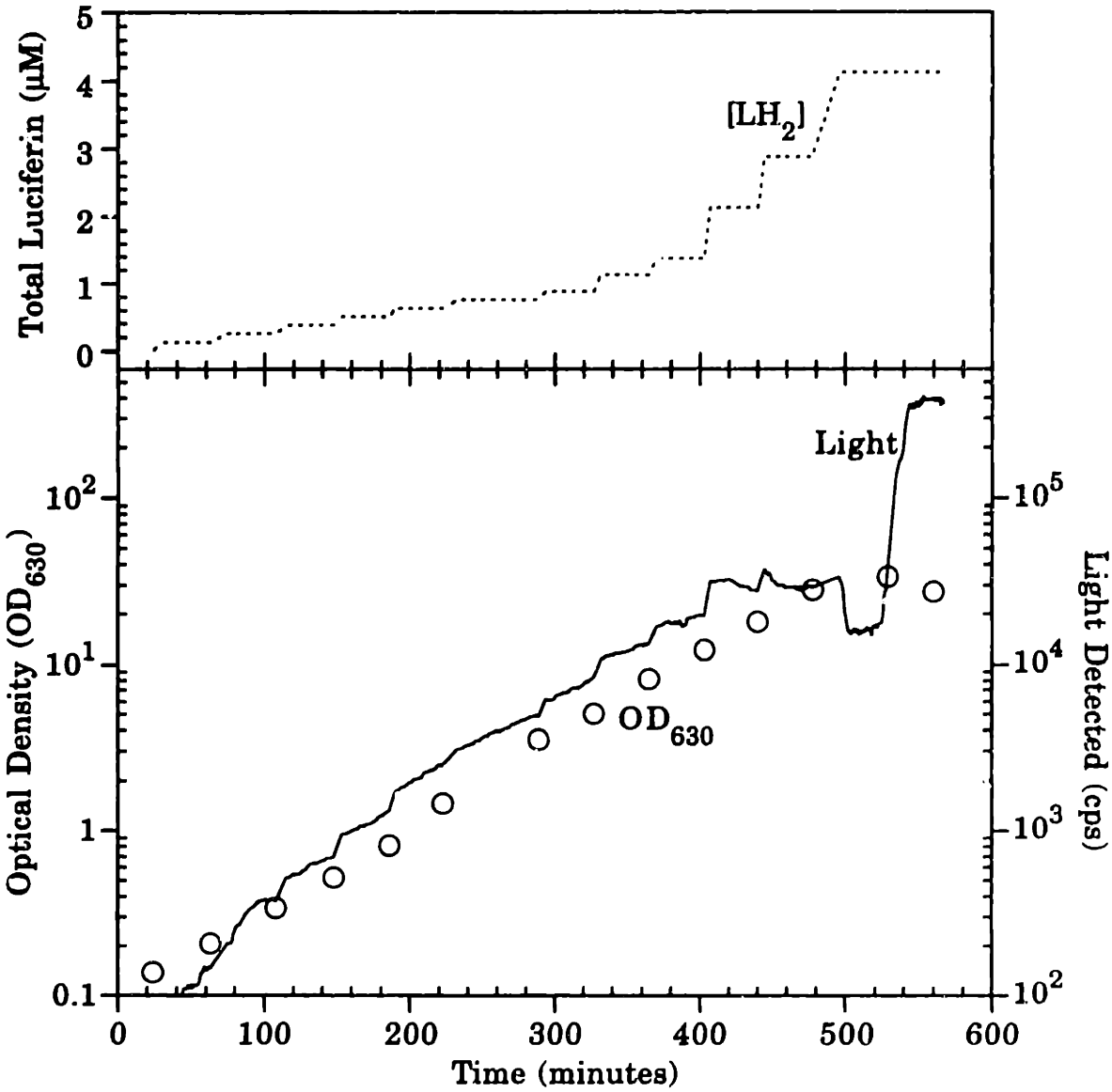

Figure 4-8: Effect of Luciferin Addition During a Fermentation

explanation are shown in Figure 4-8. In this experiment, luciferin was periodically added to the medium. Upon each addition, the light output increased rapidly but was followed by a plateau or even a decrease, supporting the idea that luciferin may be limiting. The total luciferin concentration, which was lower than the typical 15 μM in order to exaggerate concentration effects is shown in the dotted line on the top part of the figure. Overall, the light data, shown with the solid line, tracked with the cell density, shown as open circles, better than did the light data when all of the luciferin was added before inoculation. However, the light levels still fall off between each of the luciferin additions and at the higher ODs.

Even at 15 μM, luciferin is a very costly component of the defined medium used in this research. For this reason, it is used at concentrations that are close to the reported K_M, which is about 8 μM at the cytoplasmic pH of 7.1. Thus, small changes in $[LH_2]$ may have some effect on the light output from the cells. The intracellular reaction is assumed to follow Michaelis-Menton kinetics, as reported for *in vitro* behavior. The changes in $[LH_2]$ can be accounted for during a fermentation. At any given time, the light detected (I) is related to the luciferin concentration by:

$$I = \frac{V_{max}[LH_2]}{K_M + [LH_2]} \tag{4-2}$$

In this equation, V_{max} is an instantaneous value which incorporates any relevant effects that ATP concentration, luciferase activity and detector efficiency may have. With such a definition, V_{max} has little physical meaning and is subject to change at any time as the conditions change. In the case where there is a large excess of luciferin (i.e.: $[LH_2] >> K_M$), the right-hand side reduces to V_{max}. Therefore, when $[LH_2]$ is known, the actual light detected (I) can be adjusted to that value (I^*) expected if there was excess luciferin by the following equation, since V_{max} takes all other factors into account.

$$I^* = V_{max} = I\left(1 + \frac{K_M}{[LH_2]}\right) \tag{4-3}$$

Unfortunately, the exact luciferin concentration is only known in a real fermentation at the time of inoculation, and it cannot easily be measured in an on-line manner.

On the other hand, the number of photons released by luciferase is directly proportional to the number of luciferin molecules converted to oxyluciferin. These photons are collected and counted on-line as an integral part of this monitoring method. It should therefore be possible to calculate the luciferin concentration at any time during the fermentation. The measured light intensity is actually the photon flux or rate of photon impacts at the detector. It can be related to the rate of luciferin consumption by the use of Avagadro's number (N), the liquid volume in the bioreactor (V), and two efficiencies. The first is the quantum efficiency of the reaction (q), which is the average number of photons released per luciferin molecule consumed. The second is the detection efficiency (η), which is the fraction of the photons released by the cells in the bioreactor that are detected. The latter is primarily determined by the nature of the experimental apparatus. The luciferin-to-light relation is shown in Equation 4-4.

$$I = NVq\eta\frac{\partial[LH_2]}{\partial t} \tag{4-4}$$

Rearrangement and integration leads to the following expression for $[LH_2]$.

$$[LH_2]_t = [LH_2]_0 - \frac{1}{NVq\eta}\int_0^t I dt' \tag{4-5}$$

The subscripts denote the time t following the inoculation at time $t = 0$. The integration of Equation 4-4 to give Equation 4-5 requires that the liquid volume is known and that the two efficiencies remain constant during the batch fermentation (Dull 1941). Off-line measurements of the luciferin concentration at various times during a fermentation were used to evaluate the overall efficiency ($q\eta$), as shown in Figure 4-9. Because the two efficiencies are virtually impossible to separate, only their product can be accurately reported. For the equipment and conditions used in this research project, $q\eta = 9.4 \times 10^{-12}$ photons detected per molecule reacted.

This seems very inefficient at first, but a great deal of the detection losses can be accounted for by simple geometry. The probe lens has a radius of approximately 2 cm and a surface area around 12 cm^2. The total surface area of the liquid in the bioreactor is close to 1500 cm^2. The relative values for these surface areas indicate that fewer than one in 10^2 photons leaving the culture strike the lens. A similar portion of those that do

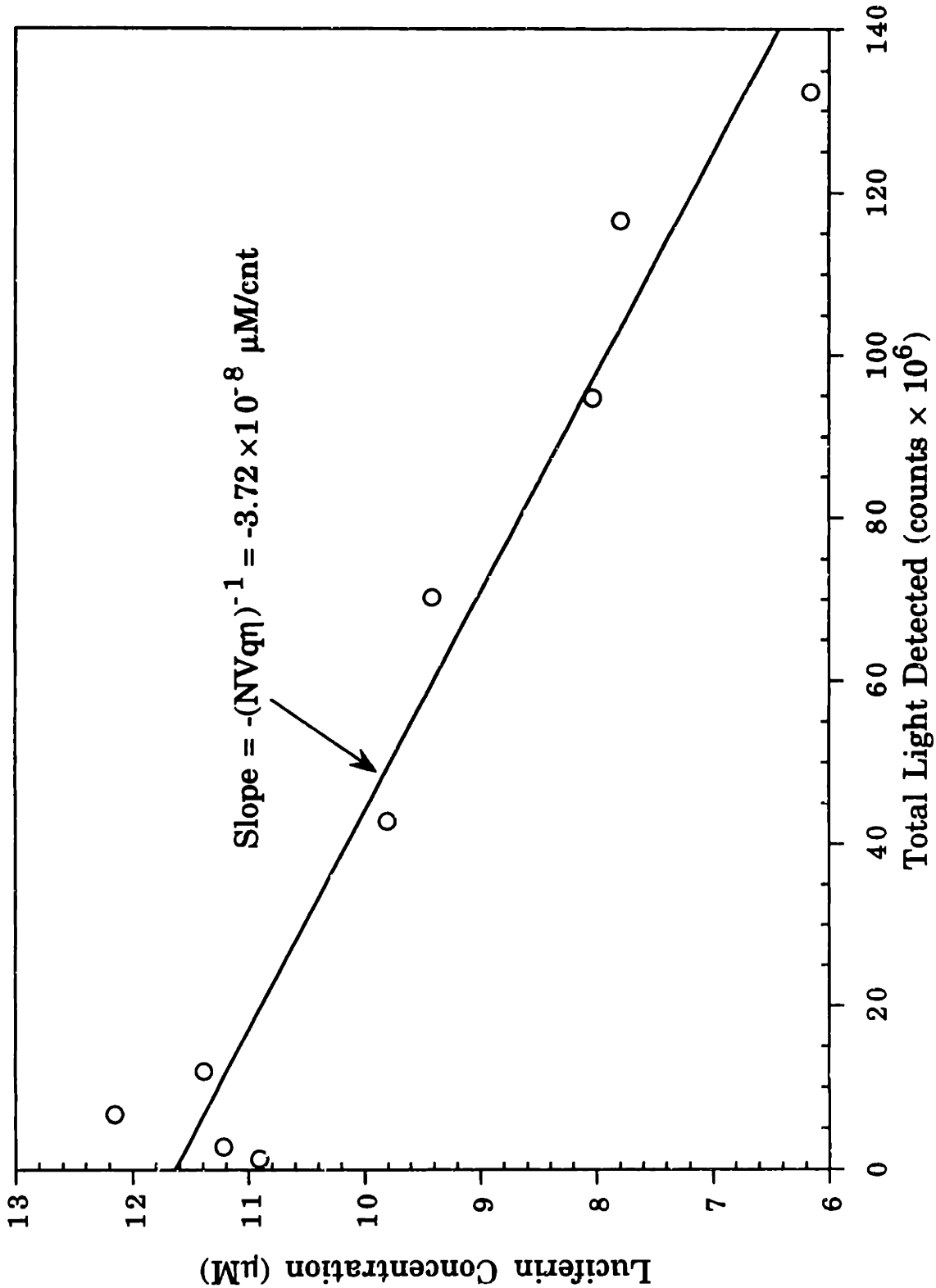

Figure 4-9: Determination of Overall Light Detection Efficiency
Total light is the time integral from the time of inoculation.

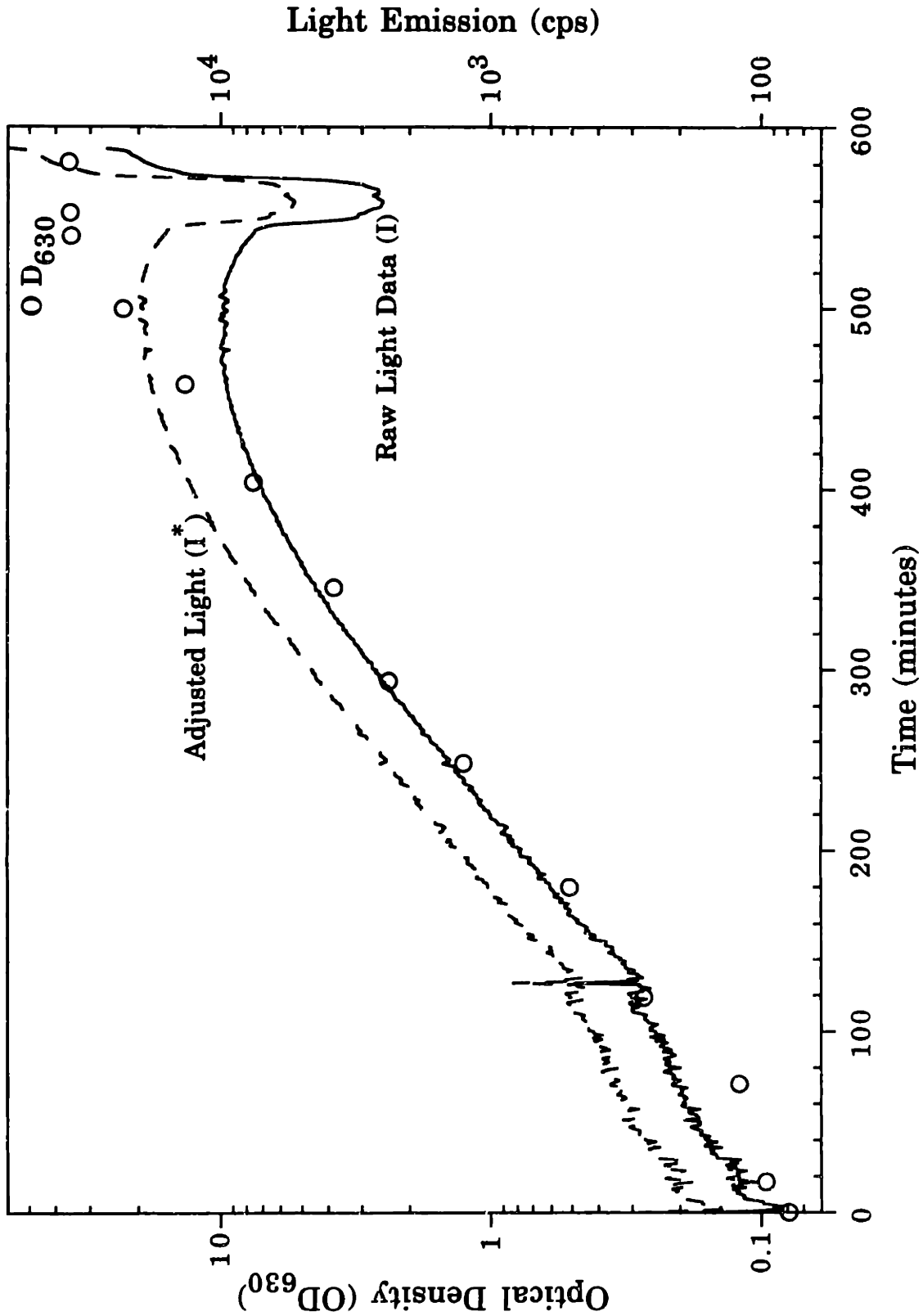

Figure 4-10: Light Adjusted for Calculated Luciferin Depletion

strike the lens will do so at the wrong angle. Thus, they will not enter the optical fiber from within the acceptance cone and will not be transmitted to the detector. Finally, the transmittance of the fiber and the quantum efficiency of the PMT each account for another order of magnitude. Thus, this analysis accounts for approximately 10^6 of the 10^{11} photons which are not detected for each that is. Fiber age and electronics may account for additional losses. In addition, the possible range for the quantum efficiency is known even though its exact value is not known. The range is from 0.2 to 0.88, according to the literature (McElroy and DeLuca 1985). The primary factor affecting the quantum efficiency is pH; so there is a possibility that it could be evaluated by duplicating cytoplasmic conditions *in vitro*.

From the data presented above, the detection efficiency (η) can be seen to be on the order of 10^{-11} photons detected per photon released, but many factors may affect its value. As the optical fiber bundle ages and is subjected to physical manipulations, individual fibers within the bundle may break. This decreases the fiber transmission and therefore the detection efficiency. Also, fouling of the lens or the watchglass on the lens assembly will lead to a decrease in the detection efficiency. Fouling is minimized by the use of the watchglass to protect the lens and of a silicone based hydrophobic coating on the watchglass. Cleaning and, if necessary, replacing fouled watchglasses between batches also helps to reduce the potential for fouling. Furthermore, some changes in η may be caused by changes in the detector electronics. A different photomultiplier tube or photon counter may have a slightly different internal efficiency. Also, the discrimination setting on the photon counter may drift. This setting is a threshold value used by the photon counter to distinguish random noise from actual photons. Small changes in the discrimination setting could thus result in line noise counting as photons or in some photons not being counted.

Visual inspections of the lens and watchglass found no discernible fouling over the course of each batch fermentation. Also, the fiber bundle and the electronic components are kept in fixed locations during each batch; so it is unlikely that any fibers are damaged or settings changed. The pH of the cytoplasm is maintained under a wide variety of conditions. So it is reasonable to expect that the overall efficiency ($q\eta$) remains constant over the course of each batch fermentation. The linearity of the fit in

Figure 4-9 supports that hypothesis. Since these values are constant, they can be used in real time to correct the light data for luciferin consumption. Avagadro's number and the liquid volume are known and constant. So by combining Equations 4-3 and 4-5, the correction becomes:

$$I^* = I\left(1+\frac{NVq\eta K_M}{NVq\eta[LH_2]_0 - \int_0^t Idt'}\right) \tag{4-6}$$

This equation was applied using the values for the efficiencies as determined in Figure 4-9, and the results are shown in Figure 4-10. The solid line shows the raw light data, and the dashed line shows the value adjusted according to Equation 4-6. This adjustment, however, does not account for all of the non-linearity in the later portion of the exponential growth phase. Additional possible reasons for this non-linearity are discussed in the next chapter.

5. ATP MONITORING RESULTS

The results presented in the previous chapter demonstrated the ability of intracellularly expressed firefly luciferase to provide a signal of cell viability. When this signal was measured on-line, it provided an accurate measure of the viable cell density at low cell densities. However, at higher densities, the light emitted no longer corresponded to the viable cell number. In this chapter, the nature of this change in per-cell light emission will be reported. Because firefly luciferase utilizes adenosine 5'-triphosphate (ATP) as a substrate, changing intracellular ATP concentration may cause these changes in the amount of light emitted by each cell.

This chapter will present experimental results which demonstrate that this is indeed the case. First, other factors which may have contributed to the changing per-cell light emission will be analyzed in order to demonstrate that these are not responsible for the observed behavior. Next, the intracellular ATP concentrations will be determined by assays samples from the fermentation broth, and the efficacy of the extraction technique will be evaluated. Finally, these ATP concentrations will be used to relate the light production from the cells with its dependence on the intracellular ATP pool.

5.1 FACTORS THAT MAY AFFECT LIGHT

Before one can conclude that the light emission is related to the ATP concentrations in the cells, other potential causes of the changing light production must be examined. In the previous chapter, it was shown that the fractional cell viability remained constant and that consumption of luciferin from the medium was not a cause for the decreased per-cell light output. Nor did a theoretical correction for the inner filter effect result in improvements in the correlation between the light emission and the cell density late in exponential growth (Figure 4-7). Several additional factors are addressed in this section, including dissolved oxygen levels, luciferase activity, luciferin transport into the cytoplasm, experimental analysis of the inner filter effect, and cell sizes.

5.1.1 Dissolved Oxygen

The bioreactor used for these studies controlled dissolved oxygen (DO) by changing the agitation speed. As shown in the previous chapter,

changing the agitation rate can have an effect on light detection (Section 4.3.2). It was therefore necessary to change the DO control system in order to examine the effect of DO independently of the effect of the agitation rate. To accomplish this, the QuickBASIC program that had been written to monitor the bioreactor using the Omega A/D board was modified to also control the DO. The modified program utilized a digital proportional, integral and differential (PID) control algorithm to maintain the DO at a given setpoint. The controller changed the oxygen content of the feed gas by adjusting the relative flow rates from air and nitrogen cylinders to the bioreactor. This control approach was independent of the total gas flow rate and the agitation rate, which were both kept constant. The controller was tuned based on calculations using an experimentally determined value for the gas transfer coefficient ($k_L a$) of the bioreactor, and then fine-tuned in experiments with a cell-free system.

Based on the light detection efficiencies presented in the previous chapter (Section 4.4), even when the light production is as high as 10^4 counts per second (cps), the oxygen required by luciferase is only 10^{-4} mmole/L/hr. These relative rates indicate that the dissolved oxygen level would have little effect on the luciferase reaction in these aerobic fermentations. Significant changes in the DO, both transient and long-term, did not cause any discernible change in the light output. The only occasion on which such a change has occurred was when the DO fell to below 15% of air saturation (Figure 5-1). As the DO, shown by the dotted line and the right axis, fell below 5%, the per-cell light detected, shown by the solid line and the left axis, changed its pattern of linearly decreasing with time. The linear decrease was extrapolated to enhance this observation as shown by the dashed line in Figure 5-1. For the fermentations performed in this research, the DO setpoint was 35% in order to avoid the anomalous behavior of light production introduced at the lower DO levels.

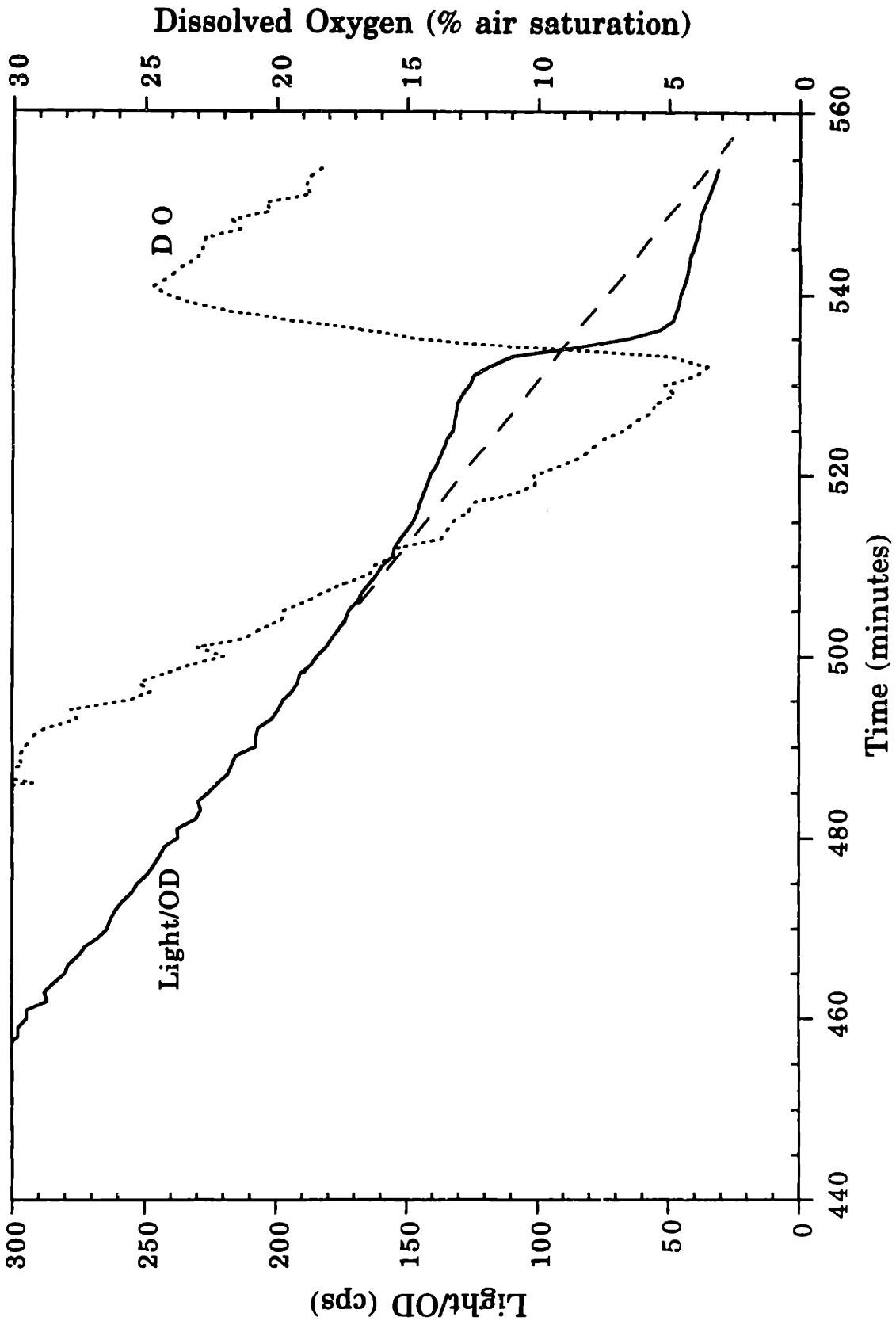

Figure 5-1: Effect of Low Dissolved Oxygen on Light Production

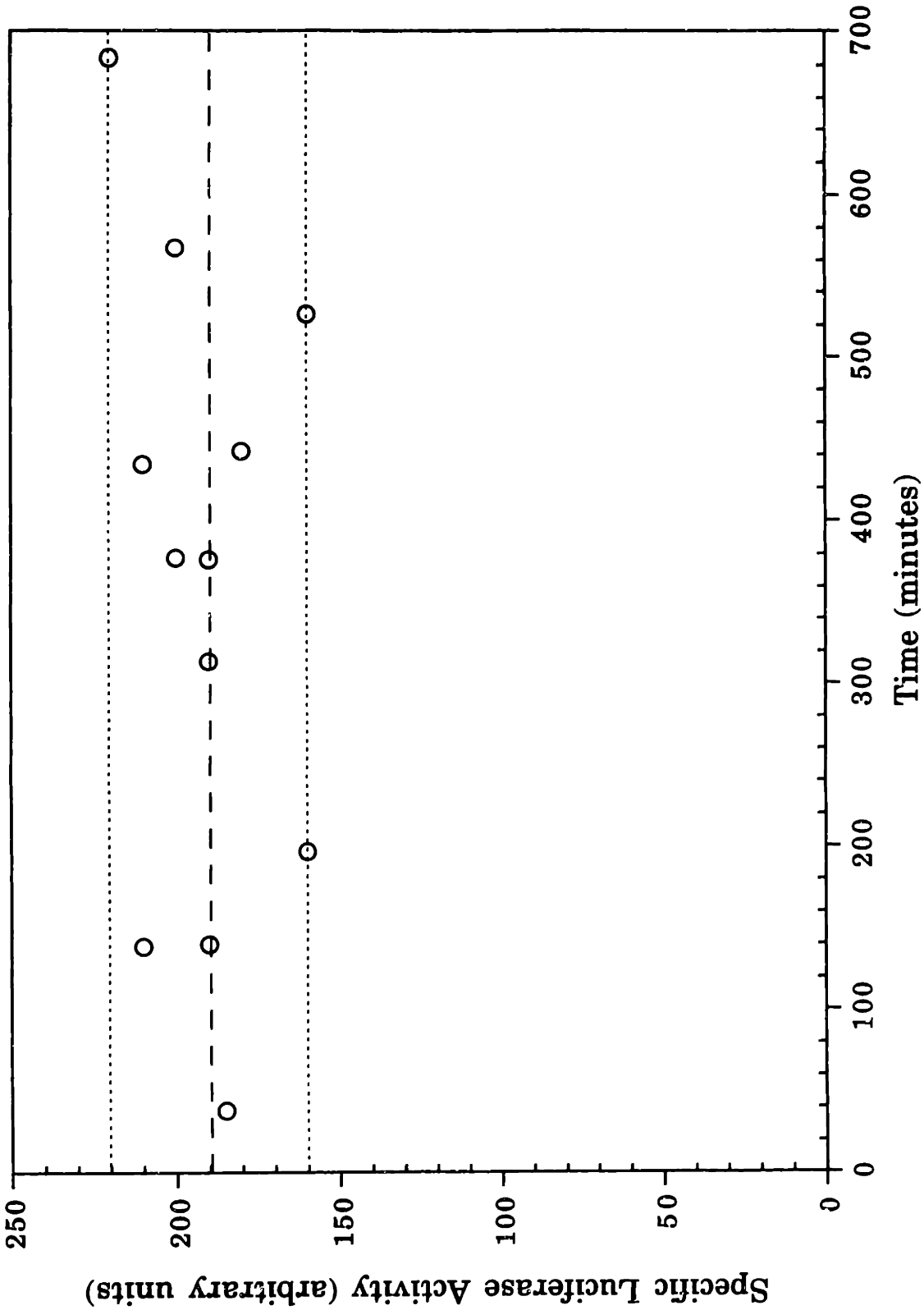

Figure 5-2: Luciferase Activity During a Typical Fermentation
The dashed line is the average value, and the dotted lines show the maximum variation of the data.

5.1.2 Luciferase activity

Unlike oxygen, the activity of luciferase in the cells is difficult to control and likely to have very significant effects on light production. Ideally, the enzyme might be self-regulated by virtue of genetic manipulations of the plasmid. While this may be possible, it would involve additional genetic techniques and is not germane to this research project. Luciferase has a half-life of about 20-30 minutes in bacteria (Thompson et al. 1991); so a constitutive promoter should lead to constant activity during exponential growth. Since both of the plasmids used in this study expressed luciferase from mammalian viral promoters which have been shown to be constitutive in bacteria, luciferase activity was expected to remain constant.

This was confirmed experimentally by assaying the luciferase activity in cell lysates of culture broth during fermentations. Lysis of the cells was accomplished by sonication. After storage at -20°C, the extracts were assayed for luciferase activity, and the results from a batch cultivation are shown in Figure 5-2. The specific luciferase activity, reported in arbitrary units, was calculated based upon its light production and the optical density of the sample. The average is shown by the dashed line, and the maximum variation of 15% seen in the data (dashed lines) is only a slightly greater than the standard error of 10% for the measurements. It is therefore concluded that the luciferase activity remains constant throughout exponential growth. The small variations in the luciferase activity do not appear follow any trend, nor does the activity decrease with growth.

5.1.3 Luciferin Transport into Cells

In the previous chapter, the effects of the luciferin concentration in the medium were addressed using an equation which corrected the light emission data. However, all of the active luciferase is inside the cells; so the luciferin must cross the plasma membrane in order to react. Literature reports have stated that luciferin enters the cells passively (Wood and DeLuca 1987), but only the uncharged luciferin molecules can be transported in this manner. At a medium pH of 7.0, nearly 99% of the luciferin exists in a charged state. For this reason the medium used in this

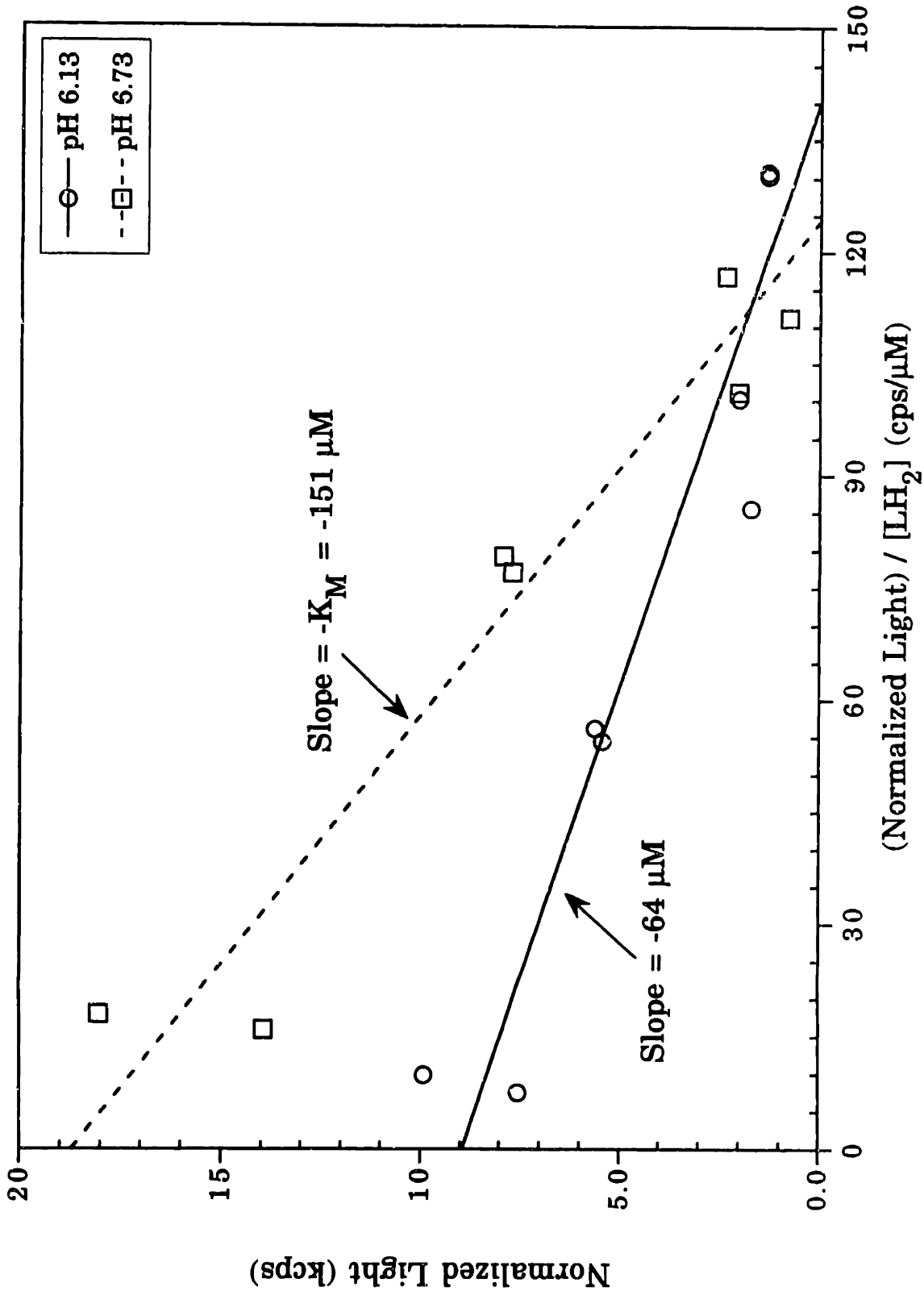

Figure 5-3: Determining $K_{M,l}$ Using an Eadie-Hofstee Plot
Light data was normalized by the optical density of the sample.

research was adjusted to a pH of 6.0, ensuring that less than 90% of the luciferin was charged. To confirm the passive diffusion mechanism for luciferin entry into the cells, the following analysis was performed.

The ratio of charged to uncharged luciferin at equilibrium is fixed both inside and outside the cells by the pK_a of luciferin (approximately 5.2) and the pH of the cytoplasm and medium, respectively. Based on the rate with which the light production rises to a maximum when luciferin is added to a sample, the rate of diffusion appears to be very fast. Also luciferase flash kinetics cause light output to reach a maximum value in less than a second and then to fall off rapidly. From these observations, one can conclude that the initial peak seen when luciferin is added to samples of intact cells is the result of the initial entry of uncharged luciferin molecules into the cell. It is rapid and transient, preceding the establishment of equilibrium, and thus would represent the concentration of uncharged luciferin outside the cells.

A series of experiments was carried out to measure the initial peak height using various concentrations of luciferin at several sample pH's. For each pH, the instantaneous K_M, or $K_{M,i}$, was calculated based on these initial maxima using an Eadie-Hofstee plot (see Figure 5-3). Because this analysis only examined the initial rate of the reaction, these $K_{M,i}$ reflect the concentration of the uncharged luciferin in the medium. That being the case, they should vary with the pH in an exponential fashion by rising tenfold for each pH unit above the pK_a of luciferin if the luciferin enters the cells by diffusion. This is because the equilibrium concentration of uncharged luciferin decreases by tenfold for each unit that the pH rises. Also, the pH of the cytoplasm remains constant; so the actual K_M inside the cells is constant.

The results from these experiments confirmed the above hypothesis (see Figure 5-4). An exponential least-squares fit of the data passed very close to the expected value (shown as a filled square) of 16 μM at a pH of 5.2. These values correspond to twice the literature K_M and the luciferin pK_a, respectively. These results confirm the literature reports that the luciferin enters the cells by passive diffusion. Thus, there is no active transport mechanism for luciferin which could cause changes in per-cell light production by moving luciferin into the cytoplasm at a different rate.

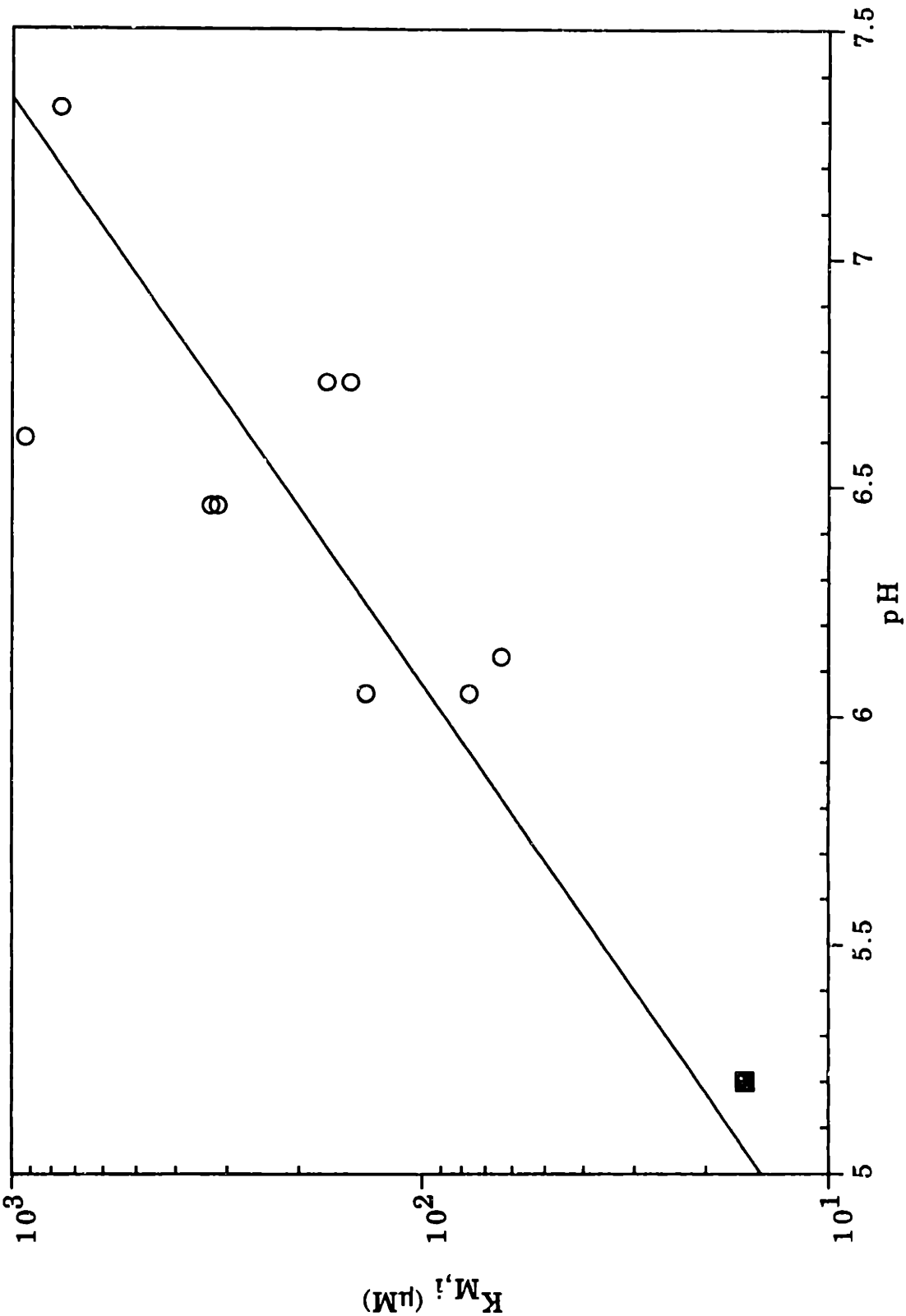

Figure 5-4: Effect of pH on the Instantaneous K_M for Intact Cells

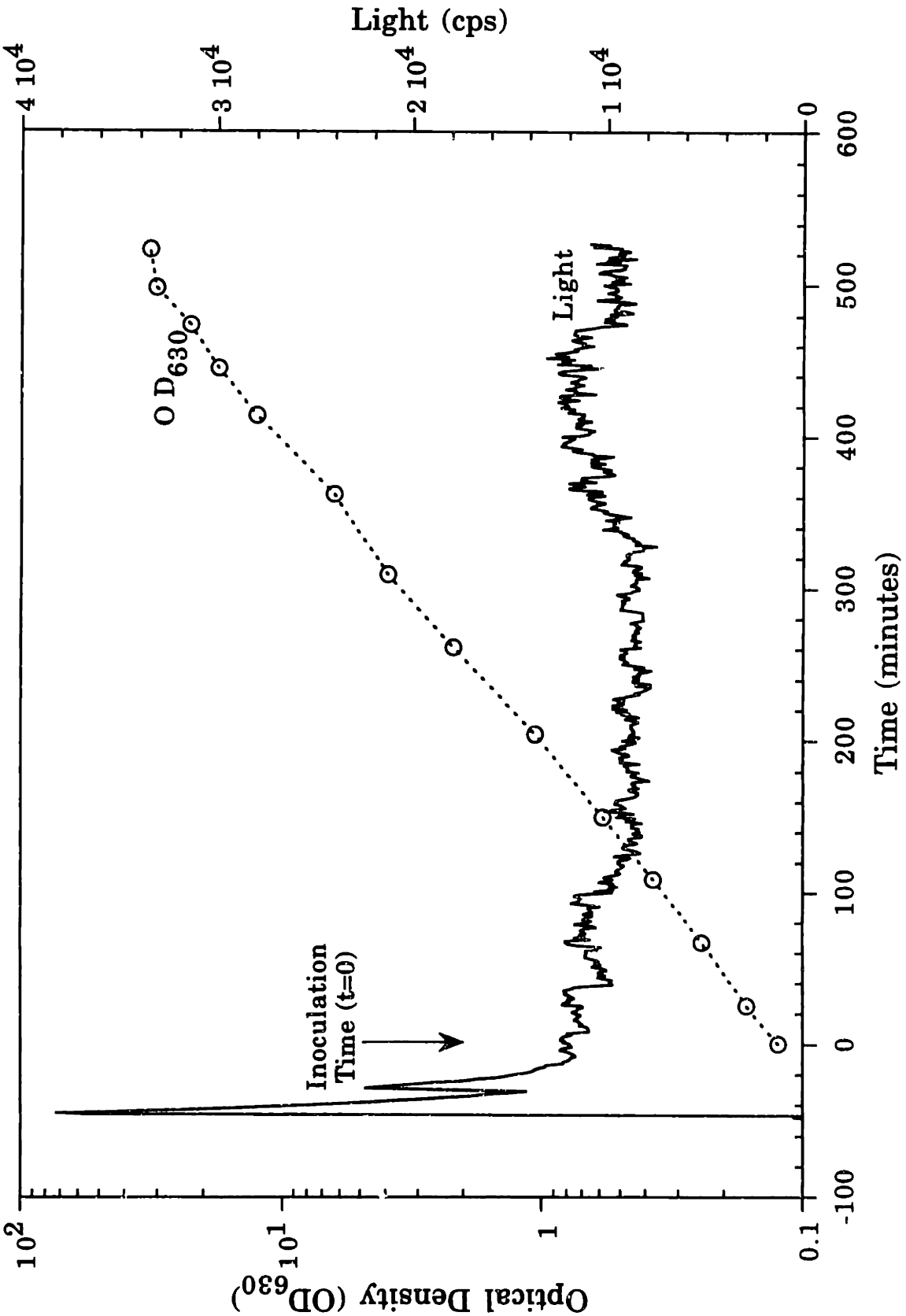

Figure 5-5: Experimental Evaluation of the Inner Filter Effect

5.1.4 Inner Filter Effect

In the previous chapter, light data from a batch cultivation was corrected for the inner filter effect using established relationships (Equation 2-9). The equation was derived from basic theories of light absorbance and scattering. However, it did not provide an adequate adjustment to the data (see Figure 4-7). This may be because this research measures light which was generated inside cells that were uniformly dispersed throughout a significant liquid volume. These same cells are the primary source of light scatter in the culture broth. The photons produced by the luciferase reaction leave the enzyme/oxyluciferin complex in random directions. The light scattering caused by particles such as cells changes the trajectory of photons, but rarely absorbs them. So it is equally likely that a scattered or unaffected photon will reach the light probe. If this is true, one could conclude that there is no inner filter effect, even though the optical density of the culture broth is quite significant.

An experiment was designed to determine if the changing density of cells in the broth had any effect on the light detection in order to validate the above hypothesis. The light source for the bioluminescence is inside the cells dispersed throughout the liquid volume in the bioreactor. Therefore a similar but constant source had to be devised for this experiment. To make the light source as similar as possible to the bioluminescent fermentations, the luciferase reaction was used, but with luciferase, ATP and luciferin all present in the medium. The medium pH was 7.0 to ensure that the reaction behaved as it would in the cytoplasm. Untransfected ATCC #15224 *E. coli* were grown in the place of the recombinant cell line so that they could not contribute any light production to the bioreactor. The ATP and luciferin concentrations were monitored so that the light production could be adjusted if they changed enough to effect the light output.

Fifty minutes prior to inoculation, luciferin, ATP and luciferase were all added to the bioreactor, resulting in normal luciferase flash kinetics followed by constant light production at about 10,000 counts per second (see Figure 5-5). The light production of a comparable fermentation of luminescent bacteria reached 6,500 cps at high cell densities; so any change large enough to affect the light detected from the luminescent bacteria would have a noticeable effect on this experiment. Once the light produc-

tion was nearly constant, the bioreactor was inoculated with the untransfected bacteria. The light emitted remained constant over the course of the fermentation. The final cell density was about 13 g/L DCW, which is typical for batch cultivations performed during this research project.

5.1.5 Cell volumes

Cell size could also have an effect on the per-cell light output from the bioreactor. While each cell changes size as it progresses though its growth cycle, but during exponential growth a steady state exists in which the cell size distribution of the population remains constant. The changing light output of the cultures could be due to the changes in cell size distributions. These distributions were therefore evaluated.

A Coulter Counter® used in combination with a Channelyzer® provided particle counts separated on the basis of size. A brief description of this apparatus and its function was presented in Section 2.1.1. The Channelyzer was calibrated using latex beads with known diameters. The particle size distributions from the samples were transferred to a computer for analysis. Size distributions from samples taken at three different times during a batch cultivation are shown in Figure 5-6. The legend gives the time at which each sample was taken, with zero being the inoculation time. The data are normalized to a total cell count of 10,000 cells.

Average cell diameters were calculated from the size distribution data in the form of number and Sauter mean diameters. Particle of sizes too small to be intact cells were not counted. The diameters remained fairly constant throughout exponential growth (Figure 5-7). The Sauter mean diameter (open squares) exhibited somewhat greater variation than the number means (open circles). Because of the greater consistency of the number mean diameters, they were used for calculations in other experiments.

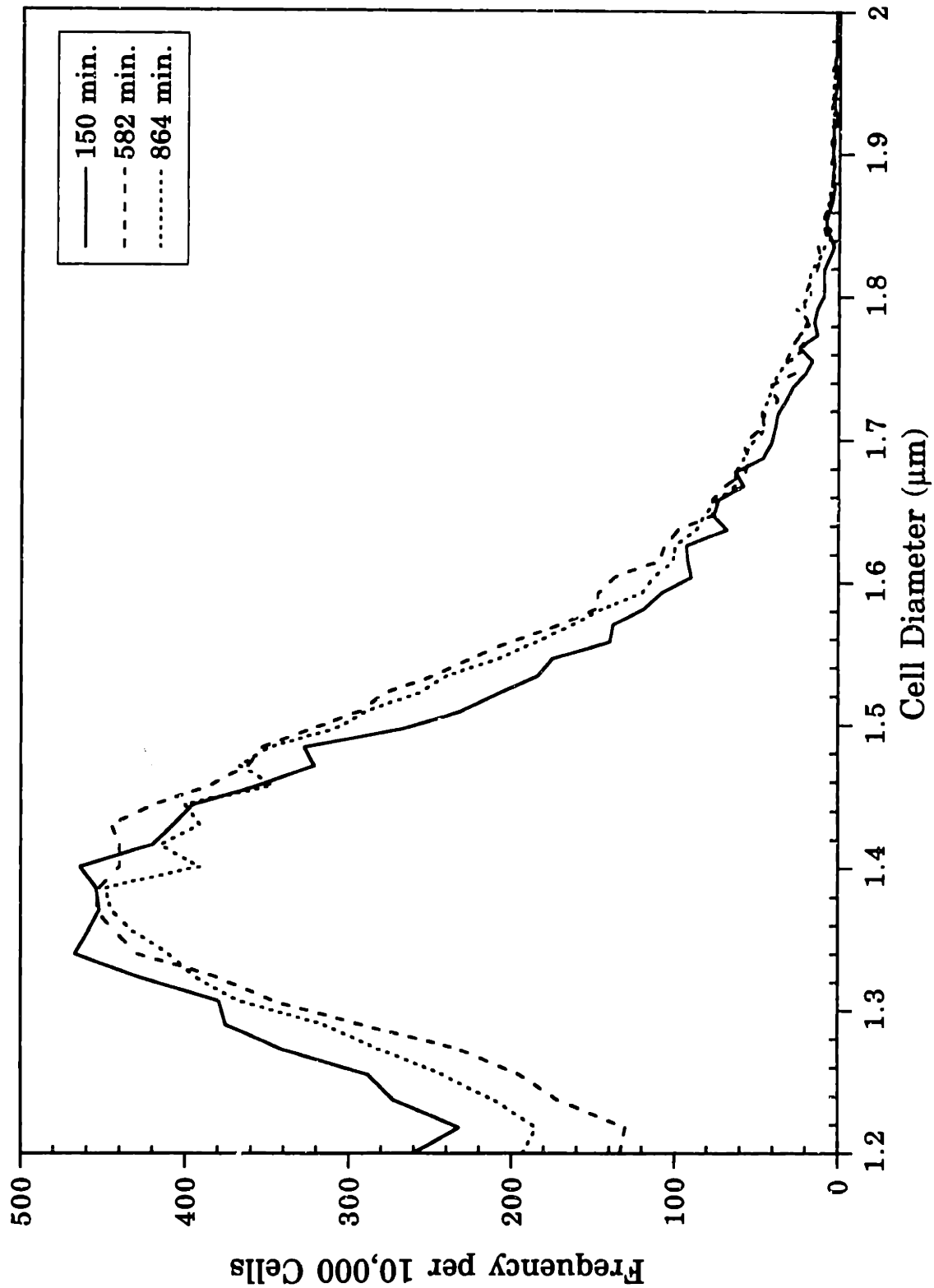

Figure 5-6: Sample Cell Size Distributions During a Fermentation

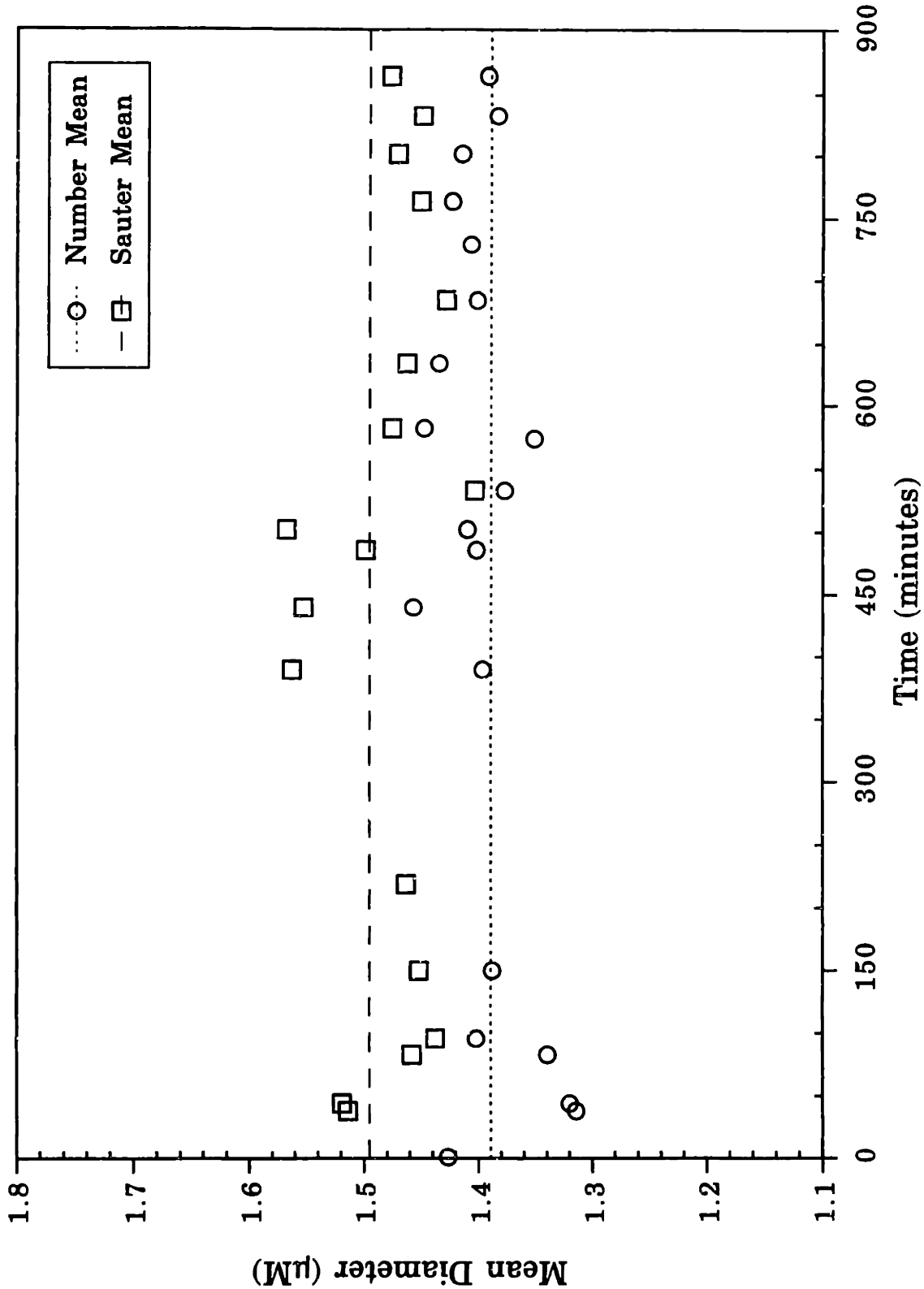

Figure 5-7: Average Cell Diameters Remain Constant
Sauter means are the open squares and number means are open circles.

5.2 ATP MEASUREMENTS

In order to fully understand the changes in the per-cell light emission, it was also necessary to determine the intracellular ATP concentrations independently. These experiments would validate that the light signal emitted from the firefly luciferase depended upon these concentrations.

5.2.1 Extraction Efficiency

Samples were prepared for ATP assays by treatment with cold perchloric acid. The acid caused lysis of the cells and denaturation of all intra- and extra-cellular proteins. Removal of the proteins in this manner ensured that no enzymatic hydrolysis of ATP would occur before the sample could be assayed. The acid extracts were then neutralized with potassium hydroxide to make certain that the extracted ATP would remain stable in the solutions until it could be assayed. The neutralized extracts were stored at -20°C for additional stability. Neutralization also facilitated removal of the perchlorate, as potassium perchlorate crystals which were removed with the cell debris by centrifugation.

A series of experiments demonstrated that this extraction procedure was less efficient when the initial OD of the sample was greater than 0.9 absorbance units. Therefore samples were diluted with filtered, spent medium to ensure that the OD was less than 0.8 prior to extraction. Spent medium was added as the diluent because it appeared to have some inhibitory effect on the luciferase reaction. Dilution with fresh medium could reduce that effect and lead to falsely high apparent ATP concentrations. The ATP standards for the assay were prepared using the same filtered, spent medium and were subject to the same perchloric acid-potassium hydroxide treatment. Reports in the literature state that perchloric acid treatment releases greater than 97% of the intracellular ATP in a consistent manner (Lundin and Thore 1975).

Another aspect of the extraction process which caused some concern was the time required to treat the samples. The time required to transfer the sample from the bioreactor to the tube containing the perchloric acid was between seven and eight seconds. Bacteria can respond to environmental stresses in less time than that time. The effect of this delay on the intracellular ATP concentration was also evaluated. While there was no

way to accelerate the sampling process, the effect of the time delay could be examined for greater delays and extrapolated to zero time to predict the value for cells in the bioreactor.

Each sample from a batch cultivation was subjected to the extraction procedure with four different delay times. The first was with the described method, and the other three preceded the treatment with perchloric acid by standing in the sampling vessel for a set time. The times were chosen so that the total time from the bioreactor to the extraction test tube was 15, 23 or 35 seconds. Results from these experiments are shown in Figure 5-8. The ATP concentrations from each sample were normalized so that the value obtained using the most rapid extraction (7.5 seconds) was set to unity. The error bars reflect the standard error of the assay combined with the error introduced by the averaging process. The amount of ATP assayed decreases by about 15% from 7.5 to 35 seconds after removal from the bioreactor. The rate of the decrease appears linear and projects a value in the bioreactor which is well within the standard error of the value at seven seconds. These results led to the use of the concentration at 7.5 seconds as an accurate representation of the value for the cells while in the bioreactor.

5.2.2 [ATP] Changes With Batch Time

After extraction and deproteination, the total ATP in each sample was assayed as described (Section 3.5.2). The maximum light production, less the background level (see Figure 3-5), provided the measure of activity. Standards were tested on each occasion that the assay was performed in order to account for any effects that freezing and storage may have had on either the samples or the reagents. ATP assays were also performed on cell-free filtrates of sample to determine the extracellular ATP concentrations. These were always found to be less than 2% of that found intracellularly. Since the standard error of the assay was approximately 6%, the total ATP concentration was treated as the intracellular value

In order to properly convert the concentration of ATP in the extracts to the intracellular value, the cell size distributions obtained using the Coulter Channelyzer® were used. The average cell volume was calculated as the volume of a sphere with the numeric mean diameter of the particles

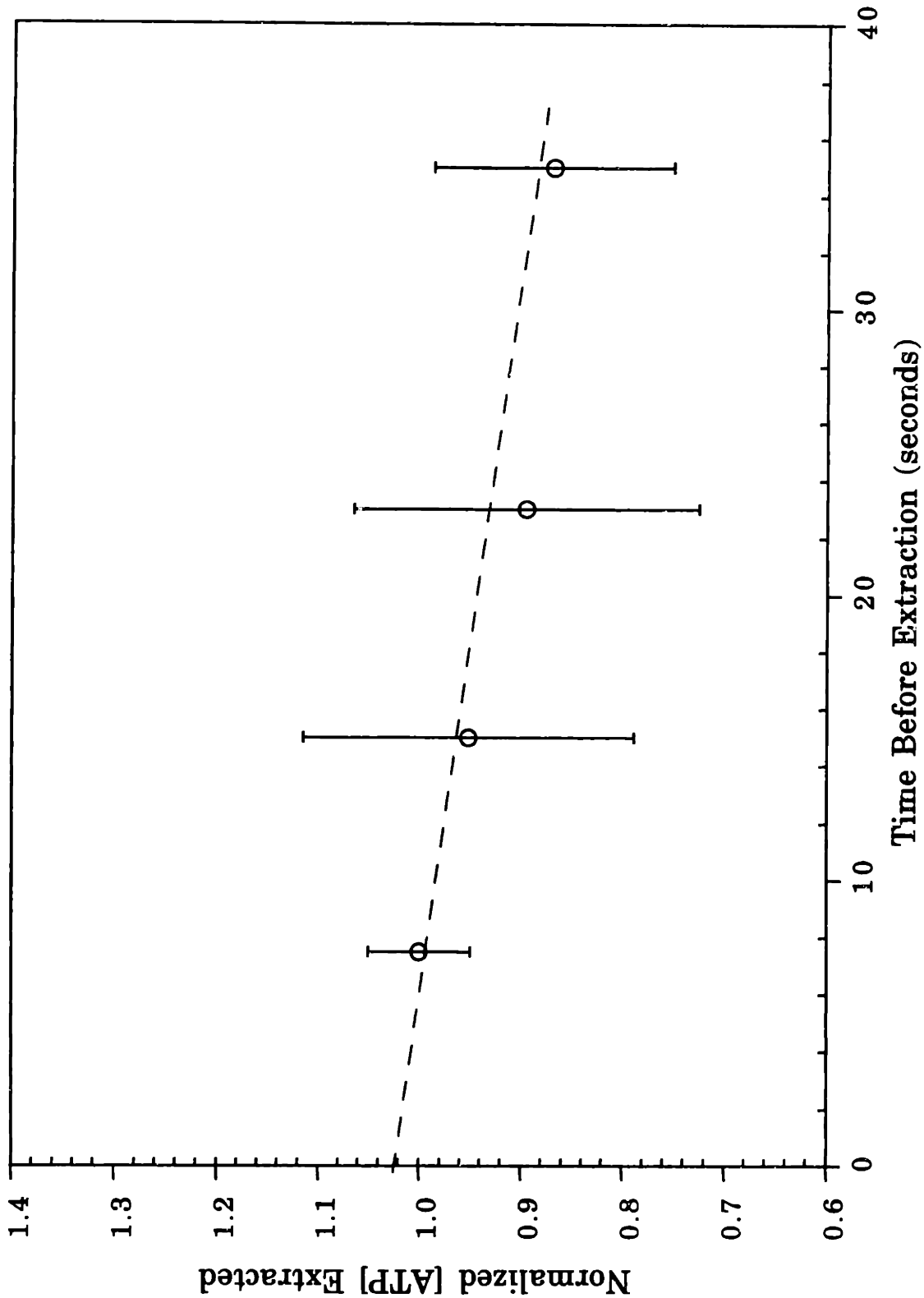

Figure 5-8: Effect of Extraction Initiation Time on ATP Release

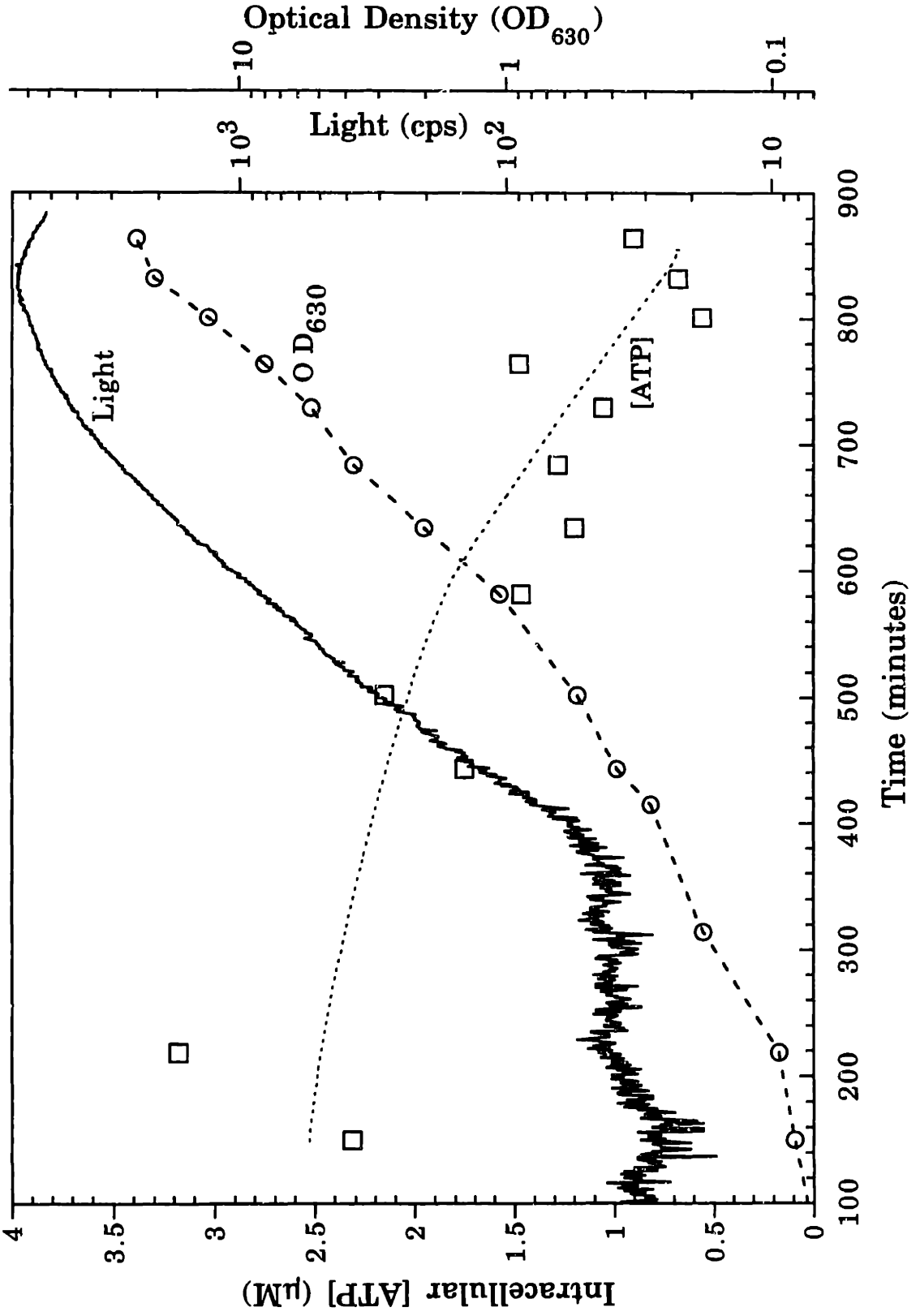

Figure 5-9: Intracellular ATP Concentration Decreases Over Time

in the sample. This volume was used with the particle counts to calculate the volume fraction of the cells in the culture broth. Dividing the experimentally determined concentration of the extracted sample by this volume fraction gave the intracellular ATP concentration. Light output could also be reported on a cell volume basis in the same manner, thereby accounting for changes in the cell density during the fermentation.

As with the light emission data presented in the previous chapter (Figure 4-4), the intracellular ATP concentration data exhibited reproducible trends. Early in each fermentation, the intracellular concentration of ATP was high, but it decreased over the course of exponential growth (Figure 5-9). In fermentations with an pronounced lag phase, the intracellular ATP concentration appeared to start at a low level and then rise to the level seen for other fermentations before following the typical pattern of decline. The apparently low level during the lag phase was seen because the conversion from the concentration in the extract to the intracellular value used on the total cell volume, but only a portion of the cells were contributing ATP.

More significant than the low levels during lag phase was the decreasing concentration as exponential growth progressed. The decline began after the cell density reached an optical density of about two absorbance units. The highest values seen for the ATP concentration are around 3 mM, or six times higher than the K_M of 0.5 mM. The lower values for the intracellular ATP concentration are very close to the K_M. The per-cell light data from the fermentations also decreased during exponential growth, although that change did not begin until the optical density reached five absorbance units. However, the similar trends in these data provide strong reinforcement of the idea that the light emission data are related to the intracellular ATP concentrations.

5.2.3 Light Emission Corresponds to [ATP]

As can be seen in Figure 5-10, the light emission per unit of cell volume varies with the intracellular ATP concentration. The standard errors of the values are approximately 7% and 3% for the intracellular ATP concentration and the light data, respectively. Reports in the literature (Lee et al. 1970) have stated that the luciferase reaction obeys Michaelis-

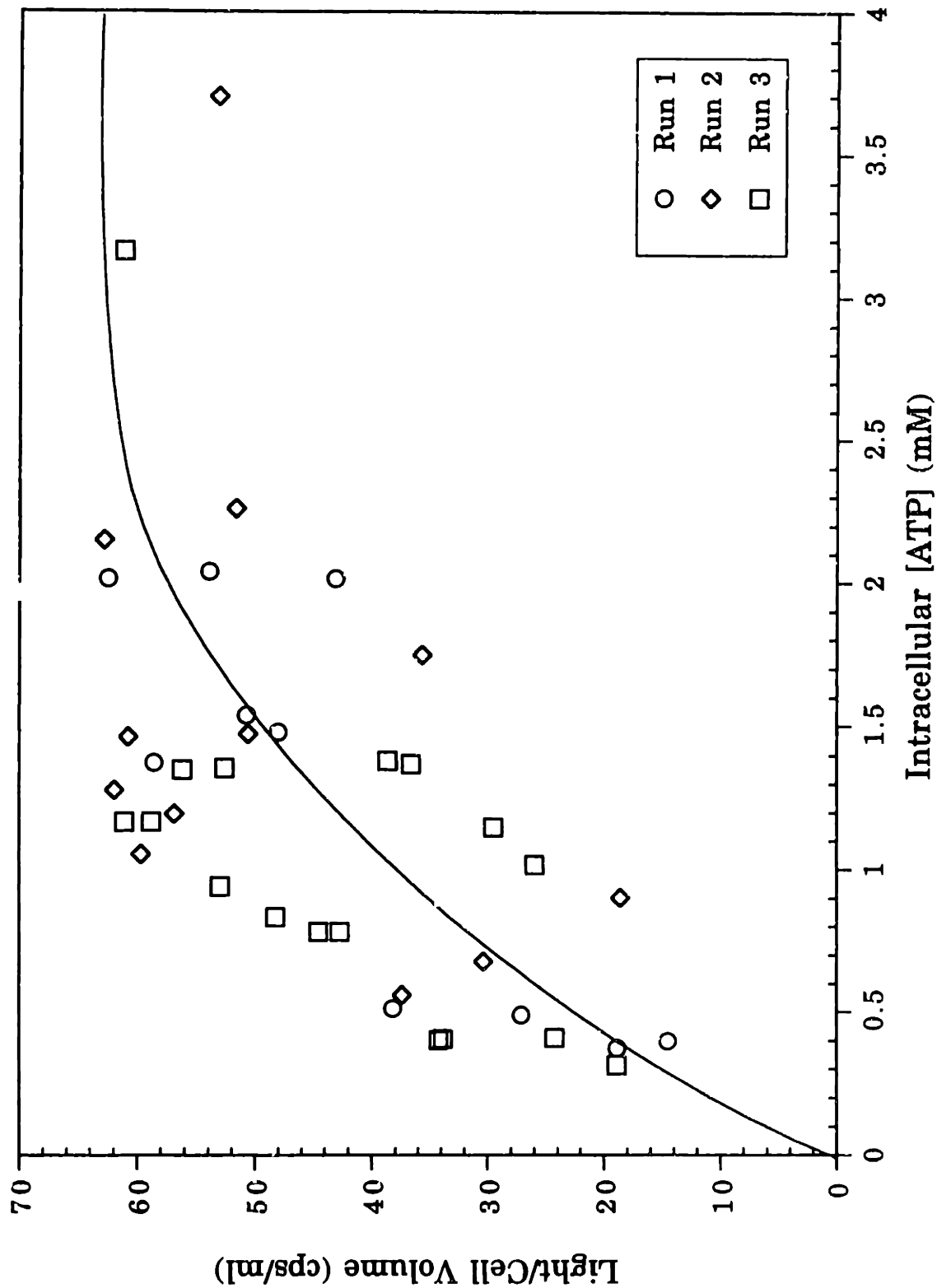

Figure 5-10: Light Emission is a Function of ATP Concentration

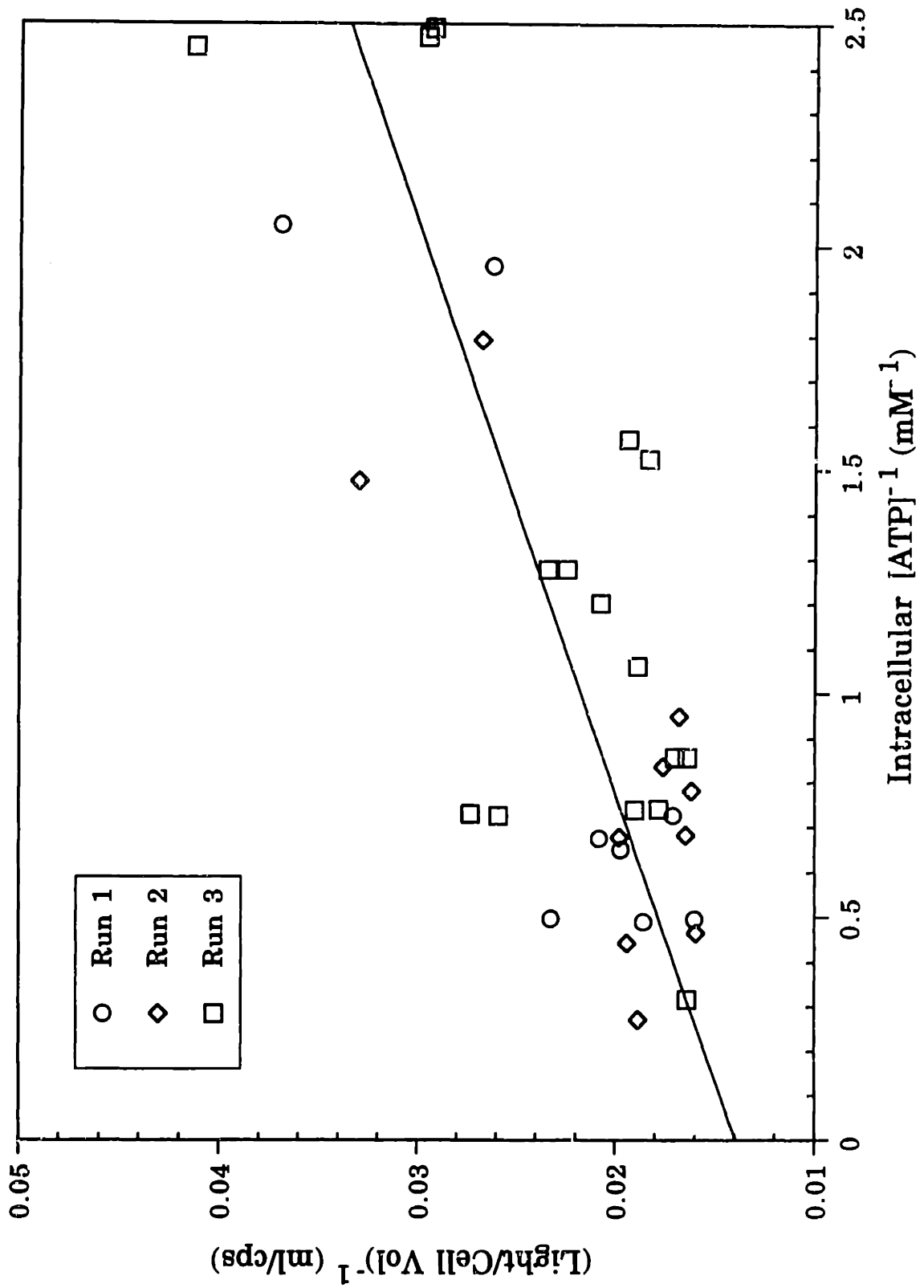

Figure 5-11: Lineweaver-Burke Plot of Light and [ATP] Data

Menton kinetics with respect to ATP. The solid curve drawn in Figure 5-10 represents the relation between the rate of light emission and the ATP concentration as predicted by these kinetics and the reported K_M of 0.5 mM. Because the overall detection efficiency has a very significant effect on the apparent maximum reaction rate, values reported for other detection systems are meaningless here. Therefore the maximum rate observed in these fermentations was used for calculating the predicted curve. The data presented are in good agreement with this prediction.

Because almost all of the data points have ATP concentrations greater than the K_M, the representation used in Figure 5-10 can be difficult to accept as confirmation of the relation between the light emission and the intracellular ATP concentrations. The data from the same three fermentations are presented in a Lineweaver-Burke plot in Figure 5-11. Here it is easier to see the correlation between the light emission and the ATP concentration. The solid line represents a linear least-squares fit of the data. Using the slope and intercept of that line, values for the K_M and V_{max} were calculated to be 0.49 μM and 71 cps per milliliter of cell volume, respectively.

6. CONCLUSIONS

This research project has successfully developed an effective, novel technique for the on-line monitoring of bacterial fermentations. It utilizes intracellularly expressed firefly luciferase to emit light from viable cells. This new method was shown to provide an easily measured light signal which, during exponential growth, corresponded to the intracellular concentration of ATP. When the ATP per cell was constant, the signal provided a measure of the number of viable cells in the bioreactor. For the media and cell lines used in this study, the light signal corresponded to the viable cell density when the optical density of the culture was below five absorbance units. Above that density, it monitored the ATP concentration. A summary of the findings from this research is presented below.

A strain of *Escherichia coli* was prepared for monitoring intracellular ATP by transfection with a plasmid containing both a selectable marker and the cDNA for firefly luciferase. The plasmids remained in the cells, as determined by assays for reversion to ampicillin sensitivity. The plasmid-containing cells maintained a constant level of luciferase activity during exponential growth, and they produced light when luciferin was added to the medium. However, only the viable cells emitted light. Neither non-viable cells nor the extracellular medium produced light. This was because only the viable cells could provide ATP, which is required for luciferase bioluminescence. In addition, it was shown that no luciferase was secreted into the medium. Any luciferase which may have been present in the medium as a result of cell lysis did not produce light. This was concluded to be due to the rapid ATP hydrolysis in the medium by both chemical and enzymatic reactions.

For bacterial fermentations, the correlation between light production and viable cell density can best be seen during the period commonly called the "lag phase." In this period, after the initial inoculation and before the apparent start of exponential growth, the light data exhibited exponential growth with time. This behavior perfectly mirrored the behavior of the viable cell number. These data show that the lag phase is actually a period of exponential growth for a small fraction of the cells. The remaining cells in the inoculum do not grow, and this caused the total number of

cells to appear to be growing very slowly. The slowly increasing optical density that is characteristic of lag phase is consistent with this concept.

However, for *E. coli* growing on glycerol minimal media, the intracellular ATP concentration begins to decrease as the cell density increases above 2.5 g/L DCW. The reason for this phenomenon is not known, but it was observed consistently over the course of this research as well as in literature reports (Cole et al. 1967). This decrease causes the light emission from the exponentially growing cells to deviate from the linear correlation with the viable cell number. The deviation begins when intracellular ATP availability decreases beyond the point where it begins affecting the rate of the luciferase reaction.

In the regime where the intracellular ATP concentration is changing, the light data correlate with ATP and not with viable cells. Cytoplasmic extracts from the growing *E. coli* were assayed for their ATP concentrations. Cell sizes that were determined using a Coulter Channelyzer® were used to convert the ATP concentration in the extracts to its actual value in the cytoplasm. The cell size distribution was found to remain unchanged over the course of the fermentations. The intracellular ATP concentration approached the reported luciferase K_M of 0.4 mM as the fermentation progressed. This caused the light production to decrease accordingly.

These results demonstrate that the luciferase light signal is actually measuring the intracellular ATP concentration. As an ATP monitoring technique, this method is a significant improvement over extraction-based methods. It is faster and does not require any operator manipulations once the cell line is established. Nor does it harm the cells or require samples to be removed from the culture. An extraction-based method using perchloric acid to lyse the cells prior to an ATP assay was used in this research project to confirm the correlation between the light data and the ATP concentrations. The extraction method required a minimum of seven seconds to initiate. This is sufficient time for bacteria to change their internal ATP concentration; so a series of extractions were performed with delays of various lengths prior to the perchloric acid treatment. Analysis of the ATP concentrations in these extracts showed that it did decrease, but quite slowly. The rate of the decrease was about 15% in 28 seconds.

This led to the conclusion that the data from the extracted samples accurately reflected what was inside the cells.

Over the course of a typical batch cultivation, around three micromoles (0.85 mg) of luciferin reacted per liter of medium. This rate of consumption is high enough to have an effect on the light output from the bioreactor due to the decreasing substrate availability. A simple model was derived to correct for the changing luciferin concentration. It uses an experimentally determined value for the overall detection efficiency ($q\eta$) and can be applied on-line in real time. If this monitoring technique is applied to longer, fed-batch fermentations, the amount of luciferin reacted will increase exponentially because the rate of luciferin consumption is proportional to the light production. This is roughly proportional to the total number of cells. In such a fermentation, it may be necessary to add luciferin in a feed stream, and the model would require modification to accommodate that addition.

Several other factors were investigated to determine their effects on the per-cell light production. These included the amount of luciferin which can enter into the cells, the luciferase activity, dissolved oxygen levels, and inner filter effects. It was shown that none of these affected the amount of light detected. Luciferase activity was shown to be constant during exponential growth. Also, no change in light production could be attributed to the dissolved oxygen level (DO) provided it remained above 15% of air saturation. When the DO fell to 5% of air saturation, a small increase in the light output was observed. Maintaining the DO at values above 30% eliminated any adverse effects it may have had on the light signal.

Reports in the literature state that luciferin enters cells passively (Wood and DeLuca 1987), and this research shows strong evidence to support that claim. The entry of luciferin into the cells was very rapid, so that the maximum light output of samples was achieved within three seconds of the luciferin addition. This was observed whether the sample was growing exponentially or was in the lag or stationary phase. The maximum output is achieved as uncharged molecules of luciferin rapidly diffuse across the plasma membrane into the cell and ordinary luciferase flash kinetics ensue. A luciferin Michaelis-Menton constant ($K_{M,i}$) was determined for luciferase in intact cells based on these maxima. The value for $K_{M,i}$ was found to depend upon the pH of the medium in the same manner

as did the amount of uncharged luciferin. This supported the hypothesis that luciferin passively diffuses into the cells, because only the uncharged molecules of luciferin can be transported into the cells. For this reason, the fermentation medium was acidified to pH 6.0 to enable the luciferin to enter the cells from the medium more easily.

Any inner filter effect caused by absorbance or light scattering by the bioluminescent cells in the bioreactor was undetectable with this monitoring system. Such effects have been documented and modeled for other fluorescent and chemiluminescent systems (Yappert and Ingle 1989). Applying those models to data collected with this system resulted in unsatisfactory "corrected" data. Observations in experiments designed to test for inner filter effects did not detect any, even at optical densities of up to 40 absorbance units. It was therefore concluded that there is no inner filter problem associated with this technique.

The detection efficiency of the *in situ*, fiber-optic luminometer assembled for this research project was low; only about one photon for every 10^{11} photons emitted by cells in the bioreactor was counted. This efficiency was sufficient, however, to easily monitor cultures with an optical density as low as 0.02 absorbance units. It would seem that an improved probe design could increase this efficiency. One possible improvement would be to eliminate the watchglass and lens combination and replace it with a clear, polished quartz window. Because the light being measured is from neither a point source nor a distant source, the lens is not being used as it was designed to be, and so it may not be accomplishing much. Simply placing the end of the fiber as close to the liquid as possible may result in more light reaching the detector.

In order to minimize the possibility that ordinary controller adjustments, such as changed agitation speed, affect the detection efficiency, it is best to keep the light-gathering surface submerged in the medium. However, this can make it more difficult to maintain the detection efficiency of the probe. If a biofilm was to form on the probe window, the efficiency would decrease resulting in a decrease in the apparent signal. The probe window must therefore be treated to prevent film formation. One approach to preventing film growth is to use a smooth, polished surface coated with a silicone-based hydrophobic coating material.

Because this technique requires genetic modification of the monitored cell line, it was important to investigate the effect of transfection on the cells' metabolism. Growth rates, β-galactosidase productivity and biomass and β-galactosidase yields on glycerol were all evaluated for both the host strain and the recombinant cell line in the presence and absence of 20 μM luciferin. Surprisingly, the recombinant cell lines exhibited equal or slightly improved behavior over the host strain for each of these parameters, although the greatest difference was less than 5%. This behavior is probably due to the screening of the recombinant clones for a rapid growth rate after transfection. Because the recombinant lines behaved the same whether or not there was luciferin in the medium, luciferase activity is clearly not a significant energy drain.

There was some concern as to the cost for this monitoring system since luciferin is more expensive than most bacterial fermentation medium components. Purchased in quantity from Molecular Probes, Inc. (Eugene, OR) luciferin cost slightly over $1,000 for 500 mg. At the typical concentration used in this research of 4.2 mg/L, this reagent cost is less than $10/L. But the amount of luciferin which actually reacted was only about 6 μM for each batch fermentation. The total cost of all the luminometer parts, including power supplies, computer boards, connectors and a spare optical fiber, was less than $5,000. The photon counter was the only part which required repair over the course of this research project.

In summary, expression of firefly luciferase in growing bacteria was shown to produce an easily measured, extracellular light signal which correlates to the intracellular ATP concentration. When the intracellular ATP concentration is constant, this becomes an effective tool for monitoring culture viability. Such a tool can be especially helpful in mammalian cell culture, where the viability is known to change during batch cultivation. The luciferase-produced light signal can easily be monitored on-line and potentially used as an input for bioreactor control systems. Because the luciferase cDNA must be inserted before cells can be monitored, this method may be more useful as an investigative tool in the laboratory.

7. RECOMMENDATIONS FOR FUTURE WORK

There are several possibilities for continuation of this research project, and these can be grouped into two broad categories. Brief summaries of the motivation and recommended approaches for some of these studies are presented below. The first category includes those aspects of utilizing and analyzing this technique which can best be investigated in a prokaryotic system. The second category is devoted to transferring this technique to mammalian cell culture and overcoming the anticipated difficulties that may ensue. Many of the more glamorous possible uses of the results of this research project involve mammalian cell culture, but bacteria can still provide a simpler model system for further development in some areas.

7.1 BACTERIAL INVESTIGATIONS

The most important aspect of this technique that remains to be determined is the exact numerical relationship between light intensity and ATP concentration. Once this is known, the ATP concentration can be calculated at any time from the light data. Ideally, this should be independent of the culture growth phase and the nutrient source used. In such a situation, any changes in per-cell light output would be attributable solely to changes in the intracellular ATP concentration, unlike in the current system, which has only been shown to be valid during exponential growth on a glycerol minimal medium.

However, both ATP and luciferase are not controlled externally and can effect the light output of the culture, thus luciferase concentrations must be well controlled to eliminate any variations in light output due to changing enzyme activity. The first step required to accomplish that control is chromosomal insertion of the luciferase gene. That would avoid any possibility of changing plasmid copy number or loss of plasmid. It also can eliminate the requirement for selective pressure during cultivation.

An additional way to control the luciferase concentration would be to use a more well-characterized promoter to control expression of the luciferase gene. The promoter should provide a constant concentration of luciferase in the cells at all times. This could be accomplished by coupling luciferase expression to expression of an inhibitor for the promoter used. One such system is the arabinose operon from *E. coli* (Schleif 1987). The *araC* gene encodes the inhibitor for the *ara* promoter. It may even be pos-

sible to create a fusion protein consisting of luciferase and *araC* so that the chimeric construct exhibits the normal activities of each domain. Placement of this fusion protein under control of the *ara* promoter would result in strict regulation of the number of molecules of the luciferase-*araC* fusion contained in each cell.

Once the luciferase activity is well controlled, the light output of a culture will be a function of two variables: the number of cells and the average intracellular ATP concentration in those cells. So any on-line cell density determining device can be combined with this improved luciferase method to instantly measure the intracellular ATP concentration at any time. Unfortunately, it would not be possible to decouple the light production from the number or density of cells in the bioreactor.

One of the more intriguing possibilities introduced by this research project is the control of fermentations based on knowledge of the ATP concentration inside the growing cells. Such knowledge could be beneficial in improving fermentation productivity. However, a great deal of research will be required to understand the relationship of intracellular ATP and productivity. Before any such control scheme could be developed, both the effect of ATP on productivity and the effect of the environment on ATP must be elucidated.

To study the effect of ATP on productivity, one must first create situations in which the ATP levels vary and measure the productivity in each case. The most frequently used bacteriological method for reducing the ATP levels in cells is addition of iodoacetamide, which covalently binds to unbound cysteines in the active site of ATP-generating enzymes, deactivating them. However, luciferase also has an unbound cysteine in its active site, and thus is rendered inactive by iodoacetamide. So alternative methods for changing the intracellular ATP concentration will be necessary. Ideally, manipulating ordinary bioreactor parameters such as dissolved oxygen level, pH and feed rates will effect changes in the ATP concentrations, because if these have significant effects, then they can be used to control the ATP.

Such environmental manipulations may lead to varied intracellular ATP levels, meaning that it will be possible to use this method for control purposes. The controller can monitor light levels, and determine intracellular ATP concentrations from them. In can then induce appropriate

changes to the bioreactor environment to change these ATP levels. Such changes should lead to improved productivity, based upon the observations recommended above regarding ATP concentrations and productivity.

It also may be very interesting to apply this monitoring technology to mixed cultures. Currently, it can be very difficult to characterize the cell content of bioreactor broth in these cultures because there is more than one kind of organism present. However, the individual strains being cultured could first be transfected with a luciferase gene so that each strain emitted light of a different color (see Section 2.2.6). Then a system of filters or monochromators would permit on-line monitoring of each organism in a mixed culture by observing only one color of light at a time.

Another area of investigation that would be appropriate in bacterial cultures would be improvements in probe design. As shown in Figure 4-9, the detection efficiency for the probe used in this investigation was very low, so that only one in 10^{11} photons was detected. It may be possible to improve that efficiency by changes in the design of the lens assembly or by changing the characteristics of the lens used. One possibility alluded to in Section 4.4 was replacing the lens assembly with a straight tube capped with a flat, clear window. This would eliminate any effects the lens may have on the light approaching the optical fiber, but it would also put the end of the fiber as close to the liquid as possible. The actual benefit of the lens is uncertain, as mentioned in Section 2.4.3, because of the diffuse nature of the light source and its proximity to the lens. For this reason it also may be useful to try using lenses of differing focal lengths and positioning them at different distances from the end of the fiber.

7.2 MAMMALIAN CELLS

In order to extend of this research project into mammalian cell culture, many anticipated problems must be overcome. A Chinese Hamster Ovary (CHO) cell line which produces human γ-interferon under dihydrofolate reductase (DHFR) amplification (Scahill et al. 1983) has been stably transfected in these laboratories with pSV2L/SVNeo. The new line has been shown to grow in the presence of G418 and to express active luciferase. It can be used to address some of these problems, including: transporting luciferin into the cells without changing the pH of the medium, obtaining reasonable and constant levels of luciferase activity, directing

luciferase to the desired subcellular organelles, and constructing smaller diameter probe that will fit into mammalian cell bioreactors.

The level of luciferase expression and the choice of organelle are genetic problems. If the expression level is constant and at an appropriate activity level in the clones of the CHO line described above, then there is no problem. But if pSV2L/SVNeo leads to poorly controlled expression levels, then a different promoter must be selected and utilized. The choice of subcellular organelle to which the luciferase should be targeted depends upon the aspect of culture activity to be studied. For instance, if one is interested in investigating the effect of ATP concentration on protein folding and/or site-occupancy glycosylation, the endoplasmic reticulum (ER) retention sequence (KDEL) should be added to the N-terminus of luciferase. Then the light signal would originate from the ER and indicate the ATP concentration there. If one is most concerned with cell viability, deletion of the peroxisome targeting sequence will result in luciferase activity in the cytoplasm. That is probably the best location for viability measurements. A more difficult and more interesting approach would insert genes for more than one color of luciferase. Properly done, this would lead to light production with different spectra generated in each kind of organelle.

However, in order to detect active luciferase, luciferin must be available. Studies have shown that luciferin esters can passively enter mammalian cells in culture. Inside the cells they are hydrolyzed to release luciferin for the luciferase reaction (Craig et al. 1991; Yang and Thomason 1993). These esters do not appear harmful to the cells. However, there may be a reduction in apparent luciferase activity in subcellular organelles because they have to cross additional membranes. So it will be important to investigate the luciferin concentrations in the organelles.

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9. NOMENCLATURE

a	Acceptance angle for an optical fiber
A/D	Analog to digital converter
ATP	Adenosine 5'-Triphosphate
c	Concentration of absorbing or scattering species
cfu	Colony forming unit
DCW	Dry Cell Weight (g/L)
DO	Dissolved Oxygen (% air saturation)
EC	Energy Charge
f	Focal length of a lens
F/#	Speed of a lens or an optical fiber
FOV	Field of view of a lens with respect to an image or target size
I	Measured light intensity (usually counts per second or cps)
I'	Light emission expected with excess luciferin but everything else the same
I_0	Light intensity before passage through sample or fiber (cps)
I_{act}	Actual light flux in absence of inner filter effects (cps)
K_M	Michaelis-Menton constant (mM or μM)
$K_{M,i}$	Instantaneous K_M as defined in Section 5.1.3 (μM)
l	Path length or distance through absorbing material (cm)
L_f	Effective focal length when light source is near lens
LH_2	Firefly luciferin
LH_2AMP	Activated adenylate ester of firefly luciferin
L_s	Distance to light source
N	Avagadro's number (6.02×10^{17} molecules/μmole)
n	Refractive index. Subscript denotes material (0 is external medium, 1 is lens or fiber core, 2 is cladding)
OD	Optical Density. Subscript denotes wavelength (absorbance units)
OD^*	Optical density at concentrations high enough that the Beer-Lambert law is invalid. $OD^* = \varepsilon' l \log(c)$
P	Packing fraction of a fiber bundle
ph/s	Photons per second
pK_a	Potential (or negative log) of the acid dissociation constant
PMT	Photomultiplier tube
q	Quantum efficiency of luminescence (photons/molecule)
R	Reflective loss parameter of an optical fiber
r_t	Radius of the target area of a lens
T	Light transmittance through a sample or optical fiber
t	Time
V	Liquid volume in the bioreactor (L)
V_{max}	Maximum reaction rate for Michaelis-Menton type kinetics
ε	Extinction coefficient in the Beer-Lambert law
ε'	Extinction coefficient at higher densities
η	Detection efficiency of the luminometer assembly (counts/photons)

λ	Wavelength (nm)
λ_{max}	Wavelength corresponding to maximum light emission
θ	Power of a lens
θ_c	Critical angle in an optical fiber. Measured from a line normal to the core-cladding boundary.

10. APPENDIX: COMPUTER CODE

The first two programs are in QuickBASIC and are reasonably well explained by the internal comments. Calls to BASDASG are utilizing routines which came on a diskette with the Omega board. The remaining programs are in APL. They are not commented as well; so a brief explanation of the program's function is provided before each one.

CONTROL.BAS

```
REM Program to use DAS-16 (Omega Inc) for DO control.
REM It is very closely related to READDATA, which monitors
REM the DAS-16 channels in the same manner but does not
REM control DO.  Control is by mixing air and N2 feeds.
REM READDATA and DOCONTRO also read the Mass Spec data periodically.
REM This program does not.  It reads ALL data channels at each
REM input point.

REM Collects data from each channel in use every 3 seconds.
REM Also counts TTL input signal every 3 seconds and computes
REM the pulse frequency.  Call statements are to DASG.LIB,
REM which is shipped with the Omega board, and should be linked
REM with this program on compiling.

REM It assumes that the board is set to 10V gain, 16 channels
REM and the board's base address is set to 300 Hex.  The C3866
REM should be set to "/10" output, and the output should be
REM wired into Counter 0 In. Do not use Ch0 In.

REM Two output files: DAS?????.OUT is averaged over each minute,
REM and LGT?????.OUT is a complete file of the light measurements.
REM ????? represents the start date in mm-dd format.
REM Each output file is in ASCII and contains the date, time
REM of innoculation and a short experiment descriptor as a header.

REM A separate file, CALIB.DAS stores default channel usage
REM and conversion data.  This can be edited with CHANIN.BAS.
REM CALIB.DAS must be in the \DAS directory.

REM Counter2 Out should be wired directly to IP2 (also the Counter 0 gate)
REM and to IP0 (also TRIG0).

REM The Air flow controller setpoint should be wired to D/A channel 0.
REM The N2 flow controller setpoint should be wired to D/A channel 1.

DECLARE SUB BASDASG (mode%, BYVAL dummy%, flag%)
DECLARE SUB BoardCall ()
DECLARE SUB CounterControl (c%, i%)
DECLARE SUB GasControl (airset)
DECLARE SUB ErrorFix ()
DECLARE SUB FileHead ()
DECLARE SUB MinuteUp ()
DECLARE SUB Mode11 ()
DECLARE SUB Mode12 ()

OPTION BASE 0
REM $STATIC
DIM dio%(15)
DIM min%(16, 19)
COMMON SHARED dio%()
REM $DYNAMIC

ReadChannel:                            'Read calibration data from calib.das
OPEN "\das\calib.das" FOR INPUT AS #1
```

```
INPUT #1, ad%, l%, o2%, air%, n2%
DIM chan$(ad%)                          'Channel usage descriptors
DIM chan(2, ad%)                        'Calibration info; max Volts, Data max
FOR i% = 1 TO ad%
  INPUT #1, chan$(i%), chan(1, i%), chan(2, i%)
NEXT i%
CLOSE #1

ShowCalib:                              'Show calibration data for confirmation
CLS
PRINT "Calibration data in use now: (#, Desc., Voltage max, Data max)"
FOR i% = 1 TO ad%
PRINT i%; "   "; chan$(i%), chan(1, i%), chan(2, i%)
NEXT i%
PRINT
PRINT "The dissolved oxygen input is on channel:  "; o2%
PRINT "The Air flow meter is on channel:          "; air%
PRINT "The Nitrogen flow meter is on channel:     "; n2%
PRINT
INPUT "Is the calibration info OK (y/n)? ", yn$
IF ((LEFT$(yn$, 1) <> "Y") AND (LEFT$(yn$, 1) <> "y")) THEN
  PRINT "Run CHANIN.EXE to create a corrected calibration file."
  END
END IF
FlowSet:
PRINT
PRINT "This program will control the DO level by varying the "
PRINT "oxygen partial pressure in the feed stream.  In order to "
PRINT "maintain a constant total gas feed rate, the program"
PRINT "will mix a nitrogen stream with an air stream."
PRINT "If the flow controller is not 5V based, a reference voltage"
PRINT "corresponding to the max must be supplied."
PRINT
INPUT "What should the total gas feed rate be (lpm)"; gasflow
PRINT

REM gets flow controller max voltage from air channel info.
flowmax = chan(1, air%)
airmax = flowmax * gasflow / chan(2, air%)    'Voltage where gas is all air
n2max = flowmax * gasflow / chan(2, n2%)      'Voltage where gas is all N2
IF ((n2max > flowmax) OR (airmax > flowmax)) THEN
  PRINT "gas flow rate too high.  Cannot feed one gas at that rate."
  GOTO FlowSet
END IF

DOSet:                    'Get and check DO setpoint
INPUT "What is the initial Dissolved Oxygen setpoint (%)"; dosp
IF (dosp < 0 OR dosp > chan(2, o2%)) THEN
  PRINT "DO set point out of range (0 <= SP <= "; chan(2, o2%); ")."
  GOTO DOSet
END IF
dovolt = dosp * chan(1, o2%) / chan(2, o2%) 'DO setpoint (volts in)

CLS
PRINT "Be sure that you have reduced the noise on the light input line"
PRINT "to less than 1000 cps before starting the program. If the noise is"
PRINT "greater than that, the program will automatically quit."
PRINT " "
INPUT "If you need to quit now, hit 'Ctrl-Brk', otherwise, hit 'Return'.", i%

DIM avg(1200, ad%)                      'Final storage for averaged data
DIM light&(24019)                       'Detailed light count storage
basadr% = &H300                         'Base address of card (300 Hex)
cnt% = 0                                'counts minutes from innoculation
```

```
samp% = 0                                   'counts samples per minute
lt% = 0                                     'counter for light data point in light&
qt% = 0                                     'quitting time flag
ot$ = CHR$(17)                              'Ctrl-Q for quitting
sp$ = CHR$(19)                              'Ctrl-S to change DO set point
fl$ = CHR$(6)                               'Ctrl-F to change gas flow rate
td% = 1                                     'Dwell time = 1/td% (sec)
wv% = 200                                   'Number of waves=200*Dwell
bck& = 0                                    'background light level
rt$ = CHR$(13)                              'return for file output
chk% = 1                                    'flag for sample routine
tc% = 19                                    'counter for 20 ms correction
cor% = 0                                    'counter until time correction

REM The following 6 values are for the DO control.
REM The eps values will be updated with each sample.
REM The others are preset according to system dynamics experimentally
REM determined by step-changing the gas feed and tracking the DO.
REM The experiments were done at many agitation rates, and the preset
REM values are good from 350 to 850 rpm.  The optimum gas feed is 5 lpm.
REM Division of or by 3 is to account for sampling frequency.

eps2 = 0                                    'DO error from -2 sample
eps1 = 0                                    'DO error from -1 sample
eps0 = 0                                    'DO error from current sample
taud = 10.4 / 3                             'Derivative time constant (piD)
taui = 3 / 1.58                             'Integral time constant (pId)
gain = 44.3                                 'Controller gain (Pid)

md% = 0                                     'mode 0 is initialization
dio%(0) = basadr%                           'base address set on card DIP switches
dio%(1) = 4                                 'interupt level = 4
dio%(2) = 3                                 'DMA level set on switch on card
flag% = 0
CALL BASDASG(md%, VARPTR(dio%(0)), flag%) 'initialize board
IF flag% THEN END
OUT basadr% + 15, &H76                      'Reset counter 1 to square wave mode
OUT basadr% + 15, &HB6                      'Reset counter 2 to square wave mode
OUT basadr% + 10, 0                         'Set counter enable
OUT basadr% + 3, 15                         'Set OP0-3 to ground voltage

CALL CounterControl(1, 100)                 'Set frequency to 100 Hz
CALL CounterControl(2, 1000)

REM Will use counters 1&2 to output a square wave to trigger 0 for
REM counting.  Counts only on high half of wave, so count time is 2/f.
REM Setting frequency to 100 Hz.  Will vary number of waves counted
REM to prevent counter overflow error.

PRINT " "
PRINT "The computer clock says it's "; TIME$; " on "; DATE$
INPUT "What is the date (mm-dd-yy)? ", dat$
IF LEN(dat$) = 0 THEN
  dat$ = DATE$
ELSE
  DATE$ = dat$
END IF
day$ = LEFT$(dat$, 5)
INPUT "What is the time (hh:mm)? ", tim$
IF LEN(tim$) = 5 THEN
  TIME$ = tim$
END IF
PRINT ""
PRINT "Enter a short description of the run (1 line max)"
```

```
INPUT desc$

md% = 10
dio%(0) = 0                                  'Set counter 0 to terminal count mode
CALL BASDASG(md%, VARPTR(dio%(0)), flag%)
IF flag% THEN END

REM Set board to read all analog channels

md% = 1                                      'mode 1 tells which channels to read
dio%(0) = 1                                  'lowest channel is 1
dio%(1) = ad%                                'highest channel in use
CALL BASDASG(md%, VARPTR(dio%(0)), flag%)  'Define channel limits
IF flag% THEN END

REM Initial DO setpoint translation and voltage output

airset = (dosp / 100) * airmax                    'setpoint (V) for air
CALL GasControl(airset)
IF flag% THEN CALL ErrorFix
IF flag% THEN END

CLS
PRINT "The A/D board is now sending DO control signals to the flow"
PRINT "controller.  Please switch the controller mode to remote."
INPUT "When you are ready to continue, hit <Enter>.", temp$

REM Determine background light levels over 3.5 min

CLS
PRINT "The program will now initialize the board and count the background"
PRINT "light levels.  The background will be recorded in the output file"
PRINT "header and subtracted from all readings.  It will clear the screen"
PRINT "in about 3.5 minutes and beep for innoculation 30 seconds later."
PRINT
PRINT "The photon counter (C3866) should be set to "; CHR$(246); "10 mode."
PRINT
PRINT "Remember the following settings on the Mass Spec:"
PRINT "     Inlet select switch on 'Auto'"
PRINT "     Reactor offgas connected to Inlet #1"
PRINT "     Reactor feed gas connected to Inlet #2"
PRINT
INPUT "Hit <Return> when ready", yn$
PRINT

WAIT basadr% + 3, 4, 4                       'Wait for square wave to go low
FOR i% = 1 TO 5                              'First few reads run high
  CALL Mode11
  IF flag% <> 0 THEN
    PRINT md%, flag%
    END
  END IF
  FOR j% = 2 TO wv%                          'Count 1 sec high (200 waves)
    WAIT basadr% + 3, 4, 0                   'Wait for count period to start
    WAIT basadr% + 3, 4, 4                   'Wait for count period to end
  NEXT j%
  WAIT basadr% + 3, 4, 0                     'Wait for count period to start
  CALL Mode12                                'Read counter
  IF flag% <> 0 THEN
    PRINT md%, flag%
    END
  END IF
NEXT i%                                      'These points not included in backgnd
```

```
h& = 0                                    'Value of highest background point
l& = 1000                                 'Value for lowest pt
FOR i% = 1 TO 107                         'Collect background light data
  CALL Mode11
  IF flag% <> 0 THEN
    PRINT md%, flag%
    END
  END IF
  FOR j% = 2 TO wv%                       'Count 1 sec high (200 waves)
    WAIT basadr% + 3, 4, 0                'Wait for count period to start
    WAIT basadr% + 3, 4, 4                'Wait for count period to end
  NEXT j%
  WAIT basadr% + 3, 4, 0                  'Wait for count period to start
  CALL Mode12
  IF flag% <> 0 THEN
    PRINT md%, flag%
    END
  END IF
  bck& = bck& + light&(lt%)
  IF light&(lt%) > h& THEN h& = light&(lt%)
  IF light&(lt%) < l& THEN l& = light&(lt%)
  CLS
  PRINT " Seconds to innoculation: "; 240 - (i% * 2)
  PRINT "Unmodified average count: "; bck& / i%
  IF ot$ = INKEY$ THEN END                'Check for Ctrl-Q request to quit
NEXT i%

bck& = bck& - l& - h&                     'Drop highest and lowest readings
IF (bck& / 105) >= 1000 THEN
  PRINT "The background levels are too high.  Adjust and restart."
  PRINT "The calculated average is: "; bck& / 105
  END
END IF

FOR i% = 1 TO 25                          'Wait out last 26 seconds
  CLS
  PRINT "Seconds to innoculation: "; 26 - i%
  FOR j% = 1 TO 100
    WAIT basadr% + 3, 4, 0
    WAIT basadr% + 3, 4, 4
  NEXT j%
  IF ot$ = INKEY$ THEN END                'Check for Ctrl-Q request to quit
NEXT i%

tim$ = TIME$                              'Get innoculation time
BEEP                                      'Signal for innoculation
PRINT "If you need to stop the program, type <Ctrl-Q>."
PRINT "It may take up to a minute for the program to acknowledge"
PRINT "     the stop keystroke. (Literally: a minute.)"
PRINT
PRINT "If you want to change the DO control set points, type"
PRINT "Ctrl-S for the DO setpoint or Ctrl-F for the gas flow rate."
PRINT "Sampling will stop until the new setting is input; so hurry."
PRINT
PRINT USING "\       \"; "T (min)"; "Light"
TIMER ON
mu = TIMER

ON TIMER(2.95) GOSUB Samples              'Sample every 3 seconds
                                          'Actually about every 3.021s
REM Corrected for with tc% and cor% by dropping about 1 sample in every 140.

SampleLoop:
spike% = 1
```

```
DO
waiting:
  ik$ = INKEY$                                'Check for quit signal
  IF ik$ = "" AND (NOT chk%) THEN             'If sample taken, chk%=0 so exit
    GOTO DoneWaiting                          'If nothings up, cycle again
  ELSEIF ik$ = qt$ THEN qt% = 1               'Set flag for quitting at minute
  ELSEIF ik$ = fl$ THEN GOSUB Flowchange      'Ctrl-F to change flow rate
  ELSEIF ik$ = sp$ THEN GOSUB Setpoint        'Ctrl-S to change DO setpoint
  END IF
  GOTO waiting

DoneWaiting:
  REM Above waits for GOSUB Samples, which releases from IFs to here.
  IF light&(lt%) > 600000 THEN                'Adjust dwell time for too much light
    IF spike% THEN                            'Is this 1st high pt this min?
      spike% = 0                              'Signal that has been a high pt
    ELSEIF td% < 20 THEN
      td% = td% * 5
      wv% = 200 / td%
      spike% = 1                              'reset high point flag
    ELSE
      td% = 200
      spike% = 1                              'reset high point flag
    END IF
  END IF
  lt% = lt% + 1
  samp% = samp% + 1
  chk% = 1                                    'Reset sample flag

REM Here are the inserted DO control calculations and call

  eps2 = eps1                                 'move errors one step down line
  eps1 = eps0                                 'and calculate current error
  eps0 = dovolt - (min%(o2%, samp% - 1) / 409.5) 'error in DO (V)

REM The following 3 lines adjust the air mix setpoint according to
REM the velocity form of the discretized PID control algorithm.

  airset = airset + gain * taud * eps2
  airset = airset - gain * (1 + 2 * taud) * eps1
  airset = airset + gain * (1 + taui + taud) * eps0
  CALL GasControl(airset)                      'send new setpoint voltages
  IF flag% THEN CALL ErrorFix
  IF flag% AND qt% THEN GOTO Finished

LOOP UNTIL samp% >= tc% + 1
CALL MinuteUp
tc% = 19
IF cor% = 6 THEN tc% = 18        'Read 1 fewer sample to get back in sync
i% = cnt% - 1
PRINT "At "; i%; " minutes, light detected is (cps): "; avg(i%, 0)
IF (qt% OR cnt% > 1200) THEN GOTO Finished
IF (avg(i%, 0) >= 500000) THEN
  IF td% < 20 THEN
    td% = td% * 5
    wv% = 200 / td%
  ELSE
    td% = 200
  END IF
END IF
GOTO SampleLoop

Finished:                                     'Save data and end
TIMER OFF
```

```
mt = TIMER
OUT basadr% + 3, 15                         'Close both inlets
IF (cnt% = 0) THEN END
tim2$ = TIME$                               'Stop time and date
dat2$ = DATE$
CLS
chktim = 1000 * (mt - mu) / lt%
PRINT "Avg interval was: "; chktim; " ms."
IF qt% THEN PRINT "User requested end of program.  Saving data."
filename$ = "DAS" + day$ + ".out"           'Main data file
CALL FileHead
FOR i% = 1 TO ad%
  PRINT #1, ", "; chan$(i%);
NEXT i%
PRINT #1, rt$;
FOR i% = 0 TO (cnt% - 1)
  PRINT #1, USING "####"; i%;
  PRINT #1, USING "_,########"; CINT(avg(i%, 0));
  FOR j% = 1 TO ad%
    PRINT #1, USING "_,##.##"; CINT(avg(i%, j%) * 100) / 100;
  NEXT j%
  PRINT #1, rt$;
NEXT i%
CLOSE #1
PRINT " "
filename$ = "Lgt" + day$ + ".out"
CALL FileHead
PRINT #1, rt$;
FOR i% = 0 TO lt% - 1
  PRINT #1, i%; ", "; light&(i%); rt$;
NEXT i%
CLOSE #1
END

REM The remaining code is Subroutines and Procedures

Flowchange:                          'subroutine for changing gas flow rate
  TIMER OFF
ResetHere:
  INPUT "What is the new total gas flow rate (lpm)? ", gasflow2
  IF (gasflow2 <= 0) THEN
    PRINT "Gas flow rate too low.  Must be > 0."
    GOTO ResetHere
  END IF
  airmax = flowmax * gasflow2 / chan(2, air%) 'New volts where gas is all air
  n2max = flowmax * gasflow2 / chan(2, n2%)   'New volts where gas is all N2
  IF ((n2max > flowmax) OR (airmax > flowmax)) THEN
    PRINT "gas flow rate too high.  Cannot feed one gas at that rate."
    GOTO ResetHere
  END IF
  airset = airset * gasflow2 / gasflow
  gasflow = gasflow2
  TIMER ON
RETURN

Samples:                             'Subroutine for gathering data at sample time
  TIMER ON
  CALL Mode11                               'Reset counter
  IF flag% THEN CALL ErrorFix
  IF flag% AND qt% THEN GOTO Finished
  IF td% = 200 THEN GOTO OneWave            'Only count one wave
  FOR j% = 2 TO wv%                         'Count (200/td% waves)
    WAIT basadr% + 3, 4, 0                  'Wait for count period to start
    WAIT basadr% + 3, 4, 4                  'Wait for count period to end
```

```
  NEXT j%
OneWave:
  WAIT basadr% + 3, 4, 0                    'Wait for count period to start
  CALL Mode12                               'Read counter
  IF flag% THEN CALL ErrorFix
  IF flag% AND qt% THEN GOTO Finished
  md% = 3
  FOR i% = 1 TO ad%
    CALL BASDASG(md%, VARPTR(dio%(0)), flag%)
    IF flag% THEN CALL ErrorFix
    IF flag% AND qt% THEN RETURN Finished
    min%(dio%(1), samp%) = dio%(0)
  NEXT i%
  chk% = 0
RETURN

Setpoint:                   'Subroutine for changing DO setpoint
  TIMER OFF
Resetpoint:
  INPUT "What is the new DO set point (%)? ", dosp
  IF (dosp < 0 OR dosp > chan(2, o2%)) THEN
    PRINT "DO set point out of range (0 <= SP <= "; chan(2, o2%); ")."
    GOTO Resetpoint
  END IF
  dovolt = dosp * chan(1, o2%) / chan(2, o2%)    'new DO setpoint (volts in)
  TIMER ON
RETURN

REM $STATIC
SUB CounterControl (c%, i%)
  SHARED basadr%
  xh% = INT(i% / 256)
  xl% = i% - 256 * xh%
  OUT basadr% + 12 + c%, xl%
  OUT basadr% + 12 + c%, xh%
END SUB

SUB ErrorFix
  SHARED cnt%, md%, flag%, qt%
  PRINT "An error has occured at minute "; cnt%
  PRINT "The MODE and error flag are: "; md%, flag%
  INPUT "Do you want to try to continue (y/n)"; yn$
  IF yn$ <> "y" AND yn$ <> "Y" THEN
    qt% = 1
  ELSE
    flag% = 0
  END IF
  cnt% = cnt% + INT(TIMER - mt + .5)
END SUB

SUB FileHead                                'Create new data file with header
  SHARED desc$, filename$, dat$, tim$, bck&, dat2$, tim2$, rt$, chktim
  PRINT "Creating Output File "; filename$
  OPEN filename$ FOR OUTPUT AS #1
  PRINT #1, desc$; rt$;
  PRINT #1, "Data collected on "; dat$; rt$;
  PRINT #1, "Innoculation at "; tim$; rt$;
  PRINT #1, "End time and date: "; tim2$; " on "; dat2$; rt$;
  PRINT #1, "Background light level was "; (bck& / 105); rt$;
  PRINT #1, "Avg interval was: "; chktim; " ms."; rt$;
  PRINT #1, "Time (m), Light (cps)";
END SUB

SUB GasControl (airset)
```

```
  SHARED flowmax, airmax, n2max, md%, dio%(), flag%
  n2set = ((airmax - airset) / airmax) * n2max   'setpoint (V) for N2
  airchan% = INT(airset * 4095 / flowmax)        'setpoint (binary) for air
  n2chan% = INT(n2set * 4095 / flowmax)          'setpoint (binary for N2
  md% = 16
  dio%(0) = airchan%
  dio%(1) = n2chan%
  CALL BASDASG(md%, VARPTR(dio%(0)), flag%) 'output appropriate voltages
END SUB

SUB MinuteUp                                  'Average data from the finished minute
  REM All data averages drop the highest and lowest values before averaging
  REM tc% used to relay the number of samples taken.  Every seven minutes,
  REM one fewer sample (18) is taken to account for the extra 20 ms per
  REM sample lost due to processor time.
  SHARED ad%, avg(), cnt%, min%(), chan(), lt%, light&(), bck&, samp%
  SHARED basadr%, qt%, tc%
  cor% = cor% + 1                                 'Counts minutes between time
  IF cor% = 7 THEN cor% = 0                         'corrections
  FOR i% = 1 TO ad%
    k% = 32767                                    'load with integer max
    h% = 0                                        'load with integer min
    dummy = 0
    FOR j% = 0 TO tc%
      IF min%(i%, j%) > h% THEN h% = min%(i%, j%)
      IF min%(i%, j%) < k% THEN k% = min%(i%, j%)
      dummy = dummy + min%(i%, j%)
    NEXT j%
    dummy = (dummy - h% - k%) / (tc% - 1)
    dummy = dummy * chan(2, i%) / (409.5 * chan(1, i%))
      ' A/D signal goes from 0 to 4095.  Board gain is set to 10V.
      ' Voltage in = (Gain * digital reading)/4095
      ' Data in = (Voltage in/max Volts)* Data max
      ' Assumes all minima are zero and board settings still same.
    avg(cnt%, i%) = dummy
  NEXT i%
  dummy = 0
  h% = lt% - (tc% - 1)
  k% = h%
  FOR i% = (lt% + 1 - tc%) TO (lt% - 1)    'Average light data less background
     IF light&(i%) > light&(h%) THEN h% = i%
     IF light&(i%) < light&(k%) THEN k% = i%
     dummy = dummy + light&(i%)
  NEXT i%
  dummy = (dummy - light&(k%) - light&(h%)) / (tc% - 1)
  avg(cnt%, 0) = dummy - (bck& / 105)
  samp% = 0
  cnt% = cnt% + 1
END SUB

SUB Mode11                                    'Resets counter 0
  SHARED md%, dio%(), flag%, basadr%
  md% = 11
  dio%(0) = -1
  WAIT basadr% + 3, 4, 4
  CALL BASDASG(md%, VARPTR(dio%(0)), flag%)  'Load to 65535 to start
END SUB

SUB Mode12                                    'Read and record Counter 0 data
  SHARED md%, dio%(), flag%, mt
  SHARED light&(), lt%, td%, basadr%
  md% = 12
  dio%(0) = 0
  WAIT basadr% + 3, 4, 4                      'Wait for counting to end
```

```
  mt = TIMER                                  'Start microsecond timer
  CALL BASDASG(md%, VARPTR(dio%(0)), flag%) 'Read counter 0
  IF dio%(1) < 0 THEN                         'converts count data to proper value.
    light&(lt%) = -1 - dio%(1)                'numbers over 32767 read as negative.
  ELSE
    light&(lt%) = 65535 - dio%(1)
    END IF
  light&(lt%) = light&(lt%) * 10 * td%  'normalize for dwell time and /10 mode
END SUB
```

CHANIN.BAS

```
REM This program creates a the calibration file and saves it as
REM a separate file, CALIB.DAS.  It stores default channel usage
REM and conversion data.

DIM chan$(15)                                 'Channel usage descriptors
DIM chan(2, 15)                               'Calibration info; max Volts, data max
st% = 1

CLS
INPUT "Is there already a calibration file? "; yn$
IF LEFT$(yn$, 1) = "Y" OR LEFT$(yn$, 1) = "y" THEN
  GOSUB ReadChannel
  GOTO ShowCalib
END IF

INPUT "Enter the number of analog channels used: ", ad%

ChannelSet:                                   'Set up channel calibration
  FOR i% = st% TO ad%
    GOSUB DataIn
  NEXT i%

ShowCalib:                                    'Show calibration data for corrections
  CLS
  PRINT "Calibration data in use now: (#, Desc., Voltage, Data)"
  FOR i% = 1 TO ad%
    PRINT i%; "   "; chan$(i%), chan(1, i%), chan(2, i%)
  NEXT i%
  PRINT
  PRINT "Highest non-Mass Spec channel: "; l%
  PRINT "Dissolved Oxygen channel:      "; o2%
  PRINT "Air flow rate channel:         "; air%
  PRINT "Nitrogen flow rate channel:    "; n2%
  PRINT
  INPUT "Is the calibration info OK? (y/n) "; yn$
  IF LEFT$(yn$, 1) = "Y" OR LEFT$(yn$, 1) = "y" THEN
    GOTO WriteChannel
  ELSE
    GOTO ChangeSet
  END IF

ChangeSet:                                    'Change calibration data
  INPUT "How many channels are in use? "; chn%
  IF chn% < ad% THEN                          'Remove last few channels
    ad% = chn%
    GOTO ShowCalib
  ELSEIF chn% > ad% THEN                      'Add new channels
    st% = ad% + 1
    ad% = chn%
    GOTO ChannelSet
  END IF                                      'Change channel data
  INPUT "What is the highest channel not used for Mass Spec data"; l%
```

```
  INPUT "What is the Dissolved Oxygen input channel"; o2%
  INPUT "What is the Air flow rate channel"; air%
  INPUT "What is the Nitrogen flow rate channel"; n2%
  INPUT "How many channels need info changed"; chn%
  IF chn% < 1 THEN GOTO ShowCalib
  FOR j% = 1 TO chn%
    INPUT "Which channel do you want to change now? "; i%
    GOSUB DataIn
  NEXT j%
  GOTO ShowCalib

WriteChannel:                          'Write calibration data to calib.das
  OPEN "\das\calib.das" FOR OUTPUT AS #1
  WRITE #1, ad%, l%, o2%, air%, n2%
  FOR i% = 1 TO ad%
    WRITE #1, chan$(i%), chan(1, i%), chan(2, i%)
  NEXT i%
  CLOSE #1
  PRINT "File CALIB.DAS written."

END

DataIn:
  PRINT "Info for Channel #"; i%
  INPUT "What is the input? "; chan$(i%)
  INPUT "What is the maximum voltage? "; chan(1, i%)
  INPUT "What is the data maximum? "; chan(2, i%)
RETURN

ReadChannel:
  OPEN "\das\calib.das" FOR INPUT AS #1
  INPUT #1, ad%, l%, o2%, air%, n2% 'Number of channels used,high nongas
  FOR i% = 1 TO ad%
    INPUT #1, chan$(i%), chan(1, i%), chan(2, i%)
  NEXT i%
  CLOSE #1
RETURN
```

The following programs were written in APL because of its versatility in manipulation of large numeric arrays. In APL, functions, routines and programs are treated the same; so any loaded program can be called from within any other. The first program, SINGLE, makes calls to several others to complete its task. That task is to translate one MCS file from the format is which it is stored (as described in the MCS manual) to a comma-delimited text format readable by most Macintosh spreadsheet and graphing software. The other programs are included below SINGLE, except for the following, which were supplied with the APL.68000 interpretter: AFE, SFGETFILE, SFPUTFILE, TRANSLATE, MREAD, MWRITE, MTIE, MUNTIE, MCREATE.

```
      ▽SINGLE;NAME;STOP;DATA;DWELL;POINTS;DATE;STARTTIME;HEADER
[1]   STOP←'F8.2,□,□,F12.2'
[2]   NAME←'Open'SFGETFILE'' ⍝DIALOG BOX FOR FILE NAME
[3]   GETHEADER
[4]   →('R'=1↑STOP)/PROBLEM
[5]   'FILE OPENED.  HEADER READ AND INTERPRETED.'
[6]   GETDATA
[7]   →('R'=1↑STOP)/PROBLEM
[8]   DATA←DATA ADDTIMES 0
[9]   'DATA READ.'
[10]  DATA←COMPRESS DATA
[11]  HEADER←'Time (s), Light (cps)'
[12]  HEADER←HEADER MAKEHEAD NAME
[13]  DATA←STOP FORMAT DATA
[14]  'DATA FORMATTED FOR OUTPUT, INCLUDING HEADER.'
[15]  (HEADER,DATA)SAVEFILE NAME,'.TRANS'
[16]  →('W'=1↑STOP)/PROBLEM
[17]  'FILE SUCCESSFULLY INTERPRETED AND WRITTEN.'
[18]  →0
[19] PROBLEM:' '
[20]  'THE FILE SYSTEM ERROR PREVENTS FURTHER PROGRAM OPERATION.'
[21]  →('R'=1↑STOP)/0
[22]  'THE FULLY FORMATTED DATA HAS SAVED AS TEXT VECTOR.'
[23]  'IT IS CALLED ''EMERGENCY''.'
[24]  'SAVE THE WORKSPACE TO GUARD THE VECTOR AND THEN FIX THE PROBLEM.'
[25]  'OUTPUT THE VECTOR BY TYPING ''EMERGENCY SAVEFILE '' FILENAME.'
      ▽
```

GETHEADER fetches the file header, reads important information from it and passes that back to SINGLE for future use.

```
      ▽GETHEADER;HEADER;ERRORCODE;PLACE
[1]   ERRORCODE←1 0 1 MTIE NAME ⍝OPEN SELECTED FILE
[2]   →(ERRORCODE≠0)/ERRORID ⍝IF ERROR, STOP AND EXPLAIN
[3]   HEADER←MREAD 1 ¯1 256 ⍝READ FIRST 256 BYTES
[4]   DWELL←HEADER[10 9 8 7] ⍝DWELL TIME AS 4BYTE INTEGER
[5]   POINTS←HEADER[12 11] ⍝NUMBER OF DATA POINTS 2BYTE
[6]   STARTTIME←HEADER[20+⍳8]
[7]   DATE←HEADER[28+⍳8]
[8]   DWELL←TEXTTOINT DWELL
[9]   DWELL←DWELL×1E¯6
[10]  POINTS←TEXTTOINT POINTS
[11]  STARTTIME←0 TRANSLATE STARTTIME
[12]  DATE←0 TRANSLATE DATE
[13]  →0
[14] ERRORID:'THERE WAS AN ERROR TRYING TO OPEN THE DATA FILE.'
[15]  'THE ERROR WAS: ',AFE ERRORCODE
[16]  STOP←'R'
      ▽
```

GETDATA fetches the two-word integer (IEEE standard) data from the file, converts it to ordinary integer format and sends it back to SINGLE.

```
      ▼GETDATA;RAWDATA;VECTOR;ERRORCODE;PLACE
[1]   PLACE←'READ'
[2]   RAWDATA←MREAD 1 ¯1,POINTS×4
[3]   →(0≠ppRAWDATA)/OK
[4]   ERRORCODE←RAWDATA
[5]   →ERRORID
[6]  OK:PLACE←'CLOSE'
[7]   ERRORCODE←MUNTIE 1
[8]   →(ERRORCODE≠0)/ERRORID ⍝IF ERROR, STOP AND EXPLAIN
[9]   RAWDATA←¯1+⎕AV⍳RAWDATA
[10]  RAWDATA←⍉(POINTS,4)⍴RAWDATA
[11]  VECTOR←1 4⍴256*¯1+⍳4
[12]  DATA←,VECTOR+.×RAWDATA
[13]  DATA←DATA+DWELL
[14]  →0
[15] ERRORID:'THERE WAS AN ERROR TRYING TO ',PLACE,' THE DATA FILE.'
[16]  'THE ERROR WAS: ',⍕ERRORCODE
[17]  STOP←'R'
      ▼
```

ADDTIMES adds a column of time data to the left of the column of light data. It is written as a function that returns a matrix to whatever program called it.

```
      ▼MATRIX←VECTOR ADDTIMES T0;DIM;TIMES
[1]   DIM←⍴VECTOR
[2]   TIMES←¯1+⍳DIM
[3]   TIMES←T0+TIMES×DWELL
[4]   MATRIX←⍉(2,DIM)⍴TIMES,VECTOR
      ▼
```

COMPRESS permits the user to compress the file by averaging the light data over several data points. The user can compress the entire file or parts of it, and can choose the extent of the compression. It is also written as a function returning a matrix to the orriginal calling program.

```
      ▽OUT←COMPRESS IN;TEMP;REGION;ANS;TSTART;TEND;DIM
[1]  LOOP1:ANS←1 ASK'DO YOU WANT TO COMPRESS THE WHOLE FILE? (Y/N) '
[2]   →(ANS≠'Y')/PARTIAL
[3]   ANS←ASKNUM'BY WHAT FACTOR DO YOU WANT TO COMPRESS THE FILE? '
[4]   TEMP←L⍟ANS
[5]   IN←TEMP SHRINKALL IN
[6]   'DONE'
[7]   →LOOP1
[8]  PARTIAL:ANS←1 ASK'DO YOU WANT TO COMPRESS A REGION OF THE DATA? '
[9]   →(ANS≠'Y')/FINISHED
[10]  ANS←ASKNUM'AT WHAT TIMEPOINT SHOULD THE COMPRESSION START (SEC)? '
[11]  DIM←⍴IN
[12]  DIM←DIM[1],(¯1+⍴DIM)⍴1
[13]  TSTART←ANS⍳,DIM↑IN
[14]  TSTART←¯1+TSTART⍳1
[15]  ANS←ASKNUM'AT WHAT TIMEPOINT SHOULD THE COMPRESSION END (SEC)? '
[16]  TEND←ANS⍳,DIM↑IN
[17]  TEND←TEND⍳1
[18]  TEMP←TEND-TSTART
[19]  TEMP←TSTART+⍳TEMP
[20]  REGION←IN[TEMP;]
[21]  ANS←ASKNUM'BY WHAT FACTOR DO YOU WANT TO COMPRESS THIS REGION? '
[22]  TEMP←⌊ANS
[23]  REGION←TEMP SHRINKALL REGION
[24]  TEMP←(TSTART,2)↑IN
[25]  TEMP←TEMP⍪REGION
[26]  IN←TEMP⍪(TEND,0)↓IN
[27]  'DONE'
[28]  →PARTIAL
[29] FINISHED:OUT←IN
      ▽
```

MAKEHEAD makes a header for the output file, and is written as a function.

```
      ▽HEADER←COLUMNS MAKEHEAD NAME
[1]   HEADER←'Data collected on ',DATE,', beginning at ',STARTTIME,'.'
[2]   HEADER←HEADER,⎕AV[14],'The Dwell Time was ',(⍕DWELL),'
sec.',⎕AV[14]
[3]   HEADER←HEADER,'The original file name was ',NAME,⎕AV[14]
[4]   HEADER←HEADER,'Some data compression may have been per-
formed.',⎕AV[14]
[5]   HEADER←HEADER,COLUMNS,⎕AV[14]
      ▽
```

SAVEFILE saves the file after translating it to ASCII from the internal APL format.

```
      ▼TEXT SAVEFILE NAME;ERRORCODE;ANS;PLACE
[1]   PLACE←'CREATE'
[2]  RETRY:ERRORCODE←'TEXT'⎕MCREATE NAME
[3]   →(ERRORCODE=0)/OK
[4]   →(ERRORCODE≠48)/PROBLEM
[5]   ANS←1 ASK NAME,' ALREADY EXISTS.  OVERWRITE IT? '
[6]   →(ANS='Y')/OK
[7]   NAME←'PLEASE NAME THE OUTPUT FILE'⎕SFPUTFILE NAME
[8]   →RETRY
[9]  OK:PLACE←'OPEN'
[10]  ERRORCODE←⎕1 0 3 ⎕MTIE NAME
[11]  →(ERRORCODE≠0)/PROBLEM
[12]  TEXT←⎕1 TRANSLATE TEXT
[13]  PLACE←'WRITE'
[14]  ERRORCODE←TEXT ⎕MWRITE 1 0
[15]  →(ERRORCODE≠0)/PROBLEM
[16]  PLACE←'CLOSE'
[17]  ERRORCODE←⎕MUNTIE 1
[18]  →(ERRORCODE=0)/0
[19] PROBLEM:' '
[20]  'THERE WAS A PROBLEM WHILE TRYING TO ',PLACE,' THE FILE.'
[21]  'THE ERROR(S) WAS (WERE): ',⎕AFE ERRORCODE
[22]  STOP←'W'
[23]  EMERGENCY←TEXT
      ▼
```

SHRINKALL compresses the entire file "IN" by the factor of "FACTOR" to one.

```
      ▼OUT←FACTOR SHRINKALL IN;DIM;RES;TEMP
[1]   DIM←⍴IN
[2]   RES←FACTOR|1↑DIM
[3]   →(RES=0)/OK
[4]   TEMP←((RES-FACTOR),1↓DIM)↑IN
[5]   IN←IN⍪TEMP
[6]   DIM←⍴IN
[7]  OK:DIM←DIM[2],(DIM[1]+FACTOR),FACTOR
[8]   RES←DIM⍴⍳⍴IN
[9]   OUT←+/RES=FACTOR
[10]  OUT←⍳⍴OUT
      ▼
```

ASK fetches a response of length "CHARS" to "QUESTION".

```
      ▼ANSWER←CHARS ASK QUESTION
[1]  LOOP:' '
[2]   QUESTION
[3]   ANSWER←⍬
[4]   →(0=⍴ANSWER)/NULL
[5]   →(0=⍴CHARS)/0
[6]   ANSWER←CHARS↑ANSWER
[7]   →0
[8]  NULL: 'YOU MUST ENTER SOMETHING (NOT JUST RETURN).  TRY AGAIN.'
[9]   →LOOP
      ▼
```

ASKNUM fetches a numeric response to "QUESTION".

```
      ▼ANSWER←ASKNUM QUESTION;DR
[1]  LOOP:ANSWER←''⎕ASK QUESTION
[2]   ANSWER←⍎ANSWER
[3]   →(3≥⎕DR ANSWER)/0
[4]   'YOU MUST GIVE A NUMERIC RESPONSE.  TRY AGAIN.'
[5]   →LOOP
      ▼
```

TEXTTOINT converts a text string to a vector of integers between 0 and 255.

```
      ▼INT←TEXTTOINT TEXT
[1]   TEXT←¯1+⎕AV⍳TEXT
[2]   INT←((⍴TEXT)⍴256)⊥TEXT
      ▼
```