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The Luminometer: A Sensitive Instrument for Measuring Low Amounts of Oxygen and some Applications to Problems Involving the Measurement of Photosynthesis

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THE LUMINOMETER: A SENSITIVE INSTRUMENT FOR MEASURING LOW AMOUNTS OF OXYGEN, AND SOME APPLICATIONS TO PROBLEMS INVOLVING THE MEASUREMENT OF PHOTOSYNTHESIS

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by

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To Carolyn

PREFACE

My graduate studies at The Rockefeller University have been a valuable experience. I am grateful to Dr. Bronk and the faculty for providing the stimulating environment and freedom that have been so important to my development here. I came to the Institute as a naive college graduate, and I am leaving considerably less naive and vastly more knowledgeable. I have had the opportunity to sample several fields of research and have added considerably to my background in mathematics, physics, chemistry, and biology. I feel that I have been "cultured in an optimal medium."

I am especially grateful to Dr. David Mauzerall, who taught me organic and photochemistry, and who introduced me to the intriguing field of photosynthesis His stimulation and guidance have been invaluable throughout my research. During my investigations, I learned from him to ask more pertinent questions and to seek better methods for finding answers to them.

I would like to thank Dr. Sam Granick for the opportunity to work in his laboratory. Through stimulating discussions with him and with Dr. Aharon Gibor, I have become familiar with many interesting biological problems. Their warm friendship has made my research a pleasant experience. The many resources of this laboratory were essential to my research. I would like to thank, too, Mrs. Rita Lau for teaching me the art of culturing Chlorella.

I am indebted to Dr. Keffer Hartline and Dr. Floyd Ratliff, in whose laboratory I gained valuable knowledge and experience in the fields of receptor physiology and physiological psychology.

I am grateful to the Electronics Shop, the Instrument Shop, and the Glass Blower, Mr. Papperitz, for their contributions to the construction of the luminometer. I would like especially to thank Mr. Paul Rosen for his valuable advice on photometric instrumentation.

I am indebted to the Illustration Service for the drawings and photographs.

I am especially grateful to Miss Carolyn Trager for her unfailing help and encouragement during the preparation of this manuscript. Her many suggestions have greatly improved the text.

ABSTRACT

A new method for the continuous measurement of low rates of oxygen production is described, which is useful for the study of photosynthetic systems. An inert carrier gas flows at a controlled rate through a cuvette, then over a solution of luminol and potassium t-butoxide in dimethylsulfoxide (DMSO). The light generated by the chemiluminescent reaction of luminol with oxygen is measured with a photomultiplier. A constant rate of oxygen production by photosynthesis in the cuvette is seen as a constant displacement from a steady baseline. The baseline is caused by the oxygen impurity in the carrier gas.

Electrolysis of water is a convenient method for the calibration of the luminometer. The response of the luminometer is linear with oxygen concentration over at least six orders of magnitude, and changes of 0.01 ppm or 5×10^{-14} moles/sec. in rate of generation of oxygen are detectable when the carrier gas contains 1 ppm oxygen impurity. The system has a response time of about 3 minutes, and a precision of better than 5%.

Various mutants of Chlorella having a variety of abnormal pigments were screened for their photosynthetic activity. Some mutants could make oxygen photosynthetically though they were discolored by abnormal quantities of chlorophyll precursors and had only about 1% of the normal chlorophyll concentrations.

Mutant 610, a carotenoid-containing mutant which, unlike normal Chlorella, cannot synthesize chlorophyll in the dark, was found to green in the light. During greening, the synthesis of chlorophyll and the development of the ability to produce oxygen were shown to be independent of cell division. On the other hand, during degreening in the dark, the loss of chlorophyll and photosynthetic ability was shown to be dependent on cell division. The luminometer was found to be capable of measuring the light-saturation curve of Chlorella at light intensities down to at least three orders of magnitude below the half saturation intensity.

Other possible ways of using the luminol detection method are discussed, and some advantages of mutant 610 for the study of greening are proposed.

CONTENTS

CHAPTER 1

INTRODUCTION

The great importance of oxygen to life can hardly be disputed. The higher forms of life depend on plants not only as a food source, but also for the oxygen they respire. The controlled reaction of the foods with oxygen in these organisms is their main source of energy. Much has been learned about the details of this oxidative respiration, but far less is known about the converse pathway in plants. Here the energy of light is converted into chemical energy useful to the plant, and, almost as a by-product, oxygen is evolved. The mechanism of this astonishingly efficient reaction has fascinated scientists for almost two centuries.

From the beginning of modern science, our knowledge of photosynthesis and respiration has grown hand in hand with the development of new methods of measuring oxygen. A revolution in chemistry and biology occurred between 1772 and 1796. In this short time, the experiments of Priestley, Ingen-Housz, Senebier, and Lavoisier revealed the relationship of oxygen to combustion, respiration, and photosynthesis (Rabinowitch, 1945).

It was Priestley who triggered the event. The techniques which he developed for measuring oxygen enabled others to extend his careful observations. With his "pneumatic trough," he made volumetric measurements of gases trapped over water or mercury. He was able to show, among other things, that five times the volume of the gas produced by heating red oxide of mercury reacted with NO as did air (Hartog, 1941). It was the physiological tests which he conducted, however, that convinced him this gas was a pure form of what he called dephlogisticated air (oxygen). He observed that this gas supported the respiration of a mouse for a longer period of time than did air, and that a candle burned brighter in the presence of the gas. Earlier, he had shown that air, "poisoned" by combustion or respiration, could be "restored" by plants if they were illuminated by the sun (Rabinowitch, 1945).

With this groundwork, Ingen-Housz extended Priestley's observations on the oxygen formed in photosynthesis and Senebier showed the importance of "fixed air" (CO_2). Lavoisier, by systematically using the gravimetric balance in checking chemical reactions, had already become familiar with combustion, and in 1775 he described the nature of Priestley's new gas as we now know it (Hartog, 1941). Finally, in 1796, in a book called Food Plants and Renovation of the Soil, Ingen-Housz re-interpreted all of these observations on photosynthesis

in terms of Lavoisier's new chemical theory. By then he understood that plants growing in the light utilize carbon from CO_2 and give off oxygen which is vital to plants in the dark and to animals (Rabinowitch, 1945).

Since that time, many other methods for measuring oxygen have been devised. Two physiological methods involve the use of luminescent bacteria (Beijerinck, 1901) and bacteria which are only motile in the presence of oxygen (Englemann, 1881). These sensitive methods were especially useful to demonstrate under the microscope the photosynthetic oxygen activity localized in a single cell.

Probably the most popular chemical method for oxygen determination has been the Winkler method (Pepkowitz, 1953). Here oxygen reacts with the system manganous chloride - manganic chloride to produce chlorine, which, in turn, is determined by titration with potassium iodide and thiosulfate.

There are today many analytical techniques for measuring oxygen. The trend, however, has been towards the use of the more convenient methods which give a continuous measure of the oxygen concentration. The properties of the methods of this latter type (which have been used in biology) are summarized in Appendix I.

The type of information gained from a measurement depends not only on the method of detection itself, but also on the way in which the method is used. On this basis, two general classes of methods used for obtaining information on the rate of oxygen production (or utilization) may be formulated. First, one may measure the integrated change in oxygen concentration and obtain the rate as the slope of the response-versus-time curve. Second, one may measure the rate of oxygen change directly.

Rate Measured as the Slope of the Response Curve

These methods are closed systems in which the changes due to respiration or photosynthesis are integrated in time. They have the disadvantage in that changes in the ambient partial pressure of oxygen, which occur during the experiment, are not under the control of the experimenter. In this class, there are two types of methods: those which have no sources or sinks for oxygen (other than the biological preparation) and those in which the detector is a sink.

Detector not a sink

The widely used Warburg manometer is an example of this type, as well as the Cartesian diver methods and the many types of microrespirometers. Good reviews of these techniques have been written by Umbreit et al. (1964), Glick (1961), and Rabinowitch (1951, pp. 845 to 849).

An advantage of these gasometric methods is that, because they measure volume changes, their sensitivity to rate is independent of the ambient concentration of oxygen. For example, the sensitivity of 10^{-16} moles per sec. of oxygen, achieved by some of the Cartesian diver methods (Glick, 1961), can be achieved equally as well at atmospheric concentrations as at low concentrations of oxygen. This method has been used to measure the photosynthesis and respiration of a single Euglena cell (Løvlie and Farfaglio, 1965).

Unfortunately, inherent in the manometric method are sources of error which lead to misinterpretations of data even when extreme precautions are taken. Rabinowitch (1951, pp. 1083 to 1118) has written an excellent discussion of the controversies which developed as a result of using manometry for the determination of the quantum yield of photosynthesis. The sources of error of this method are its sensitivity to both oxygen and carbon dioxide changes, and the slowness of equilibration of the liquid and gas phases. Also, the method is unsuitable for use in experiments on many plants since, for the most accurate measurement of quantum yield, a high pH ($\sim$ 9) is required (Rabinowitch, 1951: pp. 847 to 850).

A method of the general type under consideration which avoids the problem of diffusion lag utilizes the hemoglobin - oxyhemoglobin equilibrium. Its advantage is that the hemoglobin can be mixed in with a suspension of cells. Also, since there is no phase change in this system, the response time of the measurement is decreased to a minimum. Whittingham (1954) was limited to a response time of about 5 seconds due to mechanical difficulties, but this could probably be decreased with a few improvements. This method was a marked improvement over the Warburg manometer, and with it Whittingham demonstrated that the "induction period" for oxygen evolution in photosynthesis was an artifact of the manometric method. One disadvantage of the method is the necessity of using a measuring light, which prevents measurement of the true dark rate of oxygen change. Also, its range is limited to partial pressures between 0.007 and 0.040 atmospheres.

Detector a sink

In this type of method, the changes in oxygen concentration caused by biological activity are superimposed on changes caused by the removal of oxygen from the system by the detector. How serious a problem this is depends upon many factors, but for precise measurements of small changes at low partial pressures of oxygen this can become a serious limitation.

The oxygen cathode detector when used in an enclosed, stirred volume of liquid is an example of this type of method.* Chance (1965) recently measured cytochrome oxidase kinetics at low partial pressures of oxygen by using the oxygen cathode detector in this way. Unfortunately, the oxygen cathode is limited at the lower end of its range by a residual current equivalent to about 50 ppm of oxygen (Davies, 1962). In order to measure oxygen below the saturation level of the cytochromes, Chance (1965) and Schindler (1964) had to make use of the more sensitive bacterial luminescence as the detector. The respiration of the bacteria was not significant as a sink for oxygen.

Another example of this type of method is seen in one of the two types of arrangements used by Hoch and Kok (1963). Here, the oxygen in an enclosed suspension of algae "leaks" through a teflon membrane into the entrance port of the mass spectrometer. The mass spectrometer has the great advantage, of course, of being able to discriminate between the isotopes of oxygen. With it Hoch and Kok were able to measure separately O_2^{18} used up by respiration, and O_2^{16} produced by photosynthesis from H_2O^{16}.

Rate Measured Directly as the Response of the Detector

This technique is inherently more accurate and more useful for measuring rates of oxygen change than the method of measuring the slopes of cumulative changes. Methods of this type include two which are applicable to biology: (1) that in which the rate adds to or subtracts from an ordinarily steady rate of diffusion of oxygen from a source to a sink (the detector) and (2) that in which the rate adds to or subtracts from the oxygen partial pressure in a

* The current through the oxygen cathode detector is proportional to the rate of diffusion of oxygen to the cathode, which, in turn, is proportional to the concentration of oxygen in the liquid.

carrier fluid flowing from the biological material to the detector.

Rate adds to or subtracts from a diffusion flow

In this arrangement, the biological preparation is a thin layer of cells which are held stationary between the source and the detector. The oxygen electrode was used in this way by Fork (1961) and Vidaver (1963b). Cells or chloroplasts were spread in a thin layer on the teflon membrane covering the electrode and were held in place with a moistened dialysis membrane. A fluid with a constant oxygen concentration flowed over the membrane and served as the source of the diffusion current. The oxygen cathode was the sink. Steadily respiring cells subtracted from the diffusion flow and steadily photosynthesizing cells added to it. The result was a change in the diffusion current of the oxygen cathode, which bears a relationship to the rate of production or removal of oxygen by the cells. A very similar arrangement was used by Hoch and Kok (1963) in their mass spectrometric measurements.

The results obtained from this type of measurement should be interpreted with caution. Although the detectors used in these cases respond linearly to oxygen, the photosynthetic rate of oxygen production (especially transient rates) could add to the diffusion flow to the cathode in a non-linear way. Since the system has not been calibrated with an independent oxygen source, the method is satisfactory only for relative measurements. Its usefulness as a steady state method can be seen in the work of Vidaver (1963a).

Rate adds to or subtracts from the partial pressure of oxygen in a carrier gas

Theoretically, all of the detection methods in Appendix I can be used in this type of arrangement, with the exception of the gasometric methods. We chose to use this technique in our chemiluminescent method in order to avoid the difficulties mentioned above. Rates of oxygen production or utilization change the oxygen concentration of the flowing carrier gas. The method can easily be calibrated and gives precise quantitative data for steady-state rate measurements. The transient behavior of the biological system is damped out somewhat because of the exponential time characteristic of the approach to the steady state oxygen concentration in the gas. The time constant, however, can be decreased by increasing the flow rate, with a concommitant loss of sensitivity. A further advantage of a flow method is that the oxygen and CO_2 environment of the biological preparation may easily be controlled.

We have developed a new method applicable to the measurement of photosynthetic oxygen production at low oxygen tensions. In this method, the light produced by the chemiluminescent reaction of oxygen with luminol is measured. Oxygen produced by the photosynthetic system in one chamber is carried by high purity argon to a separate chamber containing the luminol. The "luminometer" is sensitive over an extremely wide range of oxygen concentrations and its response is linear. At the present state of development, a detection limit of 5×10^{-14} moles per second, corresponding to a 0.01 ppm (parts per million) change in concentration, has been achieved.

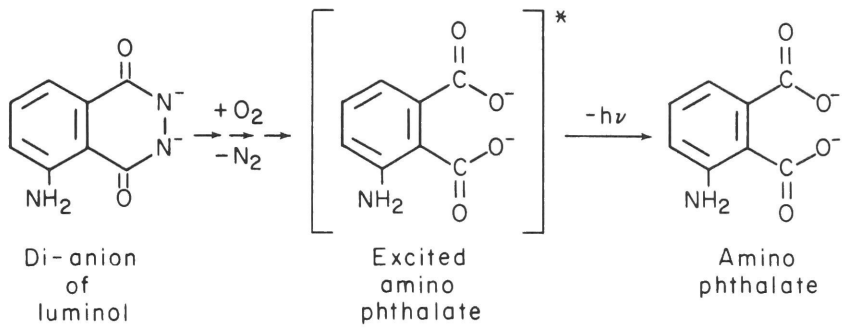
In connection with the luminometer we have developed a very useful calibration method in which oxygen is produced by the electrolysis of water. In Chapters 2 and 3 is described the construction and properties of the luminometer together with the properties of the electrolysis method. In Chapter 4 is described the use of the luminometer in the screening of mutants of Chlorella for their ability to produce oxygen photosynthetically. One mutant of Chlorella is capable of manufacturing chlorophyll only in the light. Its ability to produce oxygen after growth conditions were changed from light to dark and vice versa was also studied with the luminometer.

CHAPTER 2

DESCRIPTION OF THE OXYGEN LUMINOMETER

The Luminol Reaction

In our method, oxygen is detected by the chemiluminescent reaction of luminol in alkaline dimethylsulfoxide (DMSO). As described by White and Bursey (1964 a), the dianion of luminol reacts with oxygen to give nitrogen and the excited state of aminophthalate, the light-emitting species.



White et al. (1964 b) measured the kinetics of the reaction in the system 70 mole% DMSO, 30 mole% water, and excess NaOH and found that the rate of the overall reaction: $R = k\left[lum^{--}\right]\left[O_2\right]$. Only a low concentration of the dinegative ion ($pK \sim 13$) was present in this reaction mixture. In pure DMSO, bases stronger than the hydroxide ion can be used, and the light emission for a given oxygen concentration is greater. With potassium tert-butoxide in dry DMSO, the quantum yield of chemiluminescence is as high as 5% (White and Bursey, 1964 a). In the same solution, aminophthalate, excited by light, fluoresces at the same wave-length (485 nm) with a quantum yield of 5 - 10% (White and Bursey, 1964 a).

If a test tube containing the reaction mixture is left standing, within a few seconds the dissolved oxygen is used up and the bright blue chemiluminescence is restricted to the surface. There the oxygen is used up as fast as it diffuses into the solution from the air. This surface reaction was used in the more successful of the two types of luminol chambers we tried.

For use in the luminometer, we found the best recipe to be: 0.1 M luminol and 0.25 M potassium tert-butoxide in DMSO containing a 5% tert-butyl alcohol. The tert-butyl alcohol improved the solubility of the butoxide, and probably helped by buffering the base.

All of the reagents were dry. The solvents were redistilled, precautions being taken to exclude atmospheric water vapor, and stored over Linde Molecular Sieves Type 3A-2 (1/16 inch pellets). Less decomposition of DMSO occurred during

vacuum distillation if dry nitrogen was bubbled through it.

Potassium tert-butoxide was obtained from the Mine Safety Appliance Co., Research Corp., Callery, Pa., as the sublimed white powder. The reagent is easily contaminated by moisture and CO_2. Suitable precautions were taken to avoid this. When the butoxide occasionally became contaminated by peroxide, it was discarded. The presence of peroxide was determined by the iodine-starch method.

Luminol was recrystallized as the HBr salt from hot 48% HBr, washed with cold 48% HBr on a sintered glass filter, and washed with distilled water. In water, the fine white needles changed to a curd-like yellow precipitate. This was neutralized to pH = 6 with dilute KOH and washed thoroughly with distilled water. The neutral luminol was kept dry over P_2O_5 until use. Analysis showed zero contamination by halides.

Best results were obtained when the potassium tert-butoxide and the solvents were measured and mixed in dry nitrogen. If the relative humidity was below 40%, however, and exposure to the atmosphere was minimized, the reagents could be handled in air. The luminol and potassium tert-butoxide were dissolved separately in dry DMSO. The solutions were de-oxygenated by bubbling with pre-purified argon, which was dried by filtering through molecular sieves (Matheson Gas Purifier, Model 450, used with a Type A cartridge). Two ml of each solution were then mixed by drawing them, one after the other, into a syringe which had been flushed with argon previously. The reaction mixture was then injected into the luminol vessel. Usually such a solution remained sensitive for about a month, depending on use.

In order to obtain a linear relationship between the response and the oxygen concentration, it was necessary to "preoxidize" the freshly made luminol solution (see Chapter 3). The oxygen generated by about 10 milliamperes electrolysis current was passed through the luminometer until the response, as determined by calibration, was linear. About 2 hours of preoxidation was required. No further preoxidation was required until the luminol solution was replaced.

The Method

A carrier gas is used to transport the oxygen produced by photosynthesis to the luminol solution. The gas train is illustrated in Fig. 1. By turning stopcocks, the regulated flow of Ultra High Purity Argon (Matheson Co.) can be routed either through the bypass or through the service branch. When the service branch is in use, the carrier gas bubbles first through the electrolysis chamber, next through the cuvette containing the algal suspension, and finally passes through a drying tube before entering the luminol vessel. The 11.0 ml per min. flow rate is measured periodically to within 2% by timing with a stopwatch the displacement of one ml of water.

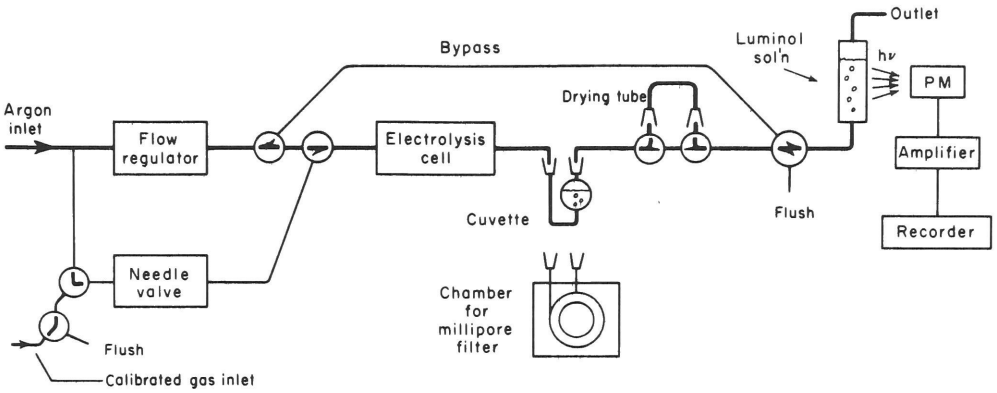


Figure 1. Flow diagram of the luminometer.

During a typical experiment, the sequence of operations is as follows. The cuvette is filled with 8 ml of a suspension of Chlorella in a buffer solution and is plugged into the gas train while the flow-regulated gas is routed through the bypass. The cuvette is flushed with argon for 5 to 10 min., and the stopcocks are turned so that the regulated flow passes through the service branch. A minute is allowed for the flow rate to stabilize before measurements are begun.

The instrument is calibrated several times during an experiment by the electrolysis of 0.001 M NaOH with a 10 microampere current. This takes only 5 minutes. At the beginning of the experiment the linearity is checked with a 10 microampere and a 1000 microampere current.

The electrolysis method is only a secondary calibration, and was checked against precalibrated gases. These entered the gas train through the calibrated-gas inlet. Analyzed mixtures of oxygen with argon were obtained from the Matheson Co. and had the following concentrations of oxygen: 2100 ppm, 525 $\pm$ 25 ppm, 60 $\pm$ 2 ppm, and 5 $\pm$ 0.5 ppm. They had been analyzed with a Lockwood and McClorie Oxygen Analyzer, which uses the Hersch cell (Phillips et al., 1964) as a detector. Calibrated gas mixtures with less than 1 ppm oxygen were not available.

A Matheson Model 19 diffusion resistant metal diaphragm pressure regulator is used to maintain the pressure between the gas cylinder and the flow regulator at 20 pounds per square inch. The flow regulator is a Brooks Instrument Co. Model 8943 ELF Low Flow Controller, which regulates by maintaining a constant pressure differential across a needle valve. In operation at 11.0 ml per min., it governs the flow to better than the 2% precision of our method of flow measurement. The needle valve, also made by the Brooks Instrument Co., is their Model 8501 ELF Low Flow Needle Valve.

The sensitivity of the luminol reaction is gradually decreased by any water vapor or carbon dioxide which is carried to the solution from the cuvette or the electrolysis cell. The trying tube, filled with granular anhydrous magnesium perchlorate (Anhydrone) and Ascarite (8 to 20 mesh), effectively removes these contaminants from the gas stream. A 7 cm long, 0.5 cm. diameter tube of the Anhydrone lasts for about 20 hours at the flow rate used.

When the luminometer is not in use, the U.H.P. Argon is left flowing through the gas train in order to prevent the accumulation of oxygen due to slow leakage.

The Construction

The arrangement of the various parts of the luminometer system is shown in Fig. 2. The stopcocks and the needle valve and flow regular controls are placed at the front where they can easily be reached. The photo-multiplier tube and the luminol chamber are located inside a light-tight cardboard box. The projector beam is reflected at a right angle so that it passes through the cuvette from behind. A 1000 RPM synchronous motor with a magnet attached to its shaft is used to drive the glass-enclosed magnetic stirring "flea" inside the cuvette.

In the construction of the gas train, the aim was to minimize the volume, and hence to minimize the travel time of the gas from point to point. This is most important between the reaction cuvette and the luminol chamber. Here the glass stopcocks, tubing, and joints all have a 1 mm diameter bore, and distances are made as short as possible. Elsewhere, they have a 2 mm bore. The needle valve and the flow regulator are connected to the pressure regulator and to the glass tubing with 1/8 inch O.D. copper tubing.

Considerable care had to be taken in the construction of all joints in order to prevent the diffusion of oxygen from the air. A leakage rate as low as 1 ml in 17 years is detectable. Swagelock tube fittings of brass were used to connect the copper tubing to the pressure regulator, flow regulator and needle valve. The copper tubing was sealed into the opening of the glass tubing with a dense thermoplastic sealing wax (Apiezon Type W wax).

The most gas-tight glass-to-glass joints for our purpose are capillary standard taper joints. It was desired, however, to have a certain amount of flexibility between the electrolysis cell and the reaction cuvette. Capillary ball and socket joints were found satisfactory provided a suitable joint grease was used. Apiezon Type N stopcock grease was used on all flexible joints and all stopcocks. Semi-permanent joints were made by cementing ball and socket joints together with Apiezon Type W sealing wax. In general, we tried to minimize the number of joints by using glass seals wherever possible.

Pressure stopcocks were necessary upstream from the electrolysis cell since, at the higher pressures that develop there, the gas leaks around the plugs of ordinary stopcocks. The pressure stopcocks of the type with an adjustable spring force were found preferable to any others.

A mercury trap was used initially to prevent air from diffusing upstream into the luminometer vessel. However, the velocity of the argon in the 4 mm I.D.

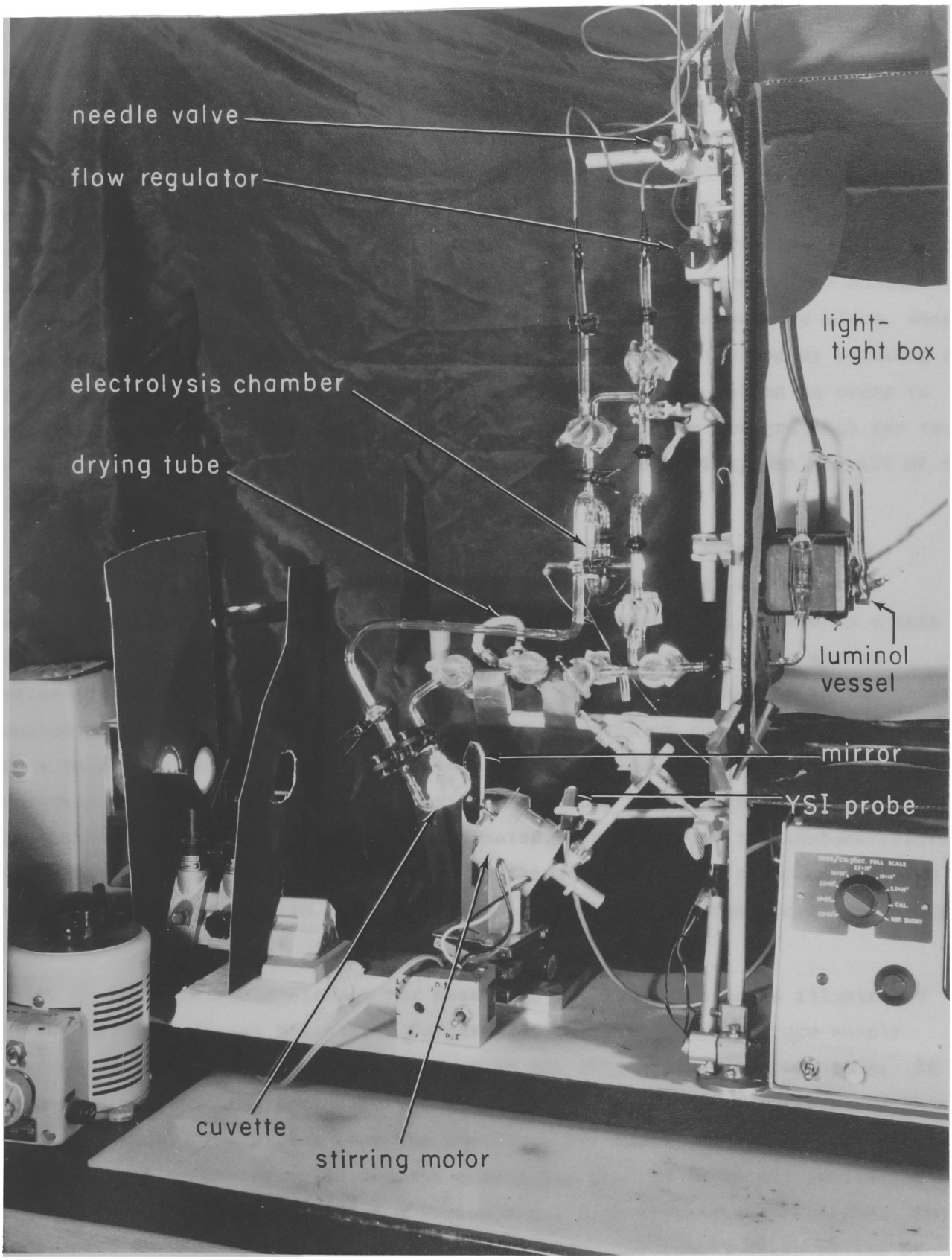


Figure 2. Photograph of the luminometer.

exit tube was discovered to be sufficient to prevent back diffusion. The mercury trap, by forcing the exit gas to bubble through mercury, caused a bothersome oscillation of the flow rate and was discarded. Two different types of vessels were used to bring the carrier gas in contact with the luminol solution. The first type tried was built in the form of a gas scrubbing column, as illustrated in Fig. 3. The fine bubbles produced permitted a large contact area per volume of gas between the two phases. For maximum light collection, the column of glass tubing was silvered on the outside with the exception of the flattened area facing the photomultiplier. A reflecting cone of aluminum foil connected the column and the photocathode. The shutter was a piece of sheet aluminum painted black, which could be moved between the luminol chamber and the photomultiplier by rotating a shaft. Care had to be exercised in the construction of the column in order to minimize the dead volume below the sintered glass disk. We are grateful for the inventive and skillful glass blowing of Mr. Wolfgang Papperitz, who did all of the glass work described.

The second, and more successful, type of luminol vessel we used consisted of a 4 cm length of 1 cm square glass tubing, which was mounted horizontally. The gas, entering and leaving at opposite ends, flowed in a thin layer over the surface of the luminol. As illustrated in Fig. 4, a mirror reflected the light produced at the surface of the luminol into the horizontally mounted photomultiplier tube. The vessel was silvered on the bottom and the two sides to obtain maximum light collection. This surface-reaction chamber had the advantage over the first vessel of having a smoother light output, since the fluctuations caused by bubbles were eliminated. In addition, the surface-reaction chamber avoided the difficulties caused by the sintered glass disk: namely, the high pressures necessary to maintain a flow through it, and the occasional clogging of the disk.

The electronic circuit for measuring the luminescence is illustrated in Fig. 5. A Northeast Scientific Corporation regulated high voltage supply provided 780 volts to the photocathode of an RCA 1P21 multiplier phototube. It was determined that the phototube has a maximum signal-to-noise ratio using this cathode voltage. The signal from the phototube was amplified by a Keithley Model 150 AR Microvolt-Ammeter. The 10 volt output was dropped to 10 millivolts and filtered before being recorded on a Varian Model G-10 strip-chart recorder. The chart speed we usually used was 4 inches per hour. The line voltage of the HV supply, the microammeter, and the recorder were regulated by a Perkin Electronics AC Line Regulator, Model MLTR 1000.

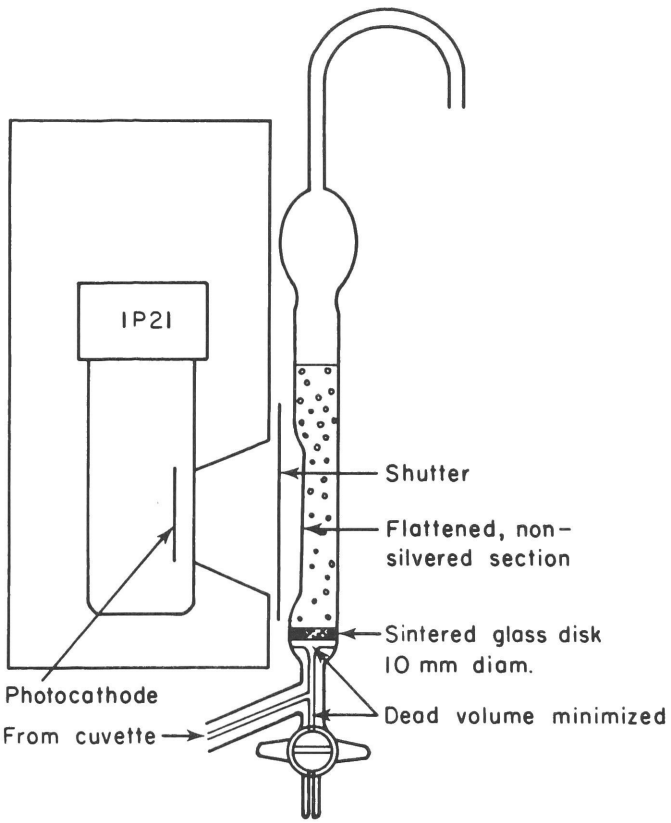


Figure 3. The bubbling column for the luminol solution and its relationship to the photomultiplier tube. Side view.

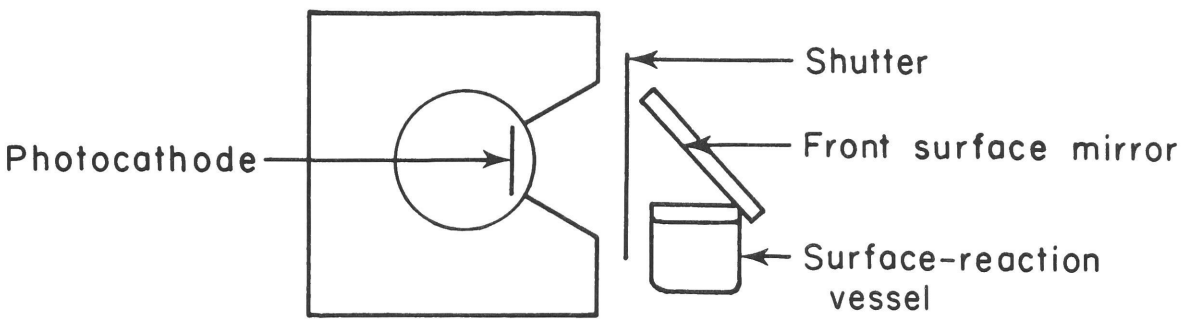


Figure 4. The surface-reaction vessel and its relationship to the photomultiplier tube. Seen end-on.

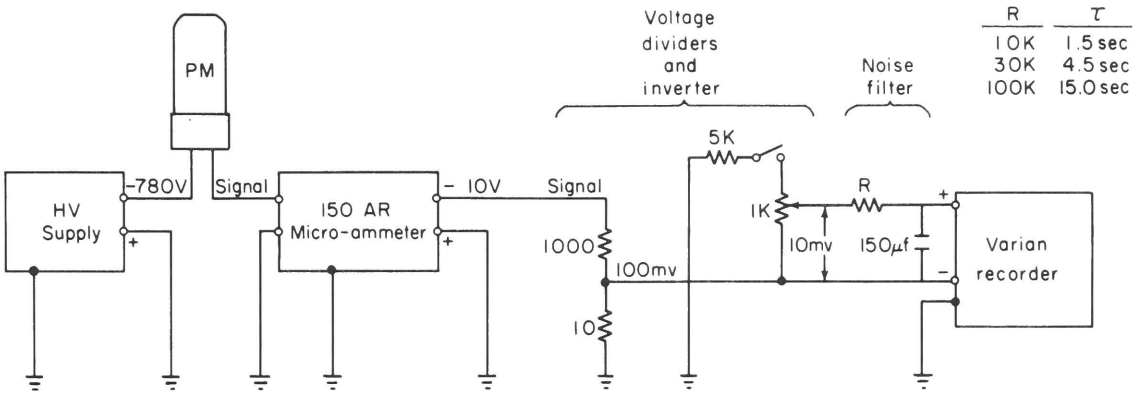


Figure 5. Electronic circuit for measuring the intensity of the luminescence.

Because of the negative-going signal put out by the microammeter, a ground loop was unavoidable in the recording circuit. However, by choosing a low, 10 ohm resistor for the voltage divider, the pick-up was made negligible. It was necessary to isolate physically the AC voltage regulator from the instrument rack, because the 60-cycle mechanical vibrations produced by the regulator affected the stability of the 150 AR microammeter.

The electrolysis chamber and its associated electrical circuit are shown in Fig. 6. The voltage was adjusted until the desired current was flowing through the chamber. At currents of 10 microamperes or less, a 1 megohm series resistor was used. In practice it was found better always to use the same polarity and to leave the electrodes unshorted when not in use. The #32 gauge platinum-iridium wire was hammered until paper thin before being sealed into the pyrex glass tubing. Otherwise, due to the different thermal expansions of the two materials, microscopic cracks developed on cooling which allowed oxygen to diffuse in from the air. The exposed platinum tip was melted into a polished ball before the tubing was sealed into the chamber.

A Keithley Model 414 Micro-microammeter and a Moseley Model 680 Chart Recorder were used. The variable voltage supply was constructed by Cellarius (1965), using five 8.4 volt mercury batteries of Type TR-146. The microammeter was operated off the regulated AC power supply.

Several types of reaction chambers were developed and are illustrated in Figs. 7 and 8. The one used in the experiments on Chlorella reported in this thesis was the quartz reaction cuvette (Fig. 7a), which was originally developed for the study of UV-excited photochemical reactions. The 25 mm diameter quartz tubing, the ground and polished optical quartz windows, and the specially made quartz "O" ring joints were obtained from Thermal American Fused Quartz Co.

The cuvettes of Fig. 7b were constructed of precision-bore square glass tubing. The tall one can be used instead of the quartz cuvette where a smaller volume ($\sim$ 1 cc) of cell suspension is desired. The other was developed for use with Acetabularia. The large single cell can be attached to a platinum wire and lowered into the solution. In the chamber of Fig. 8, the carrier gas flows over a moist Millipore filter on which algal cells are deposited. It is constructed of Lucite with the exception of a glass window which is clamped against a butyl "O" ring seal.

The light source for photo-excitation of photosynthesis is illustrated in Fig. 9 and in the photograph in Fig. 2. The beam from a 500 watt Kodak projector

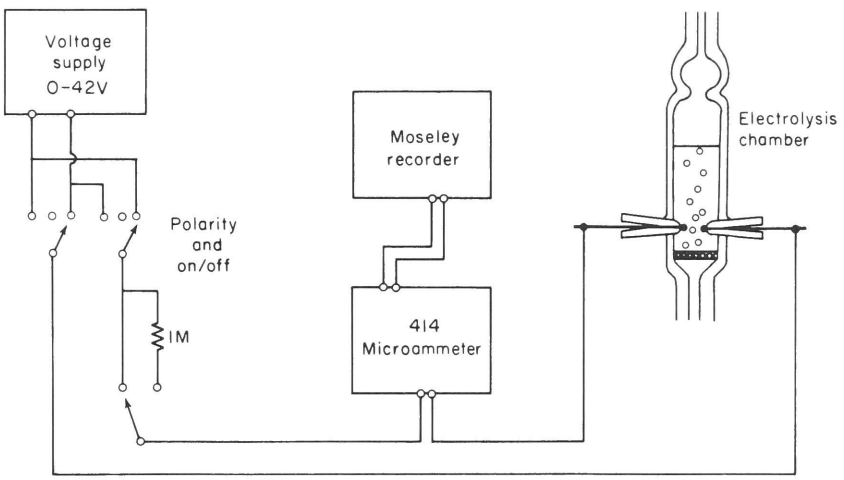


Figure 6. Electrolysis chamber and electrical circuit.

Figure 7a. Quartz Cuvette.

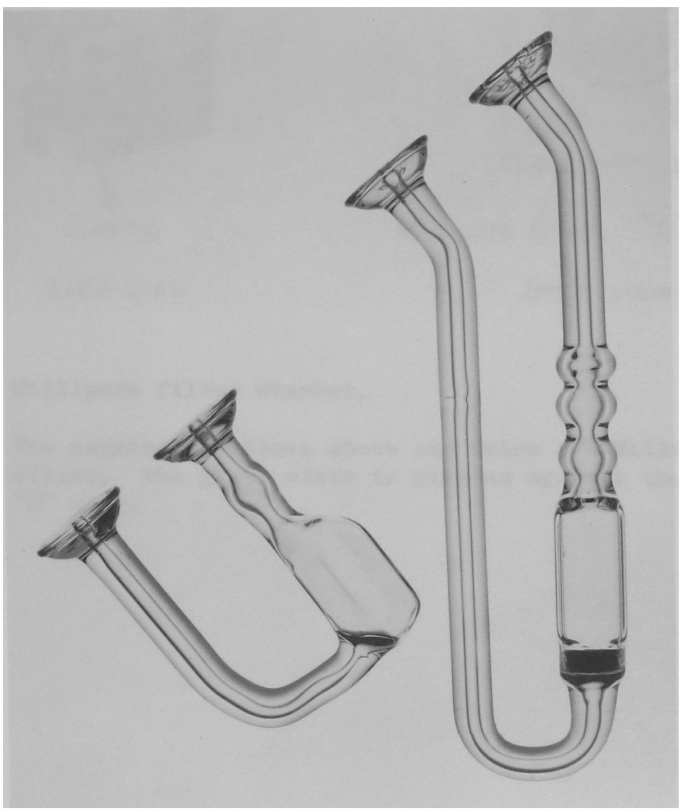


Figure 7b. Glass Cuvettes for special purposes.

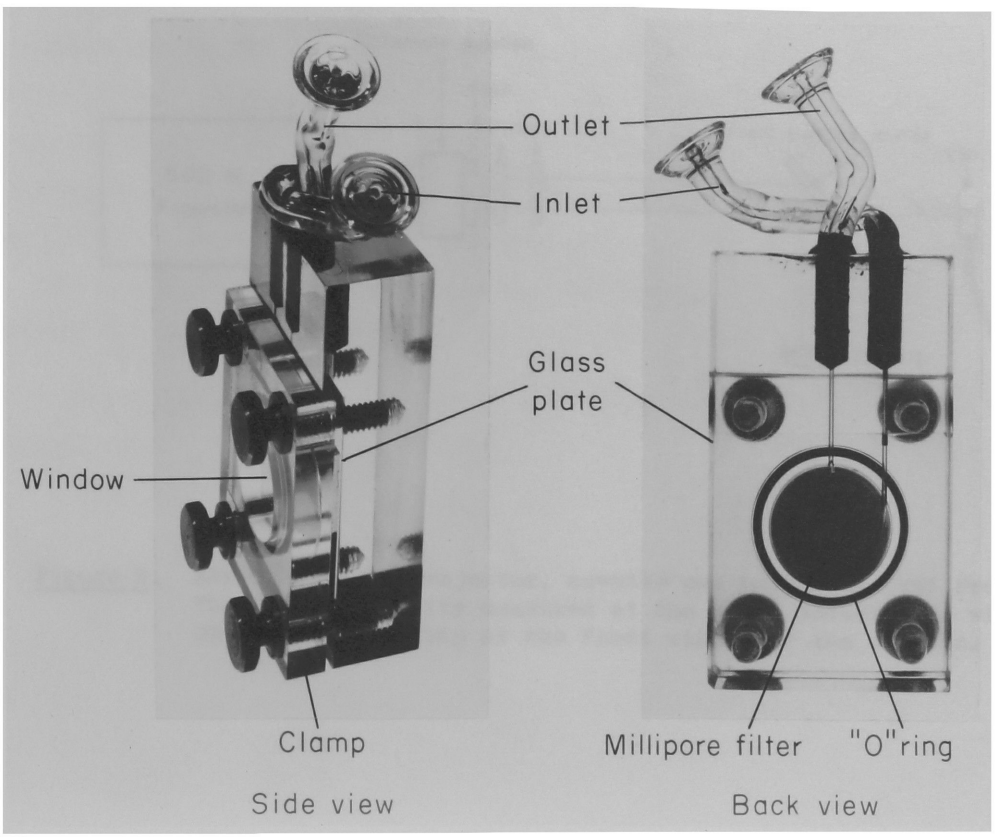


Figure 8. Millipore filter chamber.

The carrier gas flows above and below the Millipore filter. The glass plate is clamped against the "O" ring.

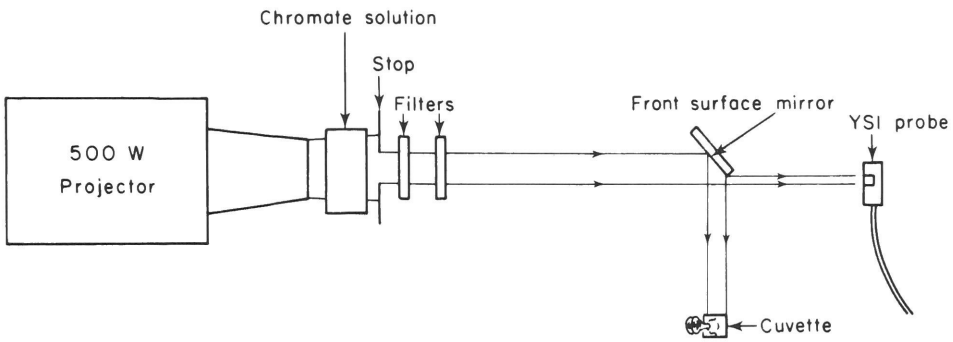


Figure 9. Arrangement of projector, cuvette and bolometer (YSI Probe). The light intensity measured at the probe location is within 5% of the intensity at the front window of the cuvette.

with a heat filter is reflected at a right angle and passed through the cuvette. The probe of a Yellow Springs Instrument Co. Model 65 Radiometer, a dual thermistor bolometer, is located equidistant from the projector in a portion of the beam that is not occluded by the mirror. The light intensity measured at that location was found to be within 5% of the intensity at the front window of the cuvette. The intensity at the front window varied over the surface by about 5%. The chromate solution filters out infra-red light, as well as all wavelengths below 480 nm. It was necessary to filter out these wavelengths in order to prevent light absorption by the benzoquinone dissolved in the solution which we used in our measurements of photosynthesis. When desired, a 660 to 670 nm band was selected by combining a Corning #2-64 cut-off filter with a Bausch and Lomb 660 nm interference filter. Neutral density screens and filters were used to attenuate the light. The projector was operated from the Perkin AC line regulator. A Powerstat transformer was used for adjusting the voltage to the lamp.

The temperature of the room was held to within 18 to 20^{o} C by an air conditioner.

CHAPTER 3

PERFORMANCE OF THE LUMINOMETER

General Description

The output of a photosynthesizing system and the response of the luminometer are inherently rates of oxygen production. A good calibration method, then, is one which produces oxygen at a comparable known rate. The generation of oxygen by the electrolysis of water is such a method, and a very convenient one to use. Simply by knowing the electrolysis current one can calculate, under ideal conditions, the rate of oxygen production.

The oxygen is produced at the anode in the following reaction:

(1) $$2OH^- \rightleftharpoons 4e + 2H^+ + O_2$$

The primary reactions at the cathode in this electrolyte (0.01 M NaOH) are

(2) $$2Na^+ + 2e \rightleftharpoons 2Na^o$$

(3) $$2Na^o + 2H_2O \longrightarrow 2Na^+ + 2OH^- + H_2 \text{ (gas)}$$

and possibly

(4) $$2H_2O + 2e \rightleftharpoons 2OH^- + H_2$$

The rate of oxygen production in moles per second is calculated from Faraday's Law on the basis of 4 electrons per oxygen molecule. The resulting increment in molar concentration is calculated by dividing by the flow rate. The increment in terms of parts per million (ppm) is calculated using the mol volume of gases with Charles' Law to adjust it to room temperature. One microampere thus produces an increment of 0.336 ppm of oxygen.

The response of the luminometer to a 10 microampere step in electrolysis current is shown in Fig. 10, and to a series of 1 microampere steps in Fig. 11. A lag of 0.4 minutes follows the start of the current. This is due to the travel time between the electrolysis chamber and the luminometer vessel. It takes a total of about three minutes to reach the new steady-state level of oxygen concentration. In all of the experiments to be described in this thesis, the steady state level was measured. The reproducibility of the response to 10 microampere and larger steps is better than 2%, and to 1 microampere steps is about 5%. The lower certainty for currents 1 microampere and below is the fault of the electrolysis method, not the luminometer, as will be shown later in this

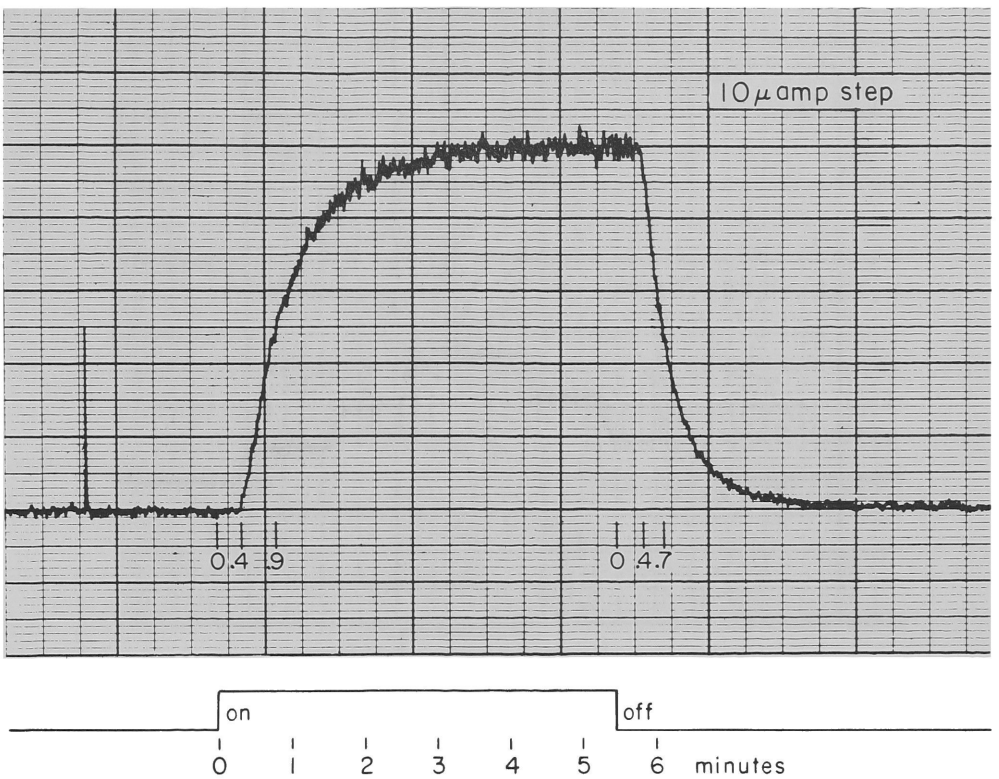


Figure 10. A response to a 10 microampere step in electrolysis current. The numbers printed on the record indicate the time after turning on or turning off the current. A 0.4 min. travel time is observed, followed by an exponential rise or fall. The half time of the rise is 0.5 min; that of the fall is 0.3 min. The chart speed was faster in this figure than in Fig. 11. The time constant of the filter circuit was 0.45 sec. The response was superimposed on a background due to the oxygen contamination of the carrier gas. The measurement was made with the bubbling column.

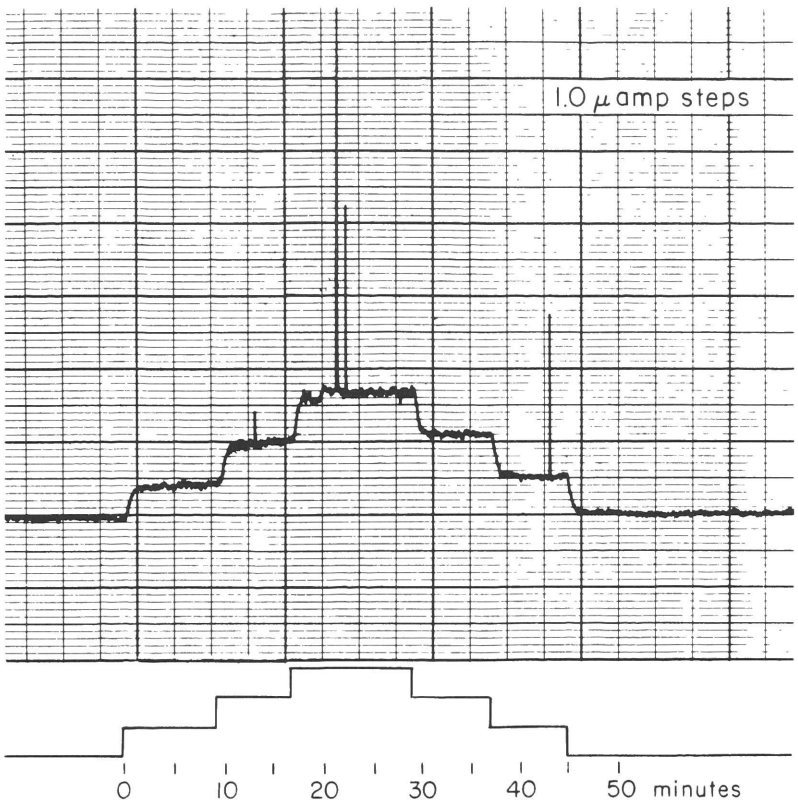


Figure 11. Responses to cascaded 1.0 microampere steps in electrolysis current. They are superimposed on a background due to the oxygen contamination of the carrier gas. The time constant of the filter circuit was 1.5 sec. The measurements were made with the bubbling column.

chapter.

The responses are superimposed on a steady baseline due to the oxygen impurity in the carrier gas plus the dark current of the photomultiplier. The dark current is about 0.2×10^{-9} amperes. The photocurrent due to the approximately 1.0 ppm background of oxygen is 1 to 3×10^{-9} amperes, depending on the efficiency of the luminol solution. By bucking out this photocurrent and amplifying, one can attain a sensitivity of about 0.3% of the baseline with the surface-reaction vessel. The sensitivity is limited by the noise, and with the noisier bubbling column the sensitivity is only 1%. The measurements of Figs. 10-12 were done with the bubbling column.

In Fig. 12 the minimum detectable variation in oxygen rate is measured. It is seen that a change of about 0.03 microamperes in the electrolysis current is just discernible. This is equivalent to a rate of oxygen production of about 5×10^{-14} moles per second, or an increment in concentration of about 0.01 ppm.

The response of the luminometer to electrolysis currents is shown in Fig. 13. This response has been tested over nearly six orders of magnitude and is seen to be linear with respect to electrolysis current. The rate of oxygen generation (moles per second) was calculated on the basis of four electrons per oxygen molecule. The line was drawn with a slope = 1.000. The points were taken on four different days using three different batches of luminol solutions. The inverted triangles were measured using the surface-reaction chamber in the experiment shown in Fig. 14. The other points were measured using the bubbling column. The standard deviation of the points from the line is 0.033 log units and is equivalent to 8.0% of the response, assuming all the error is in the response.

The response to electrolysis currents of 0.1 microamperes and lower falls below the line of Fig. 13 because of a decreased efficiency of the electrolysis current to be discussed later in this chapter. It was for this reason that the lowest three points were measured as changes superimposed on a 1 microampere current (see Fig. 12).

The lowest three points of Fig. 13 were determined in the experiment of Fig. 12. It is hoped that by using a low noise photometer, such as the EMI 6256S, and by purifying the carrier gas, the minimum detectable oxygen change can be extended one or two orders of magnitude. The response of the luminometer to 6.55 milliamperes of electrolysis current (1.6×10^{-8} moles per sec. or an oxygen concentration of 0.21%) is the highest we have tested. We expect that the luminometer will remain linear at concentrations one or two orders of magnitude higher.

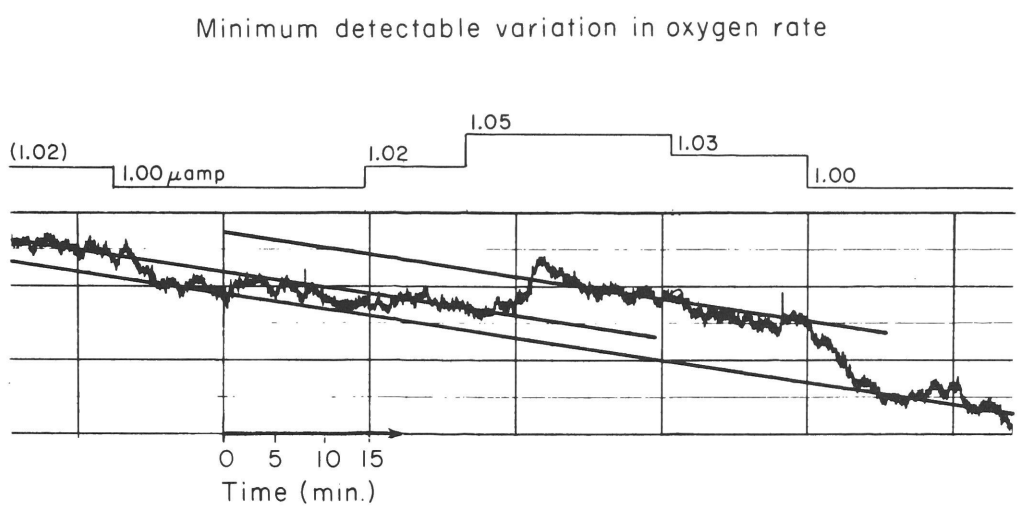


Figure 12. Minimum detectable variation in oxygen rate. A 1.02 microampere current was changed in 0.02 and 0.03 microampere steps as indicated at the top. The diagonal lines connect responses to the same current. The baseline due to the background of Figs. 10 and 11 plus the response to the ~1 microampere current has been bucked out, and the signal has been amplified 3x. The measurements were made with the bubbling column. The drift seen here was not present when the drying tube was used.

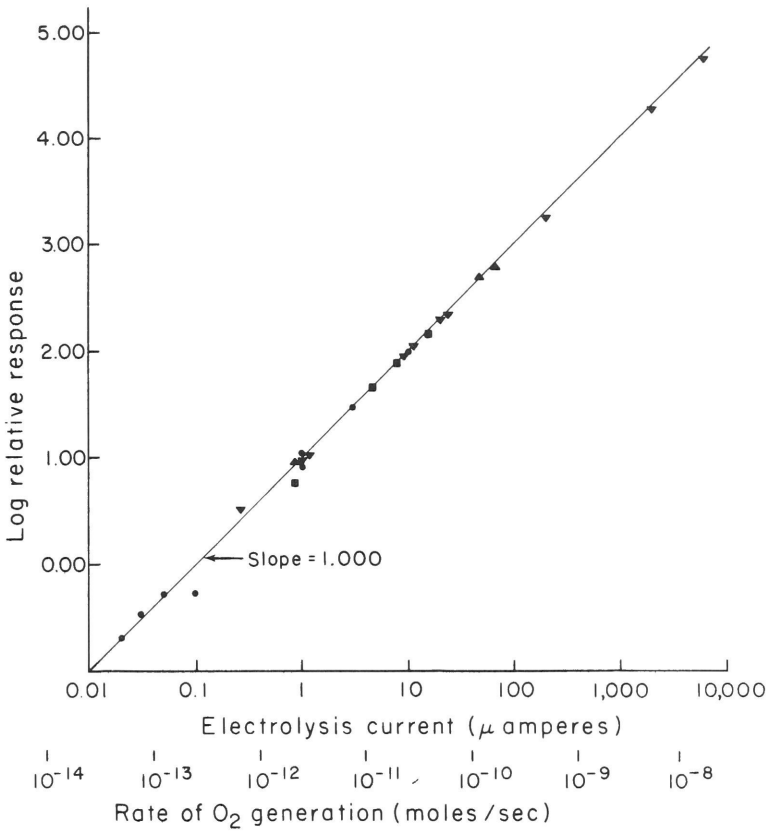


Figure 13. Log relative response plotted as a function of log electrolysis current. The rate of oxygen generation (moles per sec) was calculated from Faraday's Law assuming 4 electrons per oxygen molecule. The line was drawn with a slope = 1.000. Each type of symbol represents a measurement on a different day. The ●, ■ and ▲ were measured with the bubbling column; the ▼ were measured with the surface cell and are the same data as in Fig. 14. The lowest three points were obtained in the measurement depicted in Fig. 12. They were superimposed on a one microampere background electrolysis current. The data were normalized to the response to 10 μamperes in order to allow for differences in sensitivity.

The precision of the luminometer is better than 5.0% in any one experiment. At the moment, the absolute accuracy is limited by the accuracy of the calibrated gases used to check the electrolysis method. In Fig. 14 the responses to increments in oxygen concentration theoretically produced by electrolysis are compared with the responses to the calibrated gases. In this experiment, the electrolysis points fell along the line of slope = 0.992 with a mean deviation of the points from the line of 5.0%, and the points of the calibrated gases fell along the line of slope = 0.963 with a mean deviation of 4.5%. It is uncertain whether or not the 3% difference in slope was due to a systematic error in the electrolysis method, or in the calibration of the gases. The gases were calibrated by the Matheson Co. using the Hersch cell. The minimum detectable concentration of oxygen by this method is on the order of 1 ppm (Baker, 1959). It is possible that in an industrial application of this method the measurement of the 5 ppm gas might be inaccurate. If the Hersch cell as used by Matheson read only 25% too low, the slope of our measurements with the calibrated gases would be the same as that of the electrolysis measurement.

In addition to a small difference in slope, the curves also appear to differ in absolute oxygen concentration by about 7%. Again, this could be due to a systematic error in the calibration of the gases. The possibility of error in the electrolysis method will be discussed at the end of the chapter.

Analysis of the Luminometer Method

Preoxidation

The freshly made luminol solutions have been found not to respond linearly to oxygen concentration, but to vary as the 1.03 to 1.04 power of the concentration. The response eventually becomes linear after several weeks with the carrier gas ($\sim$ 1 ppm oxygen) flowing through the luminol vessel. This can be accomplished in a few hours by "preoxidizing" with high concentrations of oxygen obtained by electrolysis or with a gas mixture. This behavior is observed with both the bubbling column and the surface-reaction vessel.

The progress of preoxidation with a 10 milliampere current is shown in Fig. 15. The data were obtained using the surface-reaction vessel. The exponent was calculated by the relation

$$S = \frac{\log R_1 - \log R_2}{\log i_1 - \log i_2}$$

where S represents the slope of a graph such as Fig. 14, and R_1 and R_2 are the

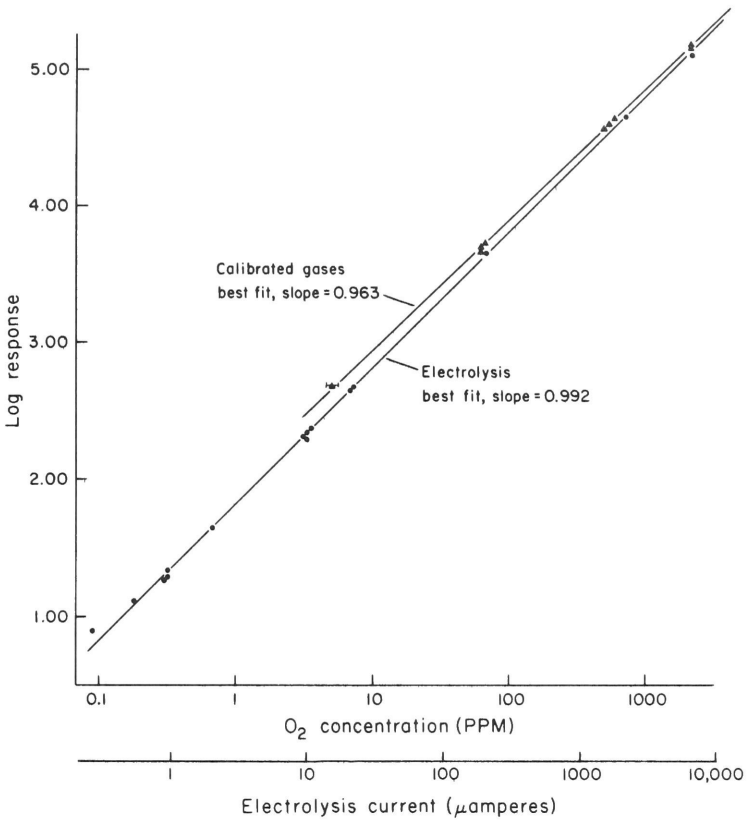


Figure 14. Log response verus log electrolysis current is compared with log response versus log number of parts per million of oxygen in the calibrating gas. The abcissas were correlated using the relation, #ppm = 0.336 i (microamperes), calculated from Faraday's Law assuming 4 electrons per oxygen molecule.

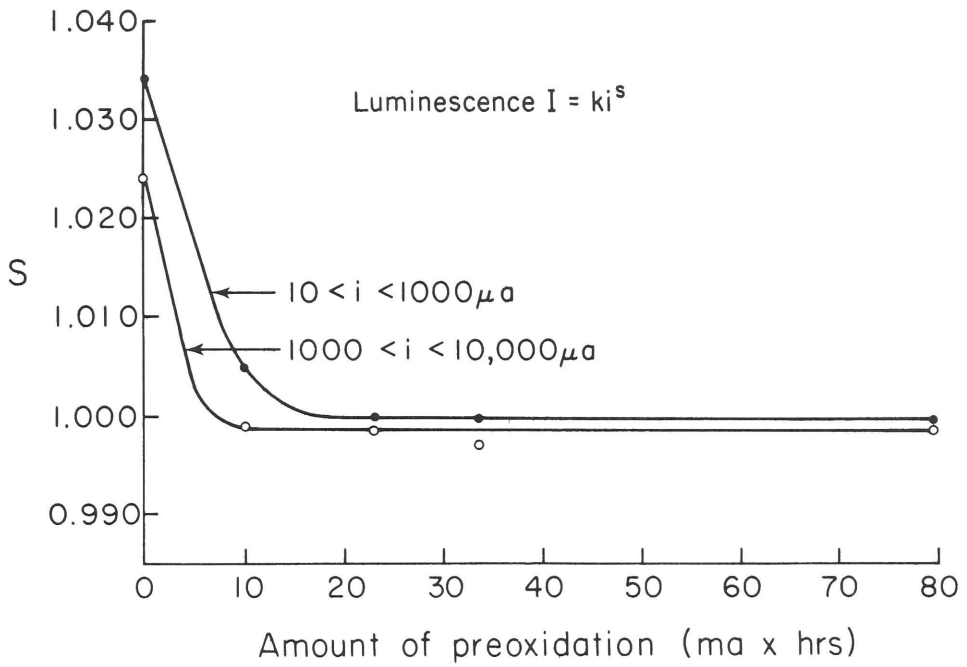


Figure 15. Approach to linearity during pre-oxidation. The exponent, S, is graphed as a function of amount of preoxidation. The upper curve was calculated from the response to currents of 10 and 1,000 microamperes; the lower one from that of 1,000 and 10,000 microamperes.

responses to electrolysis currents i_1 and i_2, respectively. These currents were about 1000 microamperes and 10 microamperes for the upper curve. The lower curve was calculated the same way, but currents of about 10,000 and 1000 microamperes were used. It is seen that about 25 milliampere-hours of preoxidation were necessary to make the response linear. This is equivalent to about 2×10^{-4} moles of oxygen generated. It is probable that a fraction of the oxygen passes right through the surface reaction vessel. If all of the oxygen reacted, about 50 milliampere-hours of preoxidation would entirely use up the luminol in the solution.

The magnitude of the response to a 10 microampere current decreased during preoxidation. The photocurrent per response declined from 10.6 millimicroamperes to 5.25 millimicroamperes after 20 milliampere-hours of preoxidation and to 3.97 millimicroamperes after 80 milliampere-hours.

These results are in contrast with results obtained when the luminol reagent had not been recrystalized. The magnitude of the response starts low, increases to a maximum and then steadily decreases. Furthermore, slopes, S, as high as 1.29 are obtained, requiring 40 milliampere-hours of preoxidation to obtain linearity.

It is evident that an impurity removed by recrystalization causes low initial responses and high slopes. An inhibitor which competes with the luminol anion for oxygen would cause this type of behavior. Presumably a trace of inhibitor which remains after recrystalization is effectively removed by preoxidation. It is expected that only a fraction of the 2×10^{-4} moles of oxygen generated during preoxidation could react with the inhibitor in competition with the luminol.

The non-recrystalized luminol was found by analysis to contain 3.7% by weight of halides. After recrystalization, none was detected. The method of analysis had a detection limit of 0.1%.

Response time

A typical response to a step in electrolysis current was described on page 17 and in Fig. 10. The 0.4 minute lag period is followed by an exponential rise or decay. The half time of the rise (0.5 minutes) is longer than the half time of the decay (0.3 minutes).

The lag period is caused by the travel time between the electrolysis chamber and the luminol vessel. This can be decreased by decreasing the volume

between the two vessels, or by increasing the flow rate.

The exponential rise to, and decay from, the new steady state is caused mainly by two slowly equilibrating processes: (1) the build-up of oxygen concentration in dead volumes between the two chambers and (2) the achievement of a steady state in the luminol vessel. Under ideal conditions, the dead volume equilibration is the limiting process, but often the luminol reaction achieves steady state at a slower rate and adds noticeably to the time constant. That a new steady state is achieved more slowly on the rise than on the decay is reasonable. When a higher steady state is approached the removal of oxygen by the luminol reaction might work against equilibration, thereby slowing the process. On the other hand, when a lower steady state is approached, the removal of oxygen by the luminol reaction might work towards equilibration, thereby speeding the process.

Other possible contributors to the rise and decay times are ruled out because their time constants are much lower than those observed. The recorder pen drive requires only one second for a full scale change, and the time constant of the filter circuit, which was 0.45 seconds during the recording of Fig. 10, was never used at greater than 15 seconds.

It cannot be ruled out, however, that some changes in the electrode reaction during electrolysis may possibly contribute to the rise time of the response. This has been noticed for electrolysis currents below 0.1 microampere. But it is doubtful if this would be a significant contribution at currents as high as 10 microamperes.

Noise and stability

The high frequency noise superimposed on the recording of Fig. 10 is not of concern because it can easily be filtered out. This was partially done in Fig. 11, in which the time constant of the imput filter circuit (see Fig. 5) was increased to 1.5 seconds, a three-fold increase over that of Fig. 10.

More serious is the noise in the signal which has a time behavior on the same order as that of the response (see Fig. 12). There are three possible sources of the fluctuations:

(1) The photomultiplier (an RCA 1P21). Its noise output, which increases with temperature and humidity, was kept constant by air conditioning. It is hoped that by using an EMI 6256S this source of noise will become insignificant.

(2) Fluctuations in the light leaving the luminol vessel. By replacing the bubbling column with the surface-reaction vessel, the sensitivity of the luminometer to small changes was improved by a factor of three. The bubbling caused fluctuations in the light output of the column. In the surface-reaction vessel eddy currents in the gas phase or convection currents in the liquid phase are possible contributors.

(3) Fluctuations in the output of electrolysis in spite of a steady current through the electrodes. This can be caused by the formation and periodic release of oxygen bubbles on the electrodes.

The strongly basic luminol solution is sensitive to both water and carbon dioxide. With the drying tube in use, however, there is only a slight drift in the sensitivity of the luminometer. This drift is probably due to the gradual decrease in luminol concentration as the luminol is used up. It is less than 10% during a day of experimentation, and by periodic calibration with a 10 microampere current, one can keep the error well within the 5.0% precision of the luminometer.

Oxygen leaks occasionally were a problem. A leak in the service branch (Fig. 1) was detected when the baseline luminescence was higher than it was when the bypass was used. Small leaks were made more noticeable by stopping the flow in that branch for a few minutes, thereby allowing the oxygen concentration to build up locally. The location of the leaks could be estimated by the travel time of the pulse of oxygen. Oxygen leaks located between the gas cylinder and the flow regulator could be detected by stopping the flow momentarily.

Fresh stopcock grease appeared to release dissolved oxygen, which took several days to diffuse entirely out. Similarly, oxygen diffuses out of teflon-coated magnetic stirring bars. Therefore, glass-covered ones were used instead.

The Luminol Reaction as a Method of Detection: Discussion

The choice of the chemiluminescent reaction of luminol for the purpose of oxygen detection has turned out to be a very good one. It is specific for oxygen, its stability is good, and the noise level is so low that a change of oxygen concentration of only 0.3% is detectable. One particularly striking feature is its linearity over the wide range of 10^{-14} to 10^{-8} moles per second. The method was originally selected because of its sensitivity. The luminometer

easily detects a partial pressure of 5×10^{-7} atmospheres (equivalent to a concentration in water at room temperature of 6×10^{-10} M) and we have estimated that a partial pressure lower than 10^{-8} atmospheres could be detected, once the carrier gas is purified. The minimum detectable rate of oxygen production, 5×10^{-14} moles per second, can probably be lowered by purifying the carrier gas and using a less noisy photomultiplier. Comparable methods of oxygen detection are the method of phosphorescence quenching developed by Frank and Pringsheim (1943), the Hersch cell, the ZrO_2 voltaic cell, and possibly the mass spectrometer.

The luminometer has a further advantage in that it can be used as a null method. The ambient oxygen concentration can be set at a certain desired level by adjusting the electrolysis rate. Then, when photosynthesis is initiated by turning on the light, the electrolysis rate can be reduced accordingly so that the ambient oxygen concentration remains at the pre-set level. The rate of photosynthesis, in this case, is calculated from the decrease in electrolysis current. The great advantage of the null method is that the oxygen concentration external to the algal cell is held constant during the experiment. The null method would be ideally suited for the study of photosynthetic rate as a function of ambient oxygen concentration.

The method of phosphorescence quenching depends upon the quenching by oxygen of the phosphorescence of a trypaflavin dye adsorbed on silica gel. As used by Pollack *et al.* (1944), oxygen produced by photosynthesis is carried past the phosphor by a flow of purified N_2 at 3 cc per minute. The time necessary for equilibration at a new oxygen concentration is 5 to 15 minutes. The method is sensitive to rates of oxygen production as low as 3×10^{-14} moles per minute. In these ways it is comparable to the luminometer. Its response, however, is neither linear nor logarithmic, and therefore it must always be compared with a calibration curve. Worse, its range is limited to concentrations below 1 part per million, it being more sensitive the lower the oxygen concentration. In addition, it appears to be relatively fussy to use.

The Hersch cell is an oxygen cathode detector adapted for measuring the oxygen concentration of a gas flowing at about 20 cc per minute. As used by Baker *et al.* (1959), its detection limit is less than one ppm, and its response is linear in the range 1 to 100 ppm. Above 100 ppm the cell eventually becomes saturated, with a half saturation point at about 1% oxygen. Daily calibration is needed and for stability the humidity of the gas must be carefully controlled (Phillips *et al.* 1964). Its sensitivity to rate of oxygen production has not been reported, but it can be estimated to be about 10^{-11} moles per second at the

flow rate of 20 cc per minute. Thus with its limited range and lower sensitivity the Hersch cell is not nearly as useful as the luminometer. As yet, it has not been applied to the measurement of photosynthesis.

The mass spectrometer has the great advantage of being able to discriminate between the various isotopes of oxygen. Hoch and Kok (1963) used it very effectively to measure at the same time both the oxygen used in respiration and the oxygen produced during photosynthesis by algal cells. Hoch and Kok were not concerned with its use at low partial pressures of oxygen. The method is capable, however, of detecting 4×10^{-14} atmospheres (Bailey, 1963). The measuring technique used by Hoch and Kok (see Chapter I) is not adaptable to precise quantitative measurement of rate of oxygen production. The mass spectrometer, however, could be used with a flow system such as that used in the luminometer.

The helium ionization detector (Berry, 1960) can detect as low as 2×10^{-8} atmospheres of oxygen, but it is also sensitive to many other gases.

One of the more promising methods of detection of very low partial pressures of oxygen is the solid electrolyte e.m.f. cell:

$$[O_2]_1 \;\Big|\; ZrO_2\ (97.5\%),\ CaO\ (2.5\%) \;\Big|\; [O_2]_2$$

The potential across the cell at 950° C. is

$$E = \frac{RT}{4F} \ln \frac{[O_2]_1}{[O_2]_2}$$

(Weissbart and Ruka, 1961).

Using a flow system which would be practical for measuring oxygen production, Mitchell (1963) has measured oxygen partial pressures of only 10^{-14} atmospheres. The minimum detectable rate of oxygen production can be estimated to be about 10^{-19} moles per second at the 17 cc per minute flow rate used by Mitchell. The stability is 3% over a 16-hour period, and the precision is very good (Weissbart and Ruka, 1961).

Analysis of the Electrolysis Method of Calibration

Observations

The electrolysis method has proven to be a very satisfactory method of calibrating the luminometer. The possible 7% inaccuracy of the method which

has been previously discussed can easily be corrected for. The lower efficiency of electrolysis at currents below 0.1 microamperes was also described. On the whole, however, the electrolysis method is far more convenient and more precise in this type of application than any other method with which we are familiar. We have found some unexpected properties, and a few limitations, of the electrolysis method. These will now be discussed.

The electrodes, immediately after use, were found to have an open-circuit voltage of 1.81 volts. After 20 minutes with the electrodes standing unshorted in the electrolyte, the polarization had decreased to 1.56 volts, and after 4 weeks of disuse the polarization had leveled off at 0.955 volts. If the current was passed in the opposite direction, the polarization had the opposite sign.

The electrodes were usually left unshorted between measurements. During electrolysis, a constant voltage is switched into the circuit of Fig. 6. The voltages necessary to drive some of the lower electrolysis currents were:

2.34 v.	10.0 ua
2.07 v.	1.00 ua
1.97 v.	.30 ua
1.90 v.	.10 ua

Thus one can see that at the lower currents, the current-voltage relationship becomes nonlinear even when the polarization voltage at zero current, 1.81 v., is subtracted. The oxygen produced by a 10 microampere step in electrolysis current was shown in Fig. 10. It was assumed that the oxygen output of the electrolysis is a square step and the exponential rise and fall of the luminometer response is due to equilibration with dead volumes in the gas train.

Although the voltage applied to the electrolysis circuit is constant, the electrolysis current varies with time, as can be seen in Fig. 16. The initial surge due to the capacitance of the electrodes is dampened somewhat by the 1 megohm resistor in the current. Following this there is a 10 microampere current which decays slowly to a fairly stable 9.8 microampere current. This initial higher current cannot be due to the capacitance of the electrodes since the time course of its decay is too slow and it is the same whether or not the 1 megohm resistor is switched into the circuit. This extra current does not appear to produce an initial burst of oxygen. Although it largely would be damped out before reaching the luminometer, the burst would cause the luminometer response to have a non-exponential rise. The extra current probably is used in the

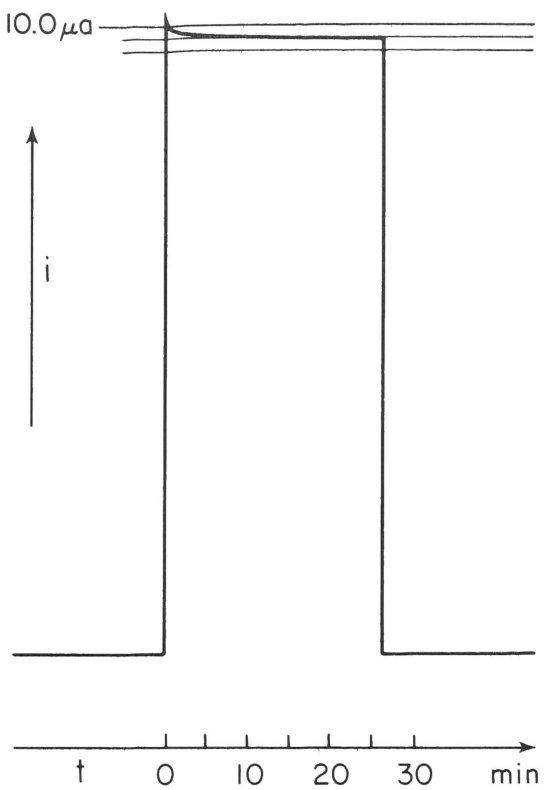


Figure 16. Electrolysis current as a function of time. At t=0 a constant voltage (2.3 volts) was switched across the electrodes. The current leveled off at 9.8 microamperes.

additional polarization of the electrode, as discussed below.

If the electrodes have been left shorted or if the direction of the electrolysis current is switched, this initial charging current is much greater and lasts longer. Furthermore, the rise of the luminometer response is considerably more sluggish and is non-exponential. This suggests that the oxygen is not produced efficiently until the electrode has become sufficiently repolarized.

The effect of platinizing was studied using a different pair of electrodes. Both electrodes were platinized until black by passing a current in alternate directions through an aqueous solution of 3.5% $H_2PtCl_6.6H_2O$ plus 0.1% Pb $(acetate)_2$. The electrodes were washed by passing current in both directions through dilute H_2SO_4 and then through 0.01 N NaOH.

Although the resistance of the electrodes was lower after platinizing, the initial charging current was even greater and the rise of the luminometer response was very sluggish and non-exponential. With the smooth platinum electrodes a decrease in efficiency of oxygen generation was noticed at currents below 1.0 microamperes. With the platinized electrodes this decrease in efficiency was somewhat greater. These results support the above observation that the polarization of the electrodes must reach a certain level before oxygen is produced. Increasing the surface area by platinizing results in an increased amount of charge necessary for polarization.

Several different electrolytes were tried. With perchloric acid the oxygen output behaved about the same as with NaOH, and with H_3PO_4 the output appeared to be slightly more efficient at low currents. In perchloric acid, however, one of the electrodes acquired a black deposit and with H_3PO_4 the solution became discolored with use. Aqueous solutions at 10^{-3} M were used.

Other workers have not had as much success with the electrolysis method. Meyer (1942) used the electrolysis method over the range of 3 to 130 microamperes for calibration of bacterial luminescence. In measuring the volume of gas produced over the electrolysis solution, he observed a sharp fall-off in efficiency below 40 microamperes. Pollack *et al.* (1944) measured the efficiency of oxygen production using the method of phosphorescence quenching. A flow system with the carrier gas bubbling through the electrolysis chamber was used. The electrolyte was dilute H_2SO_4. An 0.1% efficiency at 2 microamperes and a 10% efficiency at 70 microamperes was observed.

The possible electrode reactions

Besides the oxygen-producing anode reaction (1) on page 17, there are other possible anode reactions. Incomplete oxidation of OH^- results in the formation of peroxide and the following anode reactions therefore must be considered.

(5) $2OH^- \rightleftharpoons 2e + HO_2^- + H^+$

(6) $HO_2^- \rightleftharpoons 2e + O_2 + H^+$

(7) $2HO_2^- \rightarrow 2OH^- + O_2$

Since hydrogen produced at the cathode is dissolved in the solution to a certain extent, the anode reaction

(8) $H_2 \rightleftharpoons 2H^+ + 2e$

may have some significance.

There is good evidence that a film of platinum oxide forms on the surface of the anode during electrolysis (Anson and Lingane, 1957; Hoare, 1963; Mayell and Langer, 1964). Hoare (1963) has observed that immediately after anodization in 2 N H_2SO_4 the anode is at 1.225 volts with respect to the hydrogen electrode. This is the same as the standard emf of reaction (1). After remaining unshorted for a day or so, the anode voltage levels off at an equilibrium voltage of 1.060 ± 0.010 volts. When unoxidized platinum sites are thus exposed the reaction

(9) $Pt + H_2O \rightleftharpoons 2e + 2H^+ + Pt - O$

occurs during electrolysis in addition to the oxygen-producing reaction (1). Once the platinum surface is covered by a complete film of oxide, the layer does not thicken with continued anodization.

Mayell and Langer (1964) found by cathodic chronopotentiometric technique that the oxidation sequence in 0.1 N KOH is:

(10) $Pt \underset{.24\ v.}{\rightleftharpoons} PtO \underset{.44\ v.}{\rightleftharpoons} Pt(O)_2 \underset{.94\ v.}{\rightleftharpoons} Pt(O)_3$

They observed that the formation of $Pt(O)_2$ apparently preceeds oxygen evolution. The oxidation sequence of a platinum-black surface follows a similar sequence. However, oxidation of the platinum-black surface prior to complete coverage appears to require a greater percentage of the current. The oxygen overvoltage was found to be 0.41 volts at the smooth platinum surface, and 0.21 volts at the platinum-black surface. Their observations support ours in that the increased surface area of the platinized electrode is detrimental to the efficiency of

oxygen production at lower potentials.

Two possibly significant reactions at the cathode must also be considered. These are the cathode reduction of oxygen and of peroxide (Davies, p. 154, 1962):

(11) $O_2 + 2H_2O + 2e \rightleftharpoons H_2O_2 + 2OH^-$

(12) $H_2O_2 + 2e \rightleftharpoons 2OH^-$

Discussion

The polarization we observed is explained by the formation of the surface oxides. The initial charging current might be due to the oxidation of a few exposed platinum sites. When the electrodes are unshorted a certain amount of surface oxide may decompose. Since the polarization of the electrodes levels off at about 1.0 volt, the oxide probably remains in the higher range of platinum oxide of the reaction sequence (10).

The fall-off of oxygen efficiency observed by us at currents $\lesssim$ 0.1 microamperes and by Meyer (1942) and Pollack et al. (1944) at higher currents can have several explanations. One possibility is that the reactions (11) and (12) might, at low currents, reduce a more significant proportion of the oxygen produced at the anode. In the electrolysis arrangement of Meyer (1942) there apparently was rather poor equilibration between the electrolyte and gas phase. The resulting higher concentrations of H_2 and O_2 in the solution could lead to increased reduction of oxygen (11) and oxidation of hydrogen (8). It is not surprising, therefore, that the fall-off in efficiency occurred at currents as high as 40 microamperes.

In our electrolysis chamber the electrolyte is rapidly equilibrated with the carrier gas. The carrier gas, however, contains about 1 ppm of oxygen, which is equivalent to about 3 microamperes of current. Therefore, at electrolysis currents below 1 microampere, the oxygen concentration is approximately constant at 1 ppm. If the cathode remains more negative than -0.3 volts relative to the hydrogen electrode, the rate of oxygen reduction is proportional to the concentration of oxygen and is constant with respect to voltage. Hence the rate of oxygen reduction at the cathode during electrolysis probably remains approximately constant with decreasing current, whereas the rate of oxygen production decreases. Therefore, as the electrolysis current decreases below 1 microampere, the net number of molecules of oxygen produced per electron, i.e. the efficiency, would decrease significantly.

Another possible explanation for the fall-off in efficiency might be that the anode reaction has a lower efficiency. That is, at lower voltages, anode reactions not yielding oxygen become more and more significant. The reaction (5) producing peroxide is one possibility in NaOH solutions. The presence of peroxide in acid electrolyte was reported by Hoare (1962). The peroxide could in turn be reduced at the cathode in reaction (12). Thus some current might be carried by reactions not producing oxygen.

On the whole, the electrolysis method has, in our hands, proven to be admirably suited for generating oxygen for the purpose of calibration. Its precision is better than 5%, its response time is on the order of a minute, and it is very easy to use. A flick of a switch and a turn of a dial gives any desired oxygen rate or concentration. It can be used at oxygen rates from 10^{-12} moles per second up to at least 10^{-8} moles per second, the highest rate we have tested. In the chapter that follows its applications to the study of some problems in photosynthesis will be described.

CHAPTER 4

APPLICATION TO THE PHOTOSYNTHETIC SYSTEM OF CHLORELLA

Introduction

Chlorella are unicellular green algae. They are weeds among algae, being found growing in pond water and soil practically everywhere. They reproduce by asexual division into 2 to 16 tiny autospores which grow and develop into the adult cell with its single cup-shaped chloroplast. The adult eventually turns into a "mother cell" which releases the autospores, thus completing the life cycle (Kuhl and Lorenzen, 1964). Fig. 18a, a photomicrograph of an exponentially growing culture, shows a typical distribution of the population.

Besides their simplicity and ease of culture, Chlorella have a further advantage for experimental use --- they are heterotropic as well as autotropic (Pringsheim, 1959). That is, they can grow without light by using glucose or acetate as an energy source. This is one reason that Granick (1948) selected Chlorella in his search for mutations affecting the biosynthesis of chlorophyll: mutant cells with a genetically damaged photosynthetic apparatus could be grown on a culture medium supplemented with glucose. The ability of some of these mutants to produce oxygen photosynthetically was tested with the luminometer. These experiments will be discussed in this chapter.

That Chlorella can grow heterotropically is an advantage, in addition, for the study of chloroplast development and greening. Normal Chlorella, unlike most green plants, do not lose their ability to manufacture chlorophyll from protochlorophyllide when grown in the absence of light. Granick (1950), however, described a mutant which loses chlorophyll when grown in the dark, yet manufactures chlorophyll and grows photosynthetically when light is provided, as do the higher plants. This mutant can be grown in the dark indefinitely in a glucose or acetate medium. A similar mutant from Granick's collection was used by us to study the photosynthetic production of oxygen during chloroplast development.

In photosynthesis, the energy of light is converted into chemical energy useful to the cell. A source of electrons is needed for this process. In the higher plants the electrons are obtained from water by oxidizing it to oxygen. The electron is eventually transferred to NADP, going through many intermediate stages of reduction. ATP is formed in the process.

Since we are primarily interested in the little understood reactions resulting in oxygen evolution, the pathway to NADPH does not concern us. As a

matter of convenience we have interrupted that pathway with p-benzoquinone, i.e. we measure the "Hill reaction". Quinone is thought to accept electrons from an intermediate between chlorophyll-a and ferridoxin (Witt et al. 1965). Thus quinone acts as a sink for electrons taken out of H_2O. Quinone was chosen from among the other Hill oxidants because it is capable of passing through the membrane of whole cells. It also inhibits respiration (Clendenning and Ehrmantraut, 1950) thus simplifying the measurement of O_2 evolution.

Photosynthetic Oxygen Production by Chlorella Mutants

It would be of great interest to find a Chlorella mutant which contains little or no chlorophyll, but which would still be capable of making oxygen photosynthetically. With such a mutant, the pigment of the reaction center might be more clearly seen spectrophotometrically and a clue to the chemistry of the photosynthesizing system might be found. Due to its great sensitivity the luminometer is ideally suited for the search for oxygen production among the pigment mutants of Chlorella, which may have their photosynthetic efficiency greatly reduced.

Methods

The Chlorella mutants we investigated were kindly supplied by Professor Granick from the collection maintained in his laboratory. Cells of Chlorella vulgaris, Trelease strain, were treated with X-rays, and mutants were isolated as clones grown on agar containing glucose and inorganic salts. Selection of the mutants was made on the basis of color (Granick, 1948). These mutants have been maintained for 18 years on agar slants containing glucose and inorganic salts. They were kept in the dark at 10° C. and transferred to a fresh agar medium once a year. The cultures were checked for homogeneity at the time of the experiment by examining the colonies which developed after streaking on agar plates. All were homogeneous with respect to color; a few were heterogeneous with respect to colony size.

For the purposes of our studies, duplicate cultures of each mutant were grown in Erlenmeyer flasks, each containing 200 ml of culture medium (see Appendix II). They were shaken in a constant temperature room in the dark for 5 weeks. Standard sterile technique was used to prevent contamination by bacteria and mold. Before the experiment, one flask (L) of each culture was exposed to light for 1 day at room temperature. All cultures were then

kept in the cold room for 2 weeks, the L culture under illumination.

Samples containing 10^7 and 10^9 cells were taken at the end of log phase, centrifuged, and resuspended in 7.5 ml 0.02 M potassium phosphate buffer (pH = 6.3). A count of the number of cells per ml was made of this suspension using a Hausser "Improved Neubauer" hemocytometer. Para-benzoquinone concentration was made to 6.7×10^{-4} M, and the suspension was added to the cuvette. Measurements were made as described in Chapter 2 at I = 20.0×10^3 and 155×10^3 erg cm^{-2} $sec.^{-1}$ using the yellow light created by a chromate filtering solution (cut-off = 480 nm). The pigments were identified by their in vivo absorption, fluorescence emission and fluorescence excitation spectra in cells collected on Millipore filters. Only the cultures exposed to light were thus measured.

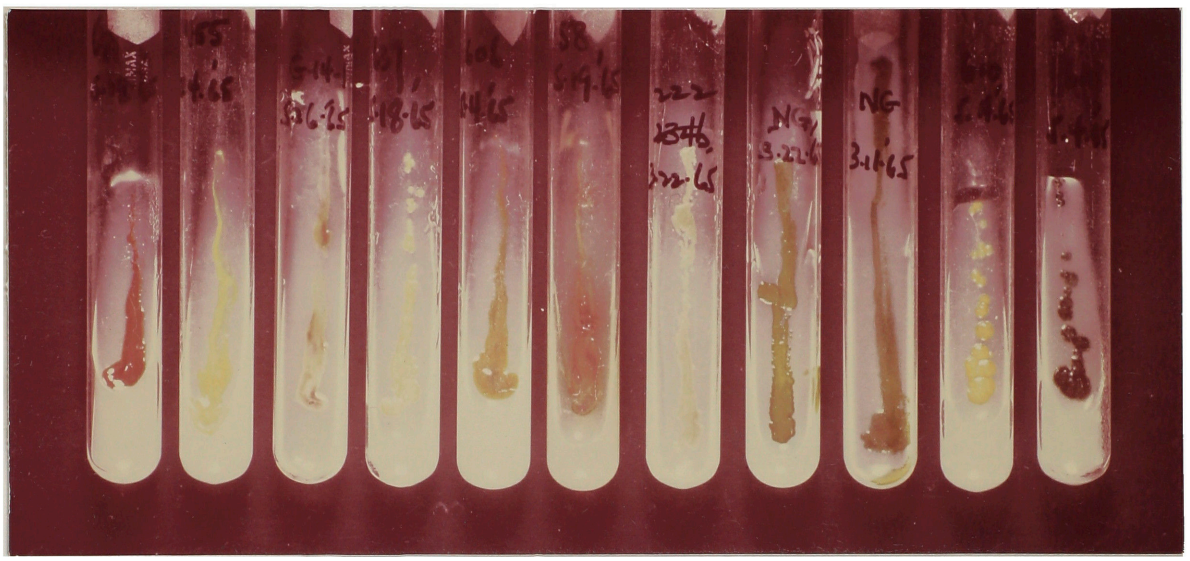
Results

The mutant colonies which were selected for this experiment are shown in Fig. 17. The results are summarized in Table I. One of the mutant cultures, #G-14-1, produced oxygen at about the same rate per cell as the wild-type, although it contained only 1% of the chlorophyll. A later experiment with younger cells gave a much lower rate per cell. Three others could also photosynthesize oxygen, but with efficiency 1% of normal. Three mutants could not produce oxygen at all, and one (# 610) could do so only when exposed to the light. In later experiments, this mutant was found to have normal oxygen rates when grown in the light for several generations. This mutant was also discovered to lose chlorophyll in the dark and grow green in the light, and as a result of this preliminary screening, it was selected for further study.

The mutant cells contained a variety of pigments, including chlorophyll precursors, in abnormally high amounts. Yet those which could photosynthesize oxygen, as would be expected, contained at least small amounts of chlorophyll. The chlorophyll was found to be distributed evenly among the cells by examining them under the light microscope and the fluorescence microscope.

Fluorescence excitation spectra, measured in vivo at selected emission wavelengths, were used to identify protoporphyrin and a metallo-porphyrin. Mutants 58-L, 222-L, and 637-L contained protoporphyrin, and a metallo-porphyrin was seen in mutant 610-L.

The mutant 222-L contains a small amount of chlorophyll and a lot of carotenoid pigment, but does not photosynthesize oxygen. It was found by fluorescence excitation measurements that the excess carotenoids present do not



681-D 455-D G-14-1-D 637-D 606-D 58-D 222-D NG-D NG-L 610-D 610-L

Figure 17. Cultures of Chlorella mutants. The mutants had been cultured in the dark at 10° C. on glucose agar slants for 1 to 2 years.

TYPE	O_2 RATE PER CELL RELATIVE TO NG-L	CHLOROPHYLL PER CELL RELATIVE TO NG-L
NG-L	100	100
G-14-1-L	100	1
681-L	6	1
681-D	9	
455-L	2	1
455-D	0.8	
610-L	0.8	1
610-D	less than 0.02	
606-L	0.3	1
606-D	0.3	
637-L	less than 0.08	0
637-D	less than 0.03	
222-L	less than 0.04	1
222-D	less than 0.04	
58-L	less than 0.04	3
58-D	less than 0.03	

Table I. Screening of Chlorella mutants. The relative rates of photosynthetic oxygen production per cell and the relative amount of chlorophyll per cell are given. The value of 100 is given to the data obtained from the wild-type (NG) Chlorella. Duplicate cultures were grown in the dark. Some cultures were pre-exposed to light (L); others were kept in the dark (D). "Less than 0.02" signifies that no oxygen was detected when the minimum detectable was 0.02 relative units per cell.

transfer their electronic excitation energy to chlorophyll, unlike the photosynthesizing mutants. On the other hand, in the non-photosynthesizing mutant 58-L, transfer of excitation energy does occur.

Conclusions

The luminometer, as used in this experiment, could have detected oxygen rates in mutants as low as 0.02% of the oxygen rate of normal Chlorella. It was learned that several mutants, though they were discolored by abnormal quantities of chlorophyll precursors and had only about 1% of the normal chlorophyll concentrations, could nevertheless make oxygen photosynthetically. The small amount of chlorophyll was distributed more or less evenly among the cells in the mutant cultures, as determined by fluorescence microscopy.

One of the carotenoid containing mutants, # 606, photosynthesized oxygen no better when pre-exposed to light than when kept in the dark. Its photosynthetic apparatus apparently was impaired by a mutation not affecting the protochlorophyll-chlorophyll conversion. Another, #610, could not manufacture chlorophyll when grown in the dark, but readily made chlorophyll and normal oxygen-photosynthesizing apparatus when light was provided.

The measurement of absorption and fluorescence spectra on cells which were collected on millipore filters is a very useful technique for identifying pigments and showing transfer of electronic excitation energy between different pigments. This allows one to draw conclusions as to the close association of these pigments in the photosynthetic unit.

Greening and Degreening of Mutant 610

Introduction

During the development of a plastid to a mature chloroplast, chlorophyll is synthesized, lamellae and grana are manufactured, and the photosynthetic apparatus is put in order (review: Granick, 1963). The process is being actively studied, not only because it is an interesting model of gene-controlled differentiation and development, but also because a few clues to the mystery of photosynthesis may be revealed. In particular, such a study could reveal answers to important questions such as: Is membrane structure necessary for photosynthesis to occur? Do chlorophyll concentration, membrane structure, oxygen production, and CO_2 reduction increase together? Is chlorophyll-b

necessary for any of these to develop?

When dark-grown plants containing no chlorophyll or chloroplast structures are exposed to light, the greening process begins. In the course of greening, three stages are seen. In the first phase, there is a light-induced rapid formation of chlorophyllide and chlorophyll from the accumulated proto-chlorophyllide and proto-chlorophyll (Smith, 1960). Only the pigment molecules which are a part of a special protein-pigment complex, the proto-chlorophyll holochrome (Smith, 1960), can react. This initial rapid phase of chlorophyll synthesis is followed by a period of little or no pigment synthesis, called the lag phase, which usually lasts 2 to 3 hours. With continued illumination, this is followed by a period of rapid chlorophyll synthesis and chloroplast development until maturity is reached. The red-infrared light effect (Hendricks, 1960) probably contributes to the stimulation of chloroplast development (Mago and Jagendorf, 1961).

Etiolated leaves of barley, bean and wheat seedlings are usually used in the study of greening. Recently the more easily controlled unicellular organisms have been used. Euglena, which loses its chlorophyll when grown in the dark, is being used by Schiff and co-workers (Ben-Shaul et al. 1965). Mutants of Chlamydomonas (Hudock et al. 1964 and Ohad et al. 1966) and Chlorella (Bryan and Bogorad, 1963) are also being used.

The Chlorella mutant 610 used in our studies has the advantage over the Chlamydomonas mutants in that it can be maintained indefinitely in the dark on glucose medium, during which time it loses all of its chlorophyll and photosynthetic activity. Furthermore, unlike the Chlamydomonas mutants, it stores a small amount of protochlorophyll, just as in the higher plants. In other words, it appears that the mutation damages only the enzyme which enables normal Chlorella to make chlorophyll in the dark (Granick, 1950).

Fig. 18 compares typical cell populations from mutant and normal cultures in the exponential growth phase. When grown in the light, mutant 610 appears to be nearly identical to the wild type - normal green (NG). When 610 has been grown in the dark for many generations, however, the cells are yellow due to the normal amounts of carotenoids present. That these cells have no chloroplast is seen in Fig. 18d. The cell is larger than normal and is filled with starch granules, identified by staining with iodine.

With the luminometer one has the great advantage of being able to measure oxygen production at an earlier time during chloroplast development

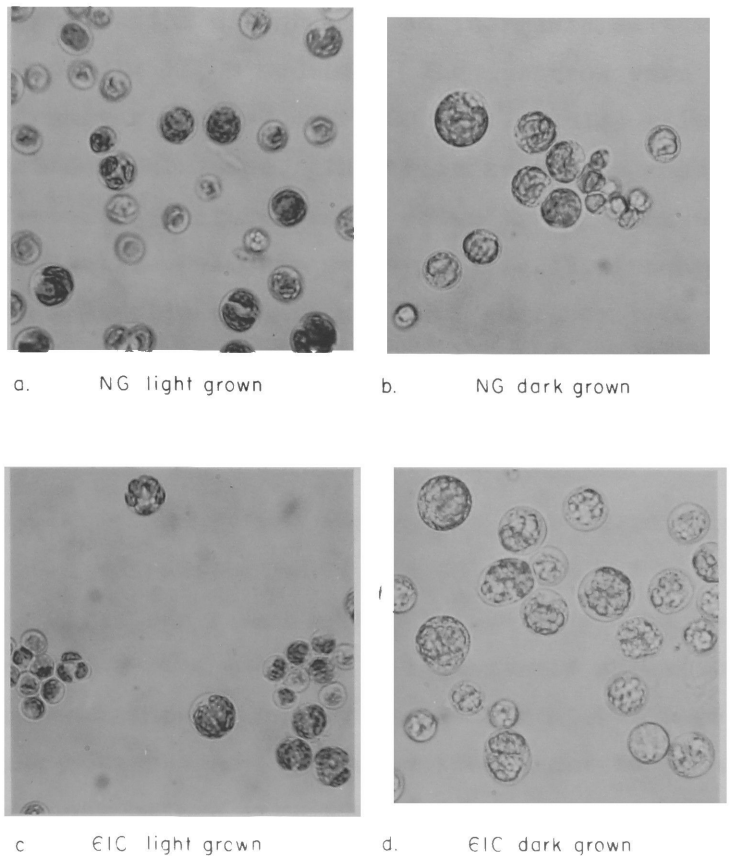


Figure 18. Cells of wild-type (NG) *Chlorella* and mutant 610 growing in log phase culture. The photomicrograph was taken using blue light which is absorbed by the chlorophyll and carotenoids of the chloroplasts. The chloroplasts appear dark in the NG light grown, NG dark grown and 610 light grown cells. The 610 dark grown cells contain starch granules and some carotenoids.

than with less sensitive methods. A further use of the luminometer was the determination of whether or not cell division is necessary for greening and degreening of the Chlorella mutant.

Methods

For experimental purposes the cultures were transplanted from slant cultures to liquid suspension cultures of an inorganic salt solution supplemented with glucose (see Appendix II, B medium). The cultures were shaken in 200 ml of media in 500 ml Erlenmeyer flasks under 800 foot-candles illumination from fluorescent and incandescent lamps. They were kept at a constant temperature of 65^{o} to 70^{o} Fahrenheit. Cultures to be grown in the dark were covered with 2 layers of aluminum foil. Cultures were periodically transferred to fresh medium using standard sterile technique. Four cultures were maintained for many generations under the above conditions: normal green in light and in dark, and mutant 610 in light and in dark. The cultures were checked for bacteria and mold by streaking each culture onto nutrient agar (Difco).

One to two days before the experiment, the cultures were inoculated into fresh medium at a concentration of 1×10^{6} cells per ml. On the day of the experiment, the cells had grown to 2×10^{6} cells per ml, a point which ordinarily would have been the start of a logarithmic growth rate continuing until a concentration of about 100×10^{6} cells per ml had been reached. At time zero the mutant cultures were switched from light to dark and vice versa. Aliquots were taken just before that time, and at various times afterwards, until the cultures reached the stationary growth phase. Measurements of oxygen production activity and chlorophyll concentration were made at each time. From the time the aliquot was taken it was handled in the dark or under a dim green safelight.

For oxygen measurement, 10 to 20 million cells were centrifuged from the culture medium and resuspended in 8 ml of 0.03 M sodium phosphate buffer (pH = 6.3). The suspension was counted on the hemocytometer and p-benzoquinone* was added to a final concentration of 1.25×10^{-3} M before

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* It was found necessary to resublime the p-benzoquinone and, for good reproducibility, to make up a fresh solution in glass distilled water for each day of experimentation. The solution was kept frozen between measurements to minimize decomposition.

putting the suspension into the cuvette. The cuvette was plugged into the luminometer and flushed for 10 to 20 minutes. Measurements were then made, as described in Chapter 2, at two intensities of light: 3.2×10^2 and 2.35×10^4 erg cm^{-2} sec^{-1}. The first intensity was in the "linear" region of the light-saturation curve, and the second was a saturating intensity. Both lights were red. The first included all wavelengths 660 - 670 nm (combining a Corning 2-64 cut-off filter and a Bausch and Lomb 660 nm interference filter), and the second contained all wavelengths $>$ 660 (Corning 2-64 filter alone). These wavelengths are in the region where both system I and system II are excited (Witt et al. 1965). Intensities were adjusted with calibrated neutral density screens.

For the purpose of chlorophyll measurement, 10 ml of the culture were centrifuged and extracted twice with 1 ml of 80% acetone containing 0.84 grams per liter of $NaHCO_3$. If the chlorophyll concentration became too high, a smaller volume of culture was used. The extracts were kept in the freezer until absorbance measurements could be made. The absorption spectra were measured on a Cary Model 14 spectrophotometer.

Results

The experiment turned out to have unusual interest because, after the cultures were switched from light to dark and vice versa, the cells stopped multiplying for a period. This afforded the opportunity of measuring the changes in chlorophyll concentration and photosynthetic oxygen activity while the cells were not dividing, and comparing these changes with those which occurred when the cells eventually started to multiply again. In Fig. 19 it is seen that the light-grown cells stopped dividing for eleven days after being put in the dark. A culture grown under identical conditions in another flask exhibited the same behavior. In Fig. 21 it is observed that there was a similar dormant period of 4 days when the dark-grown cells were placed in the light. Following these dormant periods there occurred periods of exponential growth with doubling times of 0.9 days in the light and 1.5 days in the dark.

The changes in the light-limited rate of oxygen production were practically identical with that of the maximum light-saturated rate, and are not plotted in Figs. 19 - 22. The Northeast power failure of November 9, 1965 occurred during the experiment, as indicated on the figures. There appears to be only transient effects, however, although the lights were out and the shaker was off for 12 hours.

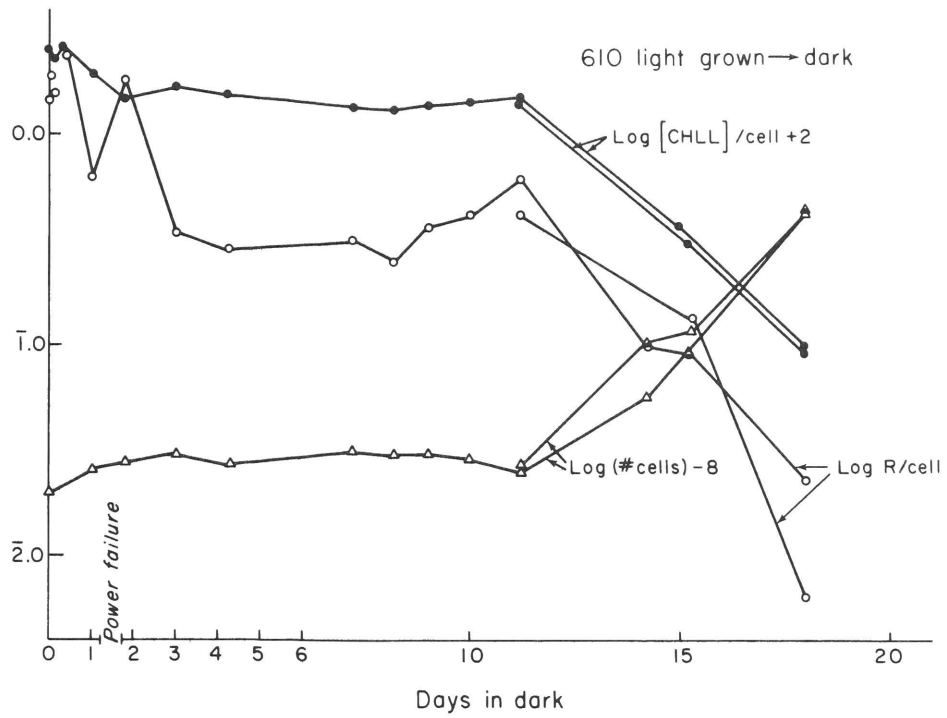

Figure 19. Degreening of mutant 610. Two cultures growing exponentially in the light were covered at time zero. Log number of cells, log oxygen rate (R) per cell, and log chlorophyll concentration per cell are plotted against number of days in the dark. The units of oxygen rate (R) are equivalent electrolysis current (microamperes), and the units of chlorophyll concentration are absorbance units.

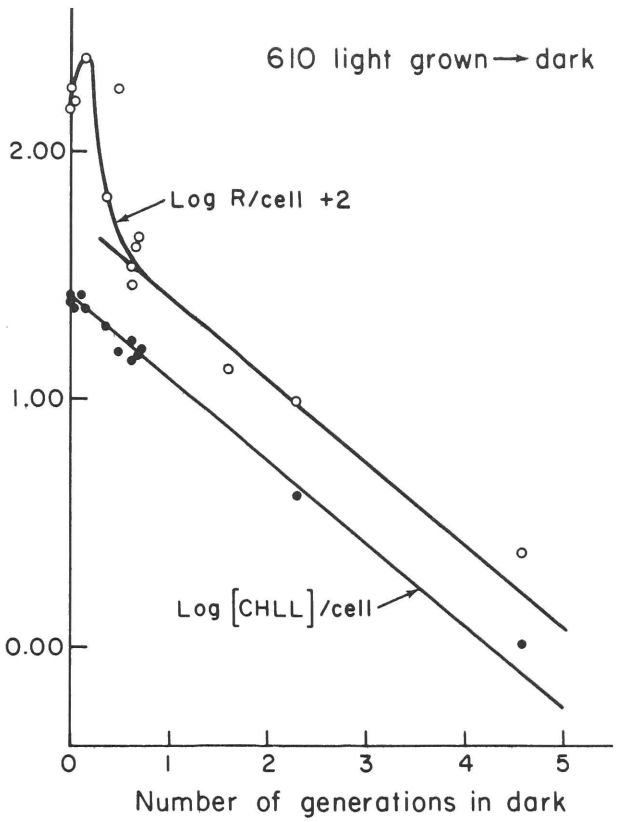

Figure 20. Degreening of mutant 610. The data of Fig. 19 are plotted against number of generations in the dark. The number of generations is defined as the number of doublings in cell concentration. The straight lines are drawn with a slope = log 1/2, i.e., the slope expected if the measurement decreases by a factor of 2 with each generation.

The changes that occurred during degreening are more clear-cut, and will be described first. The amount of chlorophyll per cell decreased gradually during the first 2 days to 60% of the original amount, and then remained constant for the remaining 9 days of the dormant period, as seen in Fig. 19. The maximum rate of oxygen production per cell is seen also to decrease gradually to a steady level during the first few days of the 11-day dormant period (this time at 21% of the original level). This did not occur until after a 60% increase in rate during the first 10 hours. In addition there was a transient burst immediately following the power failure.

During the period of exponential growth, the chlorophyll content per cell decreased exponentially, as did the rate of oxygen production. This exponential decrease with respect to growth is better shown in Fig. 20 where the same data are plotted against number of generations. One generation is defined as the amount of time required for the number of cells in the culture to double. On this type of plot the entire dormant phase is compressed into the first generation. In Fig. 20 the straight lines are drawn with slope = $\log_{10}\frac{1}{2}$. Points falling along these lines decrease in amount by $\frac{1}{2}$ with each generation. Thus it is made obvious that the chlorophyll concentration per cell decreases by one-half with each generation. The oxygen rate per cell, on the other hand, may decrease at a slightly lower rate: i.e. a factor of about 0.57 per generation after the first one and one-half generations. The significance of the trend indicated by the last three points, however, is in doubt. Other such experiments are planned. Note the initial increase, then decrease, in oxygen rate per cell during the first generation (dormant phase). More will be said about this in the discussion.

The greening process, on the other hand, was found to be independent of cell division. The changes which occurred during the greening of the dark-grown mutant are graphed in Fig. 21 and more clearly in Fig. 22. In one day the chlorophyll concentration increased to within 30% of the dormant phase level. It increased by only a factor of 2 during the exponential growth phase, during which time the number of cells multiplied by a factor of 16.

As seen in Fig. 22, the rate of oxygen production increased during the first generation to a level which probably was maintained throughout the first 3 generations. During the fourth and fifth generations, there occurred a five-fold rise to a maximum before a rapid fall. These changes will be explained in the discussion section.

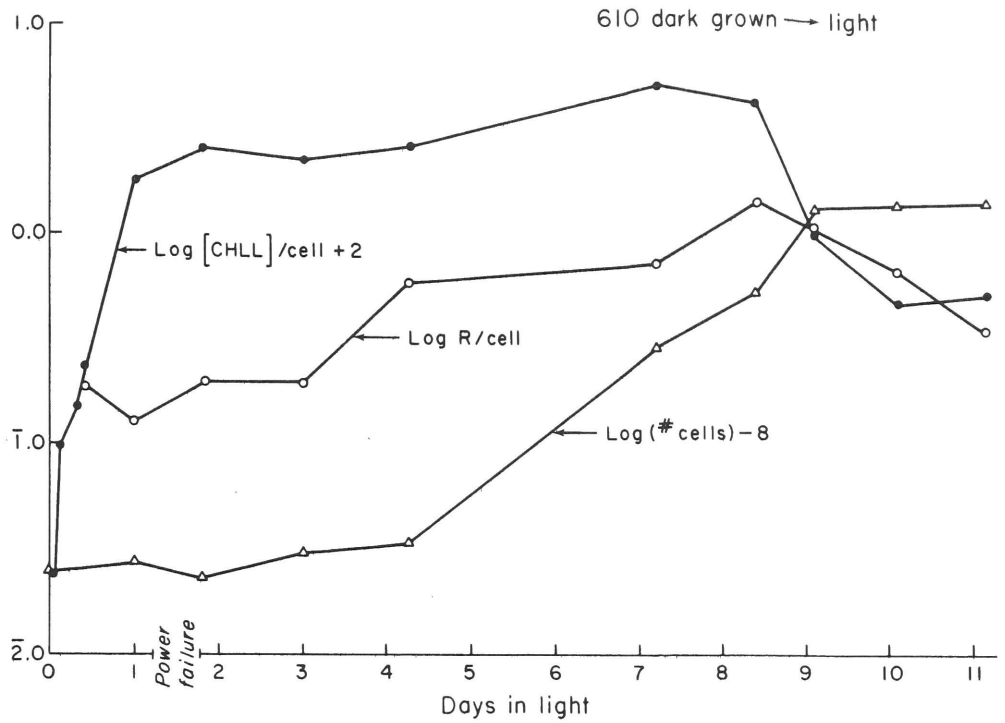

Figure 21. Greening of mutant 610. The culture, growing exponentially in the dark, was uncovered at time zero. See Figure 19 for details.

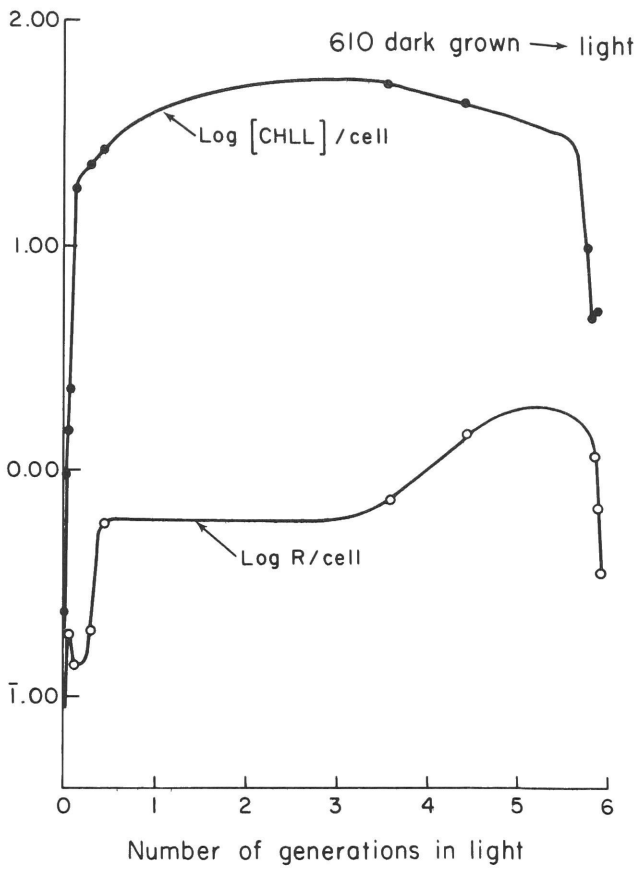

Figure 22. Greening of mutant 610. The data of Figure 21 are plotted against number of generations. The number of generations is defined as the number of doublings of cell concentration in the culture since time zero.

The biochemical changes which occur in the first 5 hours of greening were studied in another experiment and are shown in Figs. 23 and 24. The total protochlorophyll content decreases more or less exponentially as the chlorophyll increases. As shown by Koski (1950), the initial rapid phase of chlorophyll synthesis is over in the first few minutes. This was not seen in our measurements, since the first point was taken at 15 minutes. The lag in chlorophyll synthesis which has been observed in many different plants (Koski, 1950; Virgin, 1958; Ohad et al. 1966) occurs after about one hour in our system.

Discussion

This experiment shows clearly that the decrease of chlorophyll per cell, and of photosynthetic oxygen production during degreening is strongly dependent on cell division. Chlorophyll content remains constant during the dormant phase (Fig. 19) and decreases at the same rate as cell division (Fig. 20). Since no chlorophyll synthesis can occur in the dark, the chlorophyll is simply diluted out by cell division. The decrease in rate of oxygen production, however, may be slightly less than the rate of cell division. This indicates that there may be a small amount of new synthesis of chloroplast structure, particularly at sites of oxygen production, during degreening - not a simple dilution of chloroplast material by division.

This possibility is reinforced by the experiments of Ben-Shaul et al. (1965). They observed that the number of lamellae per plastid and total disks per plastid of Euglena gracilis decreased at a rate of 0.3 per generation during degreening. The volume of the plastids did not change significantly.

The initial increase, then decrease, of oxygen production during degreening probably was a transient effect of the sudden decrease in illumination. A similar effect was noticed during the greening experiment. Starting with the fourth generation (see Fig. 22), when mutual shading* would begin to be significant, (25×10^6 cells per cc), there was similarly an increase, then decrease, in the rate of oxygen production. The amount of chlorophyll per cell did not increase in either case. The chlorophyll could not, of course, have increased during degreening without light. It appears that this increase,

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* In thick cultures, the average amount of light reaching each cell is decreased significantly because of the absorption by other cells.

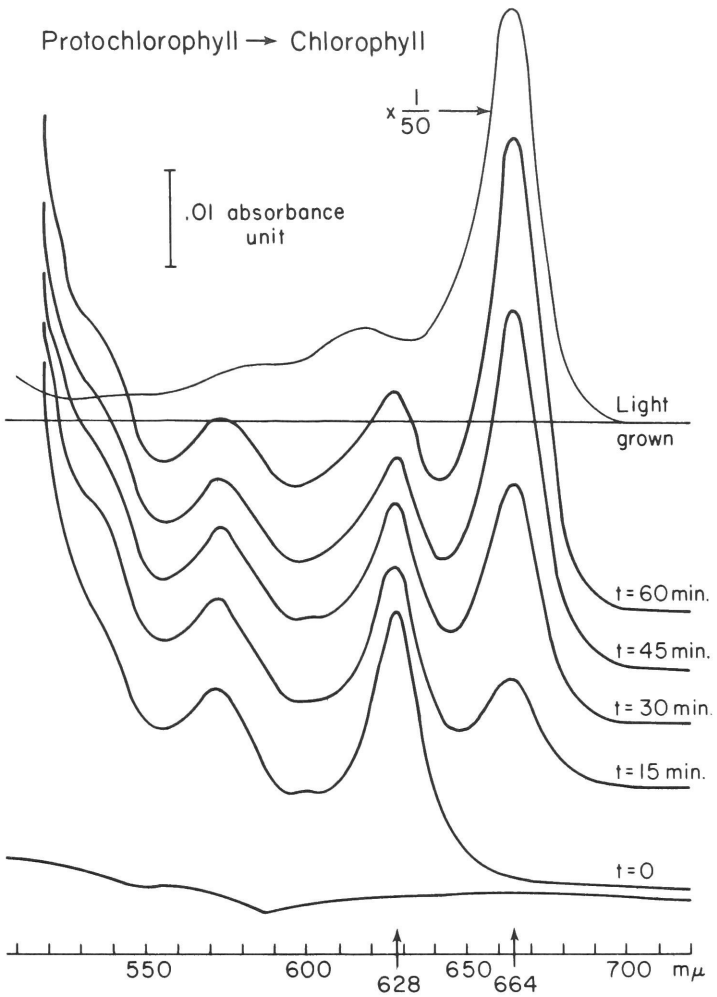

Figure 23. Conversion of protochlorophyll (λ =628) to chlorophyll (λ =664). At t = 0 an exponentially growing culture of mutant 610, grown for > 25 generations in the dark was exposed to continuous illumination. Aliquots of 32 x 10^6 cells were taken at various times of exposure, and were extracted with 5 ml 80% acetone. The top spectrum is of a similar extract of a light grown culture of mutant 610. The scale for this top spectrum is 0.5 absorbance unit.

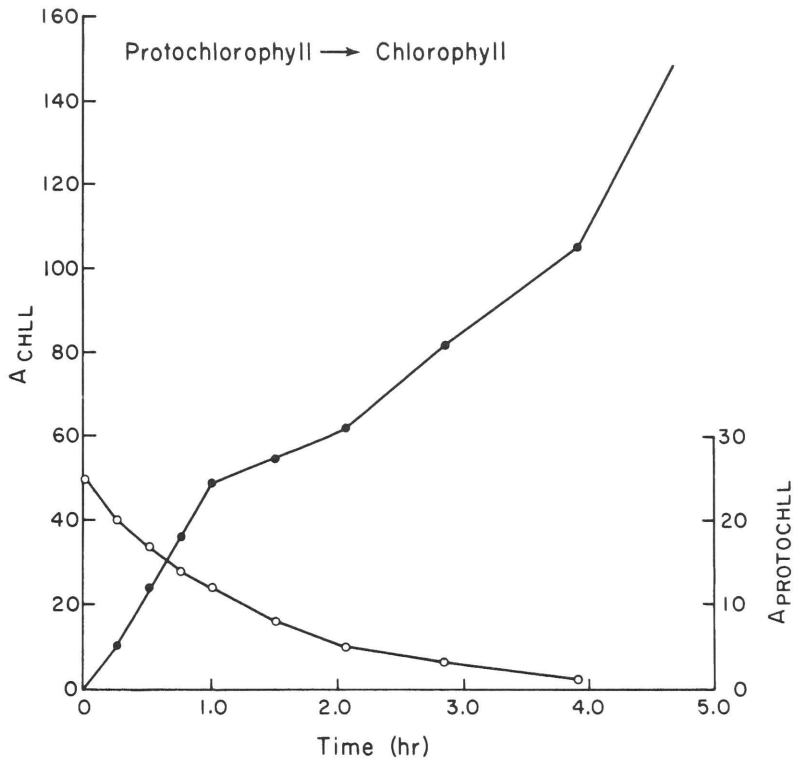

Figure 24. Conversion of protochlorophyll to chlorophyll. The measurements were made on the spectra of extracts as described in Figure 23. The protochlorophyll absorption ○, and the chlorophyll absorption ●, were plotted as a function of time of exposure to light. The contribution of chlorophyll was subtracted from the λ = 628 nm peak to give the absorbance of protochlorophyll. Vice versa, the contribution of protochlorophyll was subtracted from the λ = 664 nm to give the absorbance of chlorophyll.

then decrease, in oxygen rate is a self-regulatory response to the reduction of light intensity.

Ben-Shaul et al. (1965) noticed a related phenomenon. At the beginning of the degreening period, the plastid material continued to be manufactured during the first 1 to 2 generations, but at a diminished rate. Furthermore, in non-dividing cells, the number of disks per plastid and lamellae per plastid increased when kept in the dark. One can interpret our data and these data of Ben-Shaul as indicating that a physiological response to decreased illumination is an increase in oxygen production per cell related to additional synthesis of lamellae and grana, but not of chlorophyll.

It is clear from this experiment that the greening process, on the other hand, is largely independent of cell division. Most of the increase in chlorophyll per cell and in oxygen production per cell occurs during the dormant phase. The slight increases that occur near the end of log phase can be attributed to the mutual shading as described above.

Oxygen production was not detected until 10 hours after the start of the greening experiment; at 7-3/4 hours there was still no measurable response. It is possible that by measuring several times as many cells, and by increasing the sensitivity of the luminometer, we might be able to detect something at an earlier time. The production of oxygen has been detected after only 30 minutes of greening in etiolated barley leaves (Smith, 1954). It is probable that the explanation for the slowness of our greening system is inhibition by glucose. This "inhibition" of greening has been observed in bleached Euglena by Gibor (1966). In a preliminary experiment, oxygen production from cells greening in acetate medium appeared in 9 hours. We have planned an experiment in which we intend to transfer the dark grown cells to non-supplemented inorganic medium just before exposing them to light. It is expected that with the chemical energy sources unavailable the cells will develop their photosynthesizing capacity more rapidly.

That the cells stopped growing when the light conditions were switched is very interesting. It appears that the cells stop multiplying when the environment suddenly becomes unsuitable for growth. When they have had enough time to adapt to their new energy supply, they resume maximal growth rate.

Several experiments are suggested by these results. The effect of mutual shading could be avoided by using continuous culture technique. That is, diluting the culture daily with fresh, sterile medium to keep the cell concentration roughly constant with time. Ultimately the Chlorella cultures could be

treated so that all the cells divide synchronously as has been done by several workers (Kuhl and Lorenzen, 1964). With this system one could study the effect of division and phase of life cycle on the rate of photosynthetic oxygen production. By suspending the cells in non-supplemented inorganic media one could not only eliminate the possible inhibitory effect of glucose, as mentioned above, but also one would prevent the effect of cell division on greening and degreening, providing the CO_2 concentration is limiting. Finally it would be extremely interesting to compare our results with changes in ultrastructure of the cells visualized by electron microscopy.

Conclusion

A number of important conclusions can be drawn from these experiments. The Chlorella mutant 610, when grown in the dark, gradually loses all of its chlorophyll and its ability to produce oxygen photosynthetically. Our experiments demonstrate conclusively that these changes are dependent on cell division. In the reverse process of greening, the chlorophyll synthesis and the appearance of photosynthetic activity were shown to be independent of cell division. In the first several hours of exposure, chlorophyll is synthesized from protochlorophyll. After an initial 10 hour period when no oxygen was detected, oxygen production and chlorophyll production increase in parallel. Possibly, the development of some chloroplast structure as well as chlorophyll synthesis must occur before the Chlorella are capable of photosynthesizing oxygen.

An interesting observation is that the cells stopped multiplying when the light/dark conditions were switched. Apparently 6 to 11 days are needed under our conditions for the cells to readjust to a new source of energy. It would be worthwhile to look for changes in the structure of the mitochondria as well as of the chloroplasts with the electron microscope.

Light Saturation Curve

Since it is possible to measure the rate of oxygen production over a very large range with the luminometer, it was of interest to determine the saturation curve for light intensity. The data obtained with normal green Chlorella grown autotropically are shown in Fig. 25. We were able to measure the rate of oxygen production at light intensities at least three orders of magnitude below the half-saturation intensity. At these low light intensities the relation between response and intensity was found to have a slope slightly less than one. With the more usual methods of measuring oxygen production, the linear region is barely accessible.

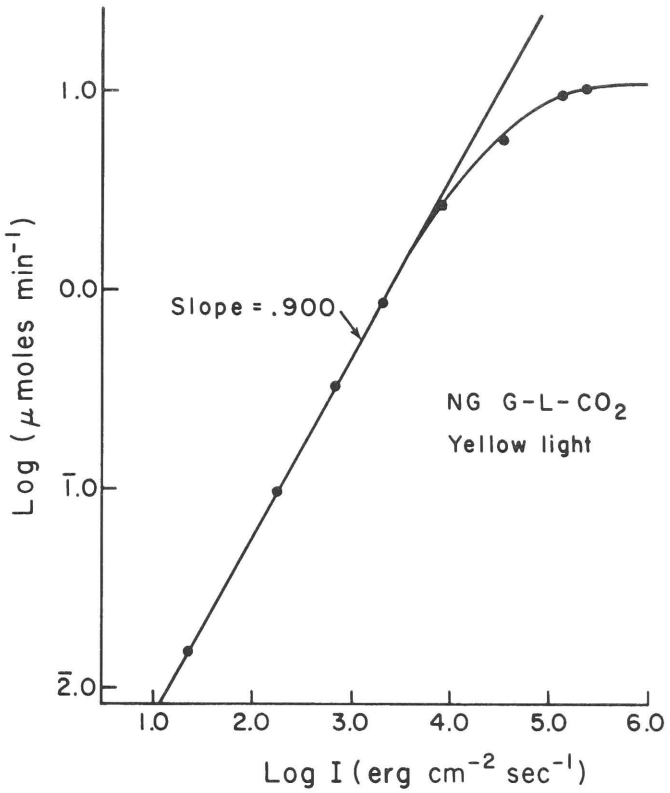

Figure 25. Light saturation curve. The log rate of oxygen production as a function of log intensity of yellow light. Wild-type Chlorella were grown autotropically in inorganic media bubbled with 5% CO_2. Measurements were made on 16.7 x 10^6 cells harvested during log phase and suspended in 8 ml 0.03 M sodium phosphate buffer, pH = 6.3, containing 1.25 x 10^{-3} M p-benzoquinone.

CHAPTER 5

GENERAL CONCLUSIONS

We have demonstrated the usefulness of the luminometer for the study of the photosynthetic production of oxygen. It is especially suited for experiments in which high sensitivity is required at low partial pressures of oxygen, although it can be used at higher levels as well.

With only a slight change in the procedure, the luminometer could also be used to study the respiration of organisms or the kinetics of cytochrome oxidase at very low oxygen tensions. The oxygen removed at a certain rate from the carrier gas could be measured as the difference between the response to the carrier gas flowing through the cuvette and that flowing through a by-pass.

There are other possible ways of using the luminol reaction to measure photosynthesis. The speed of response could be greatly increased by minimizing the dead volume and increasing the flow rate. Also, the scale could be greatly decreased. It is conceivable that the oxygen production by individual cells could be visualized in the microscope by the luminol reaction. The cells could be separated from the alkaline luminol solution by a thin teflon membrane.

The luminometer, in its present state of development, is well designed for the study of photosynthetic oxygen production as a function of ambient oxygen concentration. By using a null method, at a constant partial pressure of oxygen, one can avoid the errors due to the additional oxygen produced by photosynthesis. It might be possible to separate oxygen changes due to respiration from those due to photosynthesis by suitable analysis of the response at very low, as well as at higher, oxygen concentrations.

The mutant 610 of Chlorella was found to have greening properties similar to those of higher plants. Because of its ease of culture under reproducible conditions, it should be a good organism for the study of the biochemistry and physiology of greening. In particular, it could be a good source of the protochlorophyll holochrome. An experiment is planned to determine by the in vivo absorption spectrum how much of the extracted protochlorophyll was located in the holochrome.

The search for mutants of algae having the ability to produce at least a low rate of oxygen might be a fruitful one. Such a mutant with low concentrations of carotenoids and chlorophyll could be studied spectrophotometrically much more easily than the wild type. Also, a mutation

affecting one part of the photosynthetic pathway but not that pertaining directly to oxygen production would be of interest.

The aim of this thesis was the investigation of new approaches to an old problem. It is hoped that by the further use of the luminometer, the mutants, and the methods explored in this thesis we will come to a better understanding of the subtle reactions involved in the photosynthetic production of oxygen.

APPENDIX I

Detection Method	Useful Range (atm.)	$f(O_2)$	Specific[1] for O_2	Min. Detectable ΔPO_2 (ppm)	Min. Detectable Rate (moles/sec)	Response Time	Precision	References
Gasometric								
Warburg	any	linear	no		10^{-10}	~3 min.		Glick '61
Diver methods	any	linear	no		10^{-17}	~5 min.	<10%	Løvlie '65
Physical methods								
Mass spectrometer		linear	yes: isotopes of O_2	400	3×10^{-11}	10 sec.		Hoch '63
Phosphorescence quenching	10^{-8} to 10^{-6}	non-linear saturates at low O_2.	yes[2]	.01	3×10^{-14}	1 min.		Pollack '44
Helium ionization detector	10^{-8} to ?		no					Berry '60
O_2 paramagnetism	10^{-3} to 0.8	non-linear	yes	1000	10^{-8}	5 sec.	1%	Pauling '46
Electrochemical								
O_2 cathode, vibrating bare electrode	10^{-4} to 1.0	almost linear	yes		10^{-10}(?)	5 sec.	5%	Chance '55 Carey '65
O_2 cathode, Clark electrode with flow system								Vidaver '63
O_2 cathode, Hersch cell	10^{-6} to 10^{-2}	linear 10^{-6} to 10^{-4} atm, non-linear higher.	yes[2]					Baker '59
ZrO_2 voltaic cell	10^{-14} to 1	logarithmic	no	10^{-8}	10^{-19}		5%	Mitchell '63

APPENDIX I
(continued)

Detection Method	Useful Range (atm.)	$f(O_2)$	Specific[1] for O_2	Min. Detectable PO_2 (ppm)	Min. Detectable Rate (moles/sec)	Response Time	Precision	References
Chemical								
Hemoglobin-oxyhemoglobin	10^{-3} to .04	saturates, linear in narrow range.	yes		7×10^{-11}	5 to 8 sec.		Whittingham '54
LUMINOMETER	10^{-8} to 1	linear	yes[2]	.01 at 3	5×10^{-14}	3 min.	5%	This thesis
Biological								
Luminous bacteria	10^{-6} to 10^{-4}	saturates, linear in narrow range.	yes		10^{-11}			Schindler '64

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1 The method was considered specific for O_2 if O_2 is distinguishable from CO_2 or H_2O vapor during a photosynthetic experiment.

2 CO_2 and/or H_2O vapor must be removed from the gas phase as they affect the sensitivity of the method.

Appendix II: Growth Media

	Granick Medium (per liter)	Burr Medium (per liter)
$CaCl_2$		80 mg
$Ca(NO_3)_2 \cdot 4H_2O$	1.5 g	
KNO_3	1.5 g	1.5 g
$Na_2HPO_4 \cdot 2H_2O$	1.5×10^{-2} mole	0.75×10^{-2} mole
$NaH_2PO_4 \cdot H_2O$	1.5×10^{-2} mole	2.25×10^{-2} mole
$MgSO_4 \cdot 7H_2O$	.24 g	.24 g
$FeCl_3$	40 mg	
$FeSO_4 \cdot 7H_2O$ } as complex		7.0 mg
EDTA } as complex		12.0 mg
Glucose	7.5 g	7.5 g
$ZnSO_4 \cdot 7H_2O$	0.1 mg	0.1 mg
H_3BO_3	1.0 mg	1.0 mg
$MnSO_4 \cdot 4H_2O$	1.0 mg	1.0 mg
$CuSO_4 \cdot 5H_2O$	0.1 mg	0.1 mg
pH =	6.8-7.0	6.4-6.5

The Granick Medium was used in screening the mutants. The Burr Medium, which had little or no precipitate, was used in all other experiments. In this medium, the phosphate and the glucose were autoclaved separately from the rest of the media.

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