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THE OXYGEN LUMINOMETER. AN APPARATUS TO DETERMINE SMALL AMOUNTS OF OXYGEN, AND APPLICATION TO PHOTOSYNTHESIS*

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SUMMARY

1. The oxygen luminometer is a sensitive device for the continuous measurement of small amounts of oxygen. An inert carrier gas flows at a controlled rate through an electrolysis cell, then through a sample cuvette, and finally into a solution of luminol dianion in dimethyl sulfoxide. The light generated by the chemiluminescent reaction of the luminol dianion with the oxygen is measured with a photomultiplier. Calibration, either internally with the water electrolysis cell or externally with premixed gases, shows the response of the system to be linear over a range of greater than four orders of magnitude in oxygen concentration. The sensitivity corresponds to a rate of oxygen production of 10^{-13} mole/sec or to a change in oxygen concentration of 0.01 ppm in the gas or of 10^{-11} M in water at room temperature. The device has a response time of about 1 min and the output is a direct measure of the rate of oxygen production in absolute units.

2. The oxygen luminometer is suitable for measuring oxygen production in photosynthetic systems. A mutant of Chlorella which requires light for chlorophyll synthesis is shown to lose its chlorophyll and its oxygen-producing activity in the dark by simple dilution on cell division.

INTRODUCTION

Our knowledge of photosynthesis has grown along with the development of methods to determine oxygen. Beginning with the experiments of PRIESTLEY on 'dephlogisticated air' using his pneumatic trough, and continuing with the elegant experiments of ENGLEMANN with motile bacteria and of BEIJERINCK with luminescent bacteria, this work culminated in the quantitative manometric methods of WARBURG***. Most of our present knowledge of photosynthesis was achieved by the use of this gasometric method, supplemented recently by the more convenient oxygen polarograph. Having in mind experiments aimed at understanding the photochemical formation of oxygen, we have developed a new method to determine oxygen which is based on

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*** For an excellent discussion of this historical work see ref. 30.

the chemiluminescent reaction of oxygen and luminol dianion. It is very sensitive and is useful over a wide range of oxygen concentrations.

In a general analytical method, one can measure either the accumulated change with time of the variable, or the instantaneous rate of change at a given time. The manometric methods are restricted to the first, or integrative mode, as are other oxygen detectors when used in a closed system. As in most integrative procedures, small fluctuations tend to be averaged out, and sensitivity increases with integration time. However, to obtain the rate of a reaction, often the more significant variable, one must measure the slopes of such integrated responses from a graph; a process both time consuming and 'noisy'. In the second method, the instantaneous rate of oxygen evolution may be measured directly by allowing the oxygen to diffuse from the source to a sink. The sink may be the detector itself, or a carrier gas stream which the detector samples. We chose the rate mode because a constant quantum flux on a photosynthetic system produces, in the steady state, a constant rate of oxygen production, and thus a constant output is desirable. The carrier gas mode was chosen because of the possibility of an exact and absolute calibration method using the electrolysis of water as an alternative oxygen source. As a bonus, the method may be used in the null mode, *i.e.* to measure the rate of oxygen evolution at a pre-fixed and constant level of oxygen. Furthermore, by temporarily isolating the oxygen-generating source from the carrier gas, the method can be used in the integrative mode to increase sensitivity at the expense of rate data.

EXPERIMENTAL PROCEDURES

Description of the oxygen luminometer

Oxygen reacts specifically in a chemiluminescent reaction with luminol (5-amino-phthalhydrazide) in alkaline dimethylsulfoxide, a reaction first described by WHITE[3,4]. He and his co-workers have given evidence[5,6] that the reaction occurs between oxygen and the dianion of luminol to give mainly nitrogen and 3-aminophthalate ion in an excited state. Under optimum conditions, the reaction is limited by diffusion of oxygen into the alkaline solution. To obtain this high rate of reaction the dianon must be in high concentration. To maintain the necessary alkalinity all reagents must be dry. The dimethylsulfoxide and *tert.*-butyl alcohol were distilled from Linde molecular sieves with precautions to exclude atmospheric water vapor, and were stored over Linde molecular sieves type 3A-2. Potassium *tert.*-butoxide, sublimed, was obtained from Mine Safety Appliance, Research Corp., Callery, Pa. Luminol was recrystallized from hot 48 % HBr, and the salt neutralized with dilute KOH to pH 6 as a suspension in water, washed thoroughly and dried in vacuum over P_2O_5. Best results were obtained when the potassium *tert.*-butoxide and the solvent were mixed in a dry, inert atmosphere. Equal volumes (2 ml) of two solutions, 0.2 M luminol in dimethylsulfoxide and 0.5 M potassium *tert.*-butoxide in *tert.*-butyl alcohol–dimethylsulfoxide (1:10, v/v) were deaerated with dry argon, mixed in a syringe, and injected into the luminometer vessel.

It was usually necessary to preoxidize the freshly prepared luminol solution to obtain an accurately linear response of light emission to oxygen concentration in the gas. The oxygen generated by 10 mA of current through the electrolysis calibration cell (see below) for about 2 h was sufficient. No further preoxidation was

required during the life of the luminol solution. During the preoxydation the sensitivity of the solution to oxygen decreased by a factor of 2. With less pure luminol, more preoxidation was required and the sensitivity first increased from a low level, before slowly decreasing. Achieving exact linearity although desirable, is not crucial to the method, since an internal calibration is built in (see below). The preoxidized luminol solution remains sensitive for a month or more, depending on use.

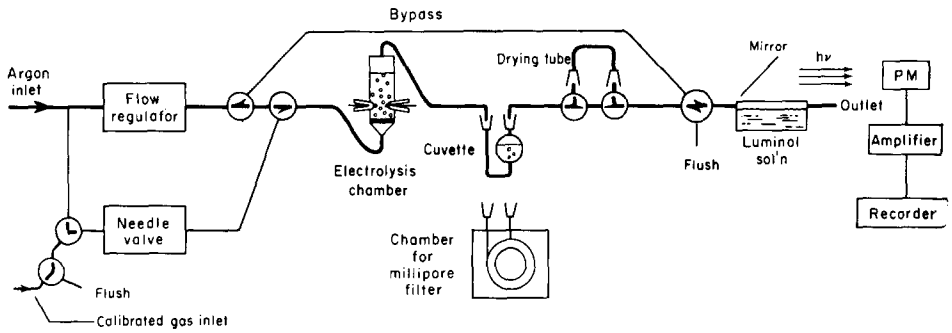

Fig. 1. Flow diagram of the oxygen luminometer.

A carrier gas is used to transport the oxygen produced by photosynthesis or electrolysis to the luminol solution. The gas train is shown in Fig. 1. By turning stopcocks, the regulated flow of ultra high purity argon (Matheson) can be routed either through the by-pass or through the service branch. When the latter is in use, the carrier gas bubbles first through the electrolysis chamber, next through the cuvette containing the algal suspension, and then through a drying tube before entering the luminol vessel. The design of the gas train is such as to minimize volume, particularly between the reaction cuvette and the luminol chamber. Here the stopcocks, tubing and joints have 1-mm bores. Considerable care had to be taken in constructing the gas train to prevent diffusion of oxygen from the air. A leakage rate of 1 ml of oxygen per 25 years is detectable. Swagelock tube fittings are used to connect 1/8 inch outside diameter copper tubing to the pressure regulator, flow regulator and needle valve. The gas train is exclusively glass and metal. Apiezon type W wax is used for glass to metal seals and for semi permanent joints. For flexibility, joints on either side of the reaction cuvette are capillary ball-and-socket joints using Apiezon N grease or 'O' ring capillary joints. Pressure stopcocks with adjustable spring force are used upstream from the electrolysis cell. A Matheson Model 19 metal diaphragm pressure regulator maintained the pressure between the gas cylinder and the flow regulator at 20 lb/inch^2. The flow regulator is a Brooks Instrument Model 8943ELF low flow controller. In operation at about 10 ml/min, it governs the flow rate to much better than 2 %. The needle valve is their Model 8501-1311 low flow needle valve.

The electrolysis chamber (3 ml) has a fritted glass disk sealed to 1 cm tubing. The No.-2-gauge platinum–iridium (85:15) wire was hammered paper-thin before sealing into pyrex tubing. The exposed tips of the electrodes were melted into polished balls about 0.5 mm in diameter before sealing the tubing into the chamber. Several types of cuvettes were constructed for a variety of experiments, including small-volume chambers and a plexiglass holder for a Millipore filter containing deposited algal cells. The cuvette used in the experiments described in this paper was a quartz

cylinder of 2 cm-light path and a volume of 15 ml with a fritted quartz disk in the inlet arm. The quartz pieces including O ring joints were obtained from Thermal American fused quartz.

To achieve maximum rate of equilibration of the solution containing the algae, and to approach complete equilibration of the oxygen in solution with that in the carrier gas, vigorous stirring and a large gas-to-liquid surface area is required. The fine bubbles produced by the sintered glass disk give the required area, and stir the cuvette contents in a vertical plane. A glass-enclosed magnetic stirrer (teflon coatings leak oxygen) produced rapid circulation in the horizontal plane, ensuring the absence of stagnant areas in the cell.

The sensitivity and rate of reaction with oxygen of the luminol solution decreases on exposure to water or carbon dioxide. The drying tube, 7 cm $\times$ 0.5 cm of Anhydrone and Ascarite remove these constituents from the gas stream. It lasts about 20 h at the flow rate usually used, 10 ml/min.

Two different types of vessels were used to bring the gas into contact with the luminol solution. The first type was in the form of a gas scrubbing column with minimal dead volume below the sintered-glass disk. The higher pressure required to force the gas through the disk, occasional clogging of the disk, and light output fluctuations caused by the rising bubbles were eliminated in the second design. A 4-cm length of 1-cm square glass tubing was mounted horizontally and nearly filled with the luminol solution. The gas, entering and leaving at opposite ends, flowed in a thin (1-mm) space over the surface of the luminol solution. The bottom and two sides of the vessel were silvered to enhance light collection and a mirror reflected the light output onto the photocathode of the photomultiplier (RCA 1P21). The luminol chamber and photomultiplier were enclosed in a lightproof box.

A Northeast Scientific regulated high-voltage supply provided 780 V (maximum signal to noise ratio) to the photomultiplier. The signal from the anode is amplified by a Keithley 150 A voltmeter. The 10-V output is divided and filtered before being displayed on a Varian G-10 recorder. All electronic apparatus and the light source were powered from a Perkin Electronic AC line regulator, MLTR 1000.

The current for the electrolysis cell is obtained from a pack of mercury batteries, with suitable dividers. A Keithley 414 ammeter measures the current through the electrolysis cell. At low currents, $< 10\ \mu A$, a 1-MΩ current-smoothing resistor is switched into series with the electrolysis cell.

The light source is a 500-W Kodak projector with heat filters. Light absorbed by *p*-benzoquinone, < 480 nm, is removed by a solution of potassium chromate. Cut-off and interference filters provide monochromatic light. Neutral density filters and screens or changing the lamp voltage serve to attenuate the light. The intensity of the light is measured by a Yellow Springs Instrument, Model 65, Radiometer at a position chosen to be equal in intensity to that incident on the front surface of the reaction cuvette. The intensity over the front surface of the cuvette varies by less than 5 %. The temperature of the room is held constant $\pm 1°$ by an air conditioner.

Calibration. The output of a photosynthesizing green plant system is inherently a rate of oxygen production. The luminometer has been designed to respond directly to this output. The ideal calibration method would also produce a rate of oxygen production directly related to some absolute and easily measurable unit. The electrolysis of water comes very close to this ideal. At a sufficiently high current density

at an 'inert' anode, 1 molecule of oxygen is evolved for every 4 electrons passing through the circuit. The current is readily measured and compared to an absolute standard. With Faraday's constant it is directly convertible to rate of oxygen production in moles/sec. The increment in molar concentration of oxygen in the gas is calculated by dividing by the flow rate and the increment in ppm by use of the gas laws to adjust to the experimental temperature and pressure.

To achieve constant proportionality between current and oxygen production down to low currents it is important that the electrode surface be kept small and that the electrolyte be very pure. As a final step the electrolyte, 1 mM NaOH, was preelectrolyzed for several hours at mA currents before transferring it to the carefully cleaned electrolysis cell. For experimental convenience the anode and cathode were not placed in separate gas streams. Back-reduction of oxygen at the cathode was minimized by vigorous sweeping of the small amount of electrolyte with the carrier gas. However, this limited the lowest useful calibrating current to 1 μA.

RESULTS

The luminometer

The response of the luminometer to a 10-μA pulse of electrolysis current is shown in Fig. 2. The response lags the oxygen production by 0.4 min which is the travel time of the gas between the electrolysis chamber and the luminometer vessel at the usual flow rate, 10 ml/min. This lag can be decreased by decreasing the volume between the two chambers or by increasing the flow rate. The rise and decay are exponential with halftimes of 0.5-min rise and 0.3-min decay. These response times depend on the dead volume between the chambers, the flow rate of the gas, and the time required to achieve a new steady state in the luminol vessel. If the luminol

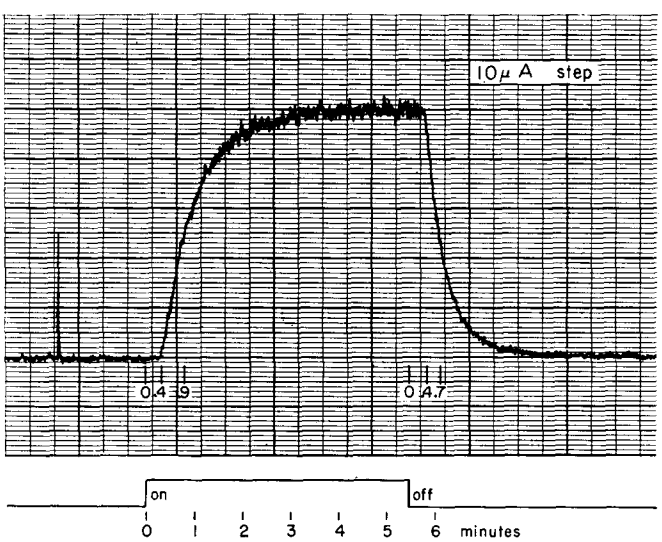

Fig. 2. Response of the luminometer to a 10-μA step in electrolysis current. The numbers on the record indicate the time in min after turning the current on or off. This step corresponds to an increase in oxygen partial pressure of about 3 ppm, superimposed on about 1-ppm background in the carrier gas. Time constant of the filter circuit was about 0.5 sec, and flow rate was 10 ml/min.

solution contains too much moisture, or is insufficiently basic, the chemiluminescent reaction itself increases the time of response.

The response of the luminometer is superimposed on a steady base line caused by the residual oxygen in the carrier gas, 1 ppm in these experiments, and by the dark current of the photomultiplier. By suppressing the base current and increasing amplifier gain, one can detect a small fraction of this residual current. The sensitivity is limited by the noise of the system, and is about 1 % of the base current with the bubbling luminol vessel and 0.3 % with the quieter surface reaction vessel. Using the bubbling column, a change of 0.03 μA on a 1-μA background was clearly measurable. This corresponds to a rate of oxygen production of 10^{-13} mole/sec or to an increment in concentration of 0.01 ppm in the gas phase.

The response of the luminometer is linear in rate of oxygen production over a wide range and is shown in Fig. 3. A log–log plot is used to cover the greater than 4 orders of magnitude range of the data. The slope of the line, determined by a least squares calculation, 0.99, shows the response to be closely linear. The standard deviation of the points from the line is 6 %. Over a more limited range, and by direct comparison with the electrolysis current, the precision of the luminometer is about

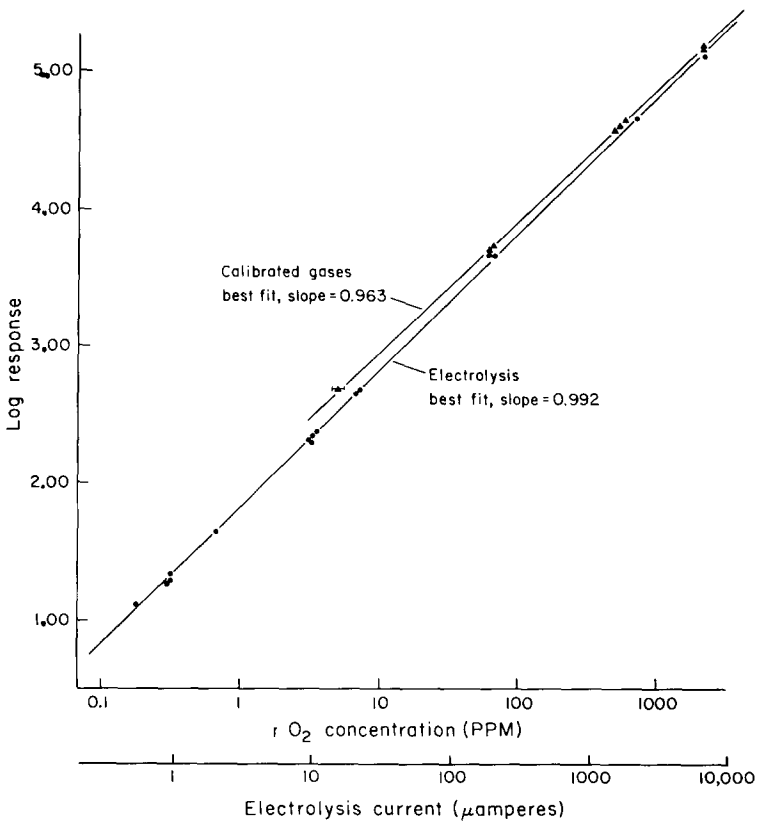

Fig. 3. Log response of the luminometer *versus* log electrolysis current or log oxygen concentration. The latter scales were indexed using Faraday's law, 4 electrons per oxygen molecule and the gas laws.

2 % for ranges above 10 μA (equivalent to 3 ppm oxygen) and about 5 % for ranges below 1 μA (equivalent to 0.3 ppm oxygen).

Earlier workers using bacterial luminescence[7] or phosphorescence quenching[8] to measure oxygen have had far less success with the electrolysis of water as a calibration method. Their coulombic efficiencies were very low even at 50 μA, presumably because of insufficient care in cell design or electrolyte purification. To be certain about our calibration, a completely independent determination was made using mixtures of oxygen in argon. The mixtures were obtained from the Matheson Company, and the Hersch cell (sensitive to about 1 ppm oxygen) was used to determine the oxygen content, with a precision of 5–10 %. The response of the luminometer to the calibrated gases is also shown in Fig. 3. The agreement is good but there are slight differences. The calibrated gases have a slope of about 0.96 instead of unity, and the discrepancy between the gases and the electrolysis increases at the lower oxygen levels. It is uncertain whether these discrepancies are caused by a small inefficiency in the electrolysis cell or by a systematic error in the calibration of the gases.

Photosynthesis

In a typical experiment the cuvette is filled with 8 ml of suspension of Chlorella cells in a buffer (0.02 M potassium phosphate, pH 6.3) and Hill oxidant (0.7 mM *p*-benzoquinone) if required and is plugged into the gas train while the regulated flow of gas is routed through the bypass. The cuvette is flushed rapidly with argon for 5–10 min and the stopcocks turned so that the regulated gas flow passes through the cuvette. The instrument is calibrated during an experiment by measuring the response on passing various currents through the electrolysis cell.

In measuring photosynthesis by oxygen evolution it is desirable to minimize the effect of respiration. For the Hill reaction, the benzoquinone inhibits respiration[9] so the measurement is simplified. In the case of carbon-dioxide-based photosynthesis, the ability to measure oxygen production at the level where it limits respiration is a greater advantage. The null method may also be used. In this mode the oxygen produced is measured at the same oxygen level as in the dark by decreasing oxygen production in the electrolysis chamber upon turning on the light so that the O_2 concentration in the carrier gas remains the same. A small gradient of oxygen does remain in the cuvette since the entrance and exit carrier gasses are necessarily at slightly different O_2 levels, but this is minimized by good equilibration.

The results obtained with a series of Chlorella mutants are given in Table I. The mutants were obtained from Dr. GRANICK[10] who had isolated them to study the biosynthesis of chlorophyll. It is seen that there is no simple correlation between oxygen produced by the Hill reaction and chlorophyll content. However, the one mutant having no detectable chlorophyll also showed no Hill activity. These particular mutants were chosen because they contained excess carotenoids and chlorophyll precursors such as porphyrins.

One of these mutants, 610, is of particular interest. It loses its chlorophyll and oxygen-producing activity in the dark and regains these in the light, an unusual behavior for Chlorella. Total carotenoids are approximately independent of light and dark growth. It is of interest to ask how does the photosynthetic apparatus turn itself off in the dark. Is the apparatus unstable and continually remade, or is it made

TABLE I

OXYGEN PRODUCTION AND CHLOROPHYLL CONTENT OF CHLORELLA MUTANTS

The value 100 is given to data obtained from wild-type (NG) Chlorella. Cultures were grown on a glucose medium[11] in the dark, then exposed to light for 1 day, except 610-D, which was kept in the dark. Keeping the other mutants in the dark had relatively little effect.

Chlorella	*Relative O_2 rate per cell*	*Relative chlorophyll per cell*
NG	100	100
681	6	1
455	2	1
610-L	100	100
610-D	<0.02	0
606	0.3	1
58	<0.04	3
222	<0.04	1
637	<0.08	0

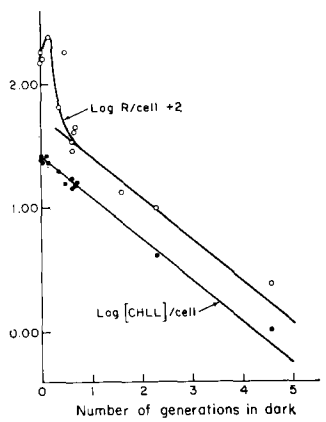

Fig. 4. Degreening of Mutant 610 in the dark. Log of rate of oxygen formation (Hill reaction) and log of chlorophyll content per cell are plotted *versus* generations in the dark. The latter is defined as the number of cell doublings determined by cell count. The straight lines have a slope of one-half, *i.e.* the slope expected if the variable decreases by a factor of 2 in each generation.

once, for the life of the cell? The answer in Fig. 4, is clear; the oxygen-evolving activity and the chlorophyll content are simply diluted out by cell division. There was also a transient in the oxygen activity immediately on turning off the light. *Vice versa*, on turning on the light on dark-adapted cultures, oxygen production and chlorophyll synthesis occurred without requiring detectable cell division. Details of these processes are now being studied.

DISCUSSION

The oxygen luminometer combines specificity and extreme sensitivity for oxygen with unprecented range of linear response to oxygen concentration or rate of synthesis. Our data cover over 4 orders of magnitude down to less than one part of oxygen

in 10^6. Since this chemiluminescent reaction has already been shown[5,6] to be linear in oxygen concentration over a 10-fold range near air levels, it is safe to assume that this reaction is linear in oxygen concentration over at least 6 orders of magnitude. The chemiluminescent reaction is specific for oxygen and this specificity to oxygen is enhanced by use of a carrier gas. The sensitivity to an oxygen source is less than 10^{-13} mole/sec. This corresponds to an increment in partial pressure of oxygen of 10^{-8} atm or to a change of 10^{-11} M in water at room temperature. The sensitivity can be increased by decreasing the flow rate and could be improved somewhat with a more elaborate light detection system. Further removal of oxygen from the carrier gas would allow operation at lower levels of oxygen.

There are several methods of determining oxygen which have a comparable sensitivity, if not the wide range, of the oxygen luminometer. We will compare several of these methods, particularly those used on photosynthetic systems.

The quenching of the phosphorescence of a trypaflavin dye absorbed to silica gel was used by POLLACK, PRINGSHEIM AND TERWOORD[8]. The method is sensitive to approx. $3 \cdot 10^{-14}$ mole of oxygen per sec with a response time of a few minutes. The sensitivity is about the same as that of the luminometer, but the response is neither linear nor logarithmic, approximately an exponential saturation, and required frequent calibration. The response saturates near 1 ppm of oxygen and so the method is restricted to this low range.

The Hersch cell is an oxygen depolarized galvanic cell adapted[12] to be sensitive to small amounts of oxygen. The method is sensitive to about 1 ppm of oxygen. At a high flow rate, 100 ml/min, its response time is about 1 min and is linear in the range 1–100 ppm of oxygen. Above this value it saturates with a half response point at about 1 % oxygen[13]. To avoid neutralizing the alkaline electrolyte (20 % KOH), carbon dioxide is best removed. WHITTINGHAM[14] has used this method to study oxygen production by Chlorella at low oxygen tension (1–500 ppm).

Although the thermodynamically ideally reversible electrode for oxygen operating at room temperature has not been found, systems close to the ideal electrode at very high temperatures are known. An example is the zirconium oxide-calcium oxide cell[15–18] operating at 1000°. The potential across this cell is proportional to the logarithm of the ratios of partial pressures of oxygen on each side of the cell. This logarithmic response is convenient to measure large spans of oxygen tension, but to reach precision of even 3 % in oxygen partial pressure requires measurements and stabilities of less than a mV. WEISSBART AND RUKA[16] claim to have achieved stabilities of this order at high (1 %) oxygen levels. At the extreme low level, 10^{-14} atm (ref. 18) the error rises to a factor of about 3. The response time is about a minute. Because of the high temperatures, water and organic vapors must be rigorously excluded; the former, because of dissociation; the latter because of reduction of oxygen. The method may have promise as a differential detector, the two sides of the cell sampling a reference and variable gas stream.

The method most simply comparable to the oxygen luminometer is that using luminous bacteria. Although very sensitive to low partial pressures of oxygen (1 ppm), the system saturates at about 100 ppm, and so is linear only over a limited range[19,20]. Its only advantage is that the luminescent bacteria may be mixed with the photosynthetic algae or chloroplasts, thus reducing transport times.

The widely used oxygen polarograph methods are far less sensitive than the

TABLE II

METHODS OF OXYGEN MEASUREMENT APPLICABLE TO PHOTOSYNTHETIC SYSTEMS

Method and ref.	*Range (atm)*	$f(O_2)$	*Sensitivity*		*Half response time*
			Δp_{O_2} *(atm)*	Δ *moles* O_2*/sec*	
Gasometric					
WARBURG[23]	1	Linear, integrative	10^{-4}	10^{-10}	3 min
Cartesian diver[24]	1	Linear, integrative	$3 \cdot 10^{-6}$	10^{-17}	5 min
Physical					
Mass spectrometer[25,26]	10^{-3}–1	Linear		$3 \cdot 10^{-11}$	10 sec
Phosphorescence quenching[8]	10^{-8}–10^{-6}	Non-linear	10^{-8}	$3 \cdot 10^{-14}$	20 sec
Electrochemical					
O_2 cathode, CHANCE[27,28]	10^{-5}–1	Linear	10^{-5}	$3 \cdot 10^{-10}$	$<$1 sec
O_2 cathode, JOLIOT[21]	10^{-6}–1	Linear		10^{-16}	?
O_2 cathode, HERSCH[22]	10^{-6}–10^{-1}	Saturates	10^{-6}	10^{-10}	5 min
O_2 voltaic, ZrO_2[18]	10^{-14}–1	Logarithmic	10^{-14}	10^{-19}	1 min
Chemical					
Hemoglobin[29]	10^{-3}–$4 \cdot 10^{-2}$	Saturates, integrative	10^{-3}	$3 \cdot 10^{-10}$	10 sec
Luminous bacteria[19,20]	10^{-6}–10^{-4}	Saturates, integrative	10^{-6}		?
Luminometer	10^{-8}–1	Linear	10^{-8}	$5 \cdot 10^{-14}$	1 min

above methods, but have the advantage of faster response times. Recently, JOLIOT[21] has made a brilliant advance with the oxygen polarograph in the form developed by HAXO AND BLINKS[22]. JOLIOT modulates the light beam exciting photosynthesis and detects only the modulated current at the electrode. A sensitivity of 10^{-16} mole of oxygen per sec is claimed.

The most often used methods for detection of oxygen in photosynthesis are listed in Table II together with pertinent characteristics. Because of inadequate data in the literature, many of the entries under *Sensitivity* are our own estimates. It can be seen that physical and electrochemical methods offer a wide choice of ranges and sensitivities and that the oxygen luminometer compares favorably with them.

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