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EFFECTS OF OXYGEN AND RED LIGHT UPON THE ABSORPTION OF VISIBLE LIGHT IN GREEN PLANTS[1,2]

BRITTON CHANCE AND BERNARD STREHLER[3]

THE JOHNSON FOUNDATION FOR MEDICAL PHYSICS, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA 4, PENNSYLVANIA AND DEPARTMENT OF BIOCHEMISTRY, FELS FUND, UNIVERSITY OF CHICAGO, CHICAGO 37, ILLINOIS

In studies of the purple bacteria, the correlation between the effects of oxygen and light provide direct information on the nature of the light reaction and assist in identifying the point in the respiratory chain at which oxidants produced in the photochemical process react (1). In addition, a comparison of the effects of respiratory inhibitors upon the absorption spectra of the pigments of the purple bacteria provide further bases for analysis of light effects (1, 2). Although spectroscopic changes due to illumination of Chlorella and other green cells have been reported from several laboratories (3 to 10), in which some studies of inhibitors and activators of the effects have been made, no dark process has been described which produces effects similar to illumination. We find that the spectra representing the differences of absorption between the aerobic and anaerobic green cells show great similarity to those representing the differences between dimly-lighted and dark cells. Such data have been obtained for two types of green algae. In brief, our data show that oxygenation or illumination of the anaerobic cells causes an absorption band to appear at 518 mμ and one to disappear at 475 mμ. Since our method is suitable for the recording of slow changes of absorption as well as rapid ones, the time course of the light and dark reactions has been studied. Three distinct light reactions are described. One reaction is the small and rapid "aerobic light effect" (phase 2) studied by previous workers (3 to 10) and a second one is observed with dim illumination of anaerobic cells (phase 3) that is related to oxygenation of the cells. The third (phase 1) is the "recovery phase" of an overshoot phenomenon (cf. 6). The nature of these reactions and their relation to the photosynthetic process is discussed. A brief report on part of this work has appeared (19).

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[3] Present address: Gerontology Branch, National Heart Institute, Baltimore City Hospitals, Baltimore, Maryland.

METHODS

Methods for measuring spectroscopic changes in photosynthetic cells are reviewed by Duysens (17) who has done pioneer work in this field. Our spectroscopic method has previously been used in photochemical studies (12) but the sample holder is similar to Witt's (7).

Figure 1 shows the apparatus especially designed for dense algal suspensions or sections of leaves. The moist chamber, which is not visible in figure 1, has a volume of 1.2 ml ($20 \times 20 \times 30$ mm). About 0.7 ml of an algal suspension containing 0.1 to 0.2 ml of cells is added and the algae are allowed to settle on the transparent base of the chamber and form a uniform layer suitable for spectrophotometric observation. The moist chamber fits into a bakelite plate which mounts directly on top of an end-on photomultiplier (fig 1). Between the algae and the photosurface is interposed a suitable filter, e.g., Wratten #44 A or Corning 5030. Moist gases containing proper proportions of nitrogen, oxygen and carbon dioxide are passed over the

surface of the liquid in the moist chamber via the connections indicated in figure 1. In order to monitor the oxygen tension and to test the photosynthetic activity of the cells, two types of platinum micro-electrodes are used. First, a micro-electrode and its associated silver reference electrode project into the base of the moist chamber and record, through suitable amplifiers, changes in oxygen concentration that occur upon oxygenation of the cells by illumination or by gas mixtures. Secondly, for more sensitive measurements, a spiral platinum wire imbedded in the lucite base of the chamber is used with reference to a calomel electrode (11).

The light for activating the photochemical systems is obtained in two ways: a) six small light bulbs are mounted directly before the 45° mirror and are painted with red nail polish which excludes, to a considerable extent, wavelengths shorter than 640 $m\mu$; b) the 45° mirror is partly silvered so that illumination from above by means of a 100 watt tungsten lamp and lens combination can be used with Corning 2403 filter. About one third of the exciting light is transmitted by the 45° mirror. The photomultiplier is protected from this red light in two ways: 1) by the chlorophyll present in the suspension studied and 2) by the filter interposed between the cell suspension and the photomultiplier. In addition, the exciting light is steady while the measuring light is flickered. Thus, light-leaks onto the phototube increase the steady component of the photocurrent and hence the noise output, but this does not cause any net displacement of the recorders responsive to the measuring signal. A full discussion of the application of these methods to photochemical reactions is given elsewhere (12, 13).

The spectrophotometer is of the double beam bichromatic type (14) and the two monochromators can be seen at the rear of figure 1. The monochromatic beams, for example 515 and 495 $m\mu$, emerging from these monochromators fall upon a 60-cycle vibrating mirror which alternately flickers light of the two wavelengths upon the 45° mirror and thence through the material under observation. Since capacitance-coupled amplifiers are used to amplify the difference in the phototube response to the two flashes of light, the output is unresponsive to the exciting light, provided it does not give signals which exceed the linear range of the amplifying circuits. Suitable controls on the freedom from artifact due to the exciting light are obtained either by turning on the exciting light in the absence of the measuring light or, alternatively, setting the wavelength of the measuring beams to the same value and then turning on the exciting light. In both cases, negligible deflection of the output of the

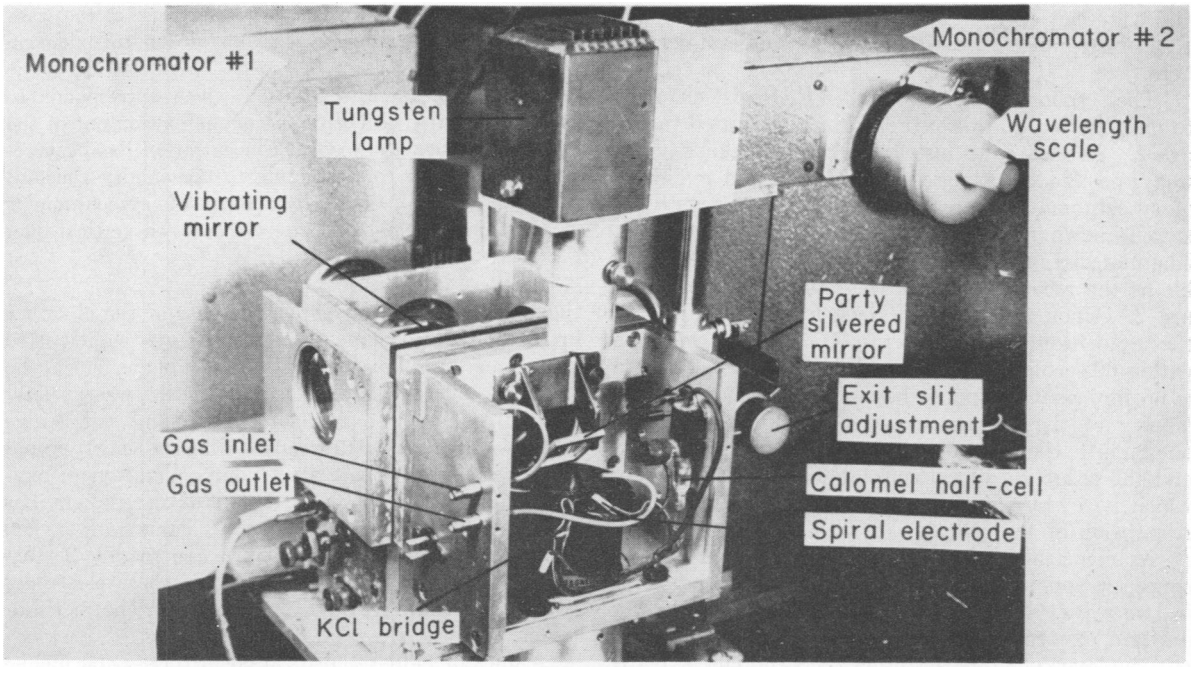

FIG. 1. A photograph of the moist chamber for spectroscopic measurements of the effects of illumination upon the green plant cells. The illustration shows in the background the monochromators of the double beam spectrophotometer, the image of the exit slits which are focused on a vibrating mirror enclosed in the box in the background. The light is reflected from the vibrating mirror onto the partly silvered 45° mirror and then downward onto the 1.5-cm^2 moist chamber housed in a bakelite disk and mounted upon the magnetic shield for the end-on photomultiplier. A 100-watt lamp and lens combination illuminates the algae by the transmission through the partly silvered mirror. The gas connection for oxygenating or disoxygenating the material in the moist chamber is shown. The connections for the platinum micro-electrode and its silver reference electrode are also shown. A cover encloses the assembly to avoid stray light. (FA 35).

recorders occurs under the conditions used in these experiments (12, 13).

Spectroscopic measurements are usually made with respect to a neutral wavelength at which no absorption changes have been found to occur. For the conditions of our experiment, 495 mμ is used as a reference wavelength. The measuring wavelength can be adjusted as desired in order to record the light absorption changes with respect to the reference wavelength. The sensitivity of the apparatus normally used is about 1 % change of absorption for full scale of the recorder. The noise level is very near the theoretical value and the records included here show fluctuations less than 10^{-4} in optical density with samples of adequate transparency. The spectral interval used is less than 3 mμ and is often 1.5 mμ. This double beam method gives a clearer result than that obtained by the compensating methods used by Duysens (3) and this is best summarized by a comparison of original experimental records shown here and in reference 17.

Kok (10) has recently had some success with a method of electrical sampling or "gating" of the photocurrent at arbitrary times after the flash. Such a method is suitable for accurate measurements at these arbitrary times, which must be chosen on the basis of the entire time course of the reaction kinetics. The instrument does not, however, plot out such kinetics and they can only be obtained with difficulty. Thus Kok's instrument is unsuitable for the studies described here.

The response of the material in the moist chamber to oxygenation and disoxygenation is indicated in figure 2. In this case, instead of algae, Baker's yeast was used and the kinetics of oxidation and reduction of cytochrome c are recorded, since this pigment has a well-known response to changes of oxygen tension. The material in the moist chamber is initially anaerobic as the record begins on the left-hand edge of figure 2. Upon admission of oxygen, the oxidation of the cytochrome proceeds rapidly and is complete within 60 seconds. Upon admission of nitrogen, there is no immediate response because of the high oxygen affinity of the cytochrome system. However, after one minute, the oxygen tension reaches a critical value and the reduction of cytochrome c proceeds at a rate which is set by the exhaustion of the oxygen by the respiration of the cells.

As mentioned above, the measurement of oxygen concentration in the moist chamber is accomplished in two ways. For prolonged illuminations in which a uniform oxygen concentration is established throughout the layer of liquid covering the Chlorella, a platinum micro-electrode polarized at −0.6 V inserted in the side of the moist chamber is satisfactory. But in order to measure transient changes of oxygen concentration in the layer of Chlorella settled upon the lucite bottom of the moist chamber, a platinum spiral has been embedded in the lucite. This spiral was of sufficient diameter (~ 1 cm) to sample a large portion of the cell population. Also the projection of

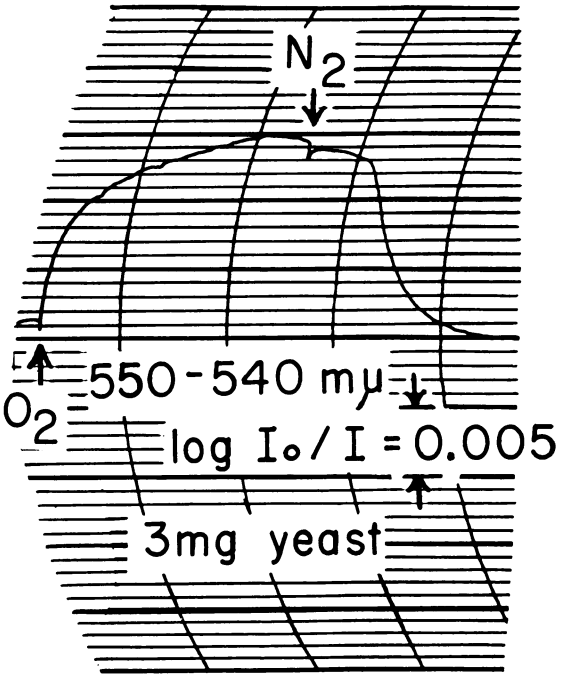

FIG. 2. An illustration of the response of cytochrome c of yeast cells to oxygenation and nitrogenation in the moist chamber of figure 1. The upward rise of the trace immediately following oxygenation is due to oxidation of reduced cytochrome c of yeast. There is a lag in the response following nitrogenation which corresponds to the time required to flush most of the oxygen out of the chamber. When the oxygen concentration has been reduced to a value corresponding to the affinity constant for cytochrome oxidase, reduction of cytochrome c occurs rapidly. The time interval between vertical lines is 60 sec. (632).

the electrode diameter above the base of the chamber in the horizontal plane (~ 0.1 mm) was sufficient to give some sampling in the vertical plane, since the thickness of the layer of settled cells was usually about 0.3 mm. A calomel electrode and salt bridge were used with this electrode. Thus, a sensitive and rapid response to the intracellular generation of oxygen is obtained as soon as the diffusion gradient has moved outside the cell surface. Changes of oxygen concentration of less than 10^{-7} M can readily be registered. The current from either of these electrodes is amplified and recorded by means of a chopper amplifier.

Values of light intensity used in these experiments are included only in order to allow others to approximate our experimental conditions. The values of lux given are those measured with a G.E. type DW-58 foot-candle meter and are the intensity values actually incident upon the lucite bottom of the moist chamber illuminated by the tungsten source via filters and mirrors as used. The light intensity was varied by neutral filters that were calibrated at 670 mμ.

The cell concentrations used here were comparable to those used by Duysens (17) who obtained absorbancy changes at 520 mμ about equal to those of figures 3 to 5. About 85 to 95 % of the light incident at 520 mμ is absorbed or scattered. These highly absorbing cell suspensions, which are necessary for obtaining adequate signal-to-noise ratio, lead to unhomogeneity of the red illumination, but this is minimized in this apparatus by the 400-mm² area of the moist chamber.

Materials

Chlorella pyrenoidosa was grown in a salt medium in daylight. This medium contained citrate as a carbon source, salts (Mg^{++}, NO_3^{--}, PO_4^{--}) and the usual trace elements, and was adjusted to pH 6.0. The culture flasks were one-liter " Shaker " type and only 200 ml of the medium was added. *Chlamydomonas reinhardii* (15) were grown in the light in salts, both with and without an acetate supplement. The algae were concentrated by centrifugation and were resuspended in the growth medium.

Results

Spectroscopic Effects of Illumination: In order to present the various spectroscopic effects of illumination in an understandable fashion, a number of separate experiments are presented under experimental conditions which are believed to represent separate effects clearly. In order to accomplish this, it has been necessary to correlate the spectroscopic effects, as measured by the double-beam spectrophotometer, and the extracellular oxygen concentration, as measured by the platinum electrode. Effects of illumination so far reported for Chlorella are mostly under aerobic conditions, particularly those described in the studies of Witt (7) and Strehler and Lynch (6). Thus, the first experiment to be considered is one which is intended to duplicate their conditions. In figure 3 B, the cells have been oxygenated by a succession of illuminations prior to the experiment reproduced here and the oxygen concentration at the beginning of the experiment corresponds roughly to 0.2 micromolar, a concentration which is adequate for the phenomenon being observed.

Illumination with 35 lux produces an abrupt downward deflection of the spectrophotometric trace corresponding to an increase of absorption of about 0.002 at 515 mμ measured with respect to 495 mμ. The oxygen trace indicates a transient rise and then a steady increase to 0.6 micromolar. Upon cessation of illumination, both traces return abruptly to the dark levels. The cell suspension remains aerobic. This small and rapid decrease of absorption caused by cessation of illumination is termed " the aerobic light effect " and is defined as a phase 2 transition.

If the cells are initially anaerobic due to flushing with 95 % N_2 - 5 % CO_2 gas mixture, their absorbancy at 515 mμ decreases from the level of figure 3 B to the level indicated in the left-hand edge of figure 3 A. Illumination with 35 lux now causes a much larger increase of absorbancy than in figure 3 B. There follows a slight decrease of absorbancy during the first 20 seconds following illumination. After brief transient, the extra-cellular oxygen rises to approximately 0.3 micromolar and upon cessation of illumination falls to zero in about half a minute. When the light is turned off, the spectroscopic trace shows a

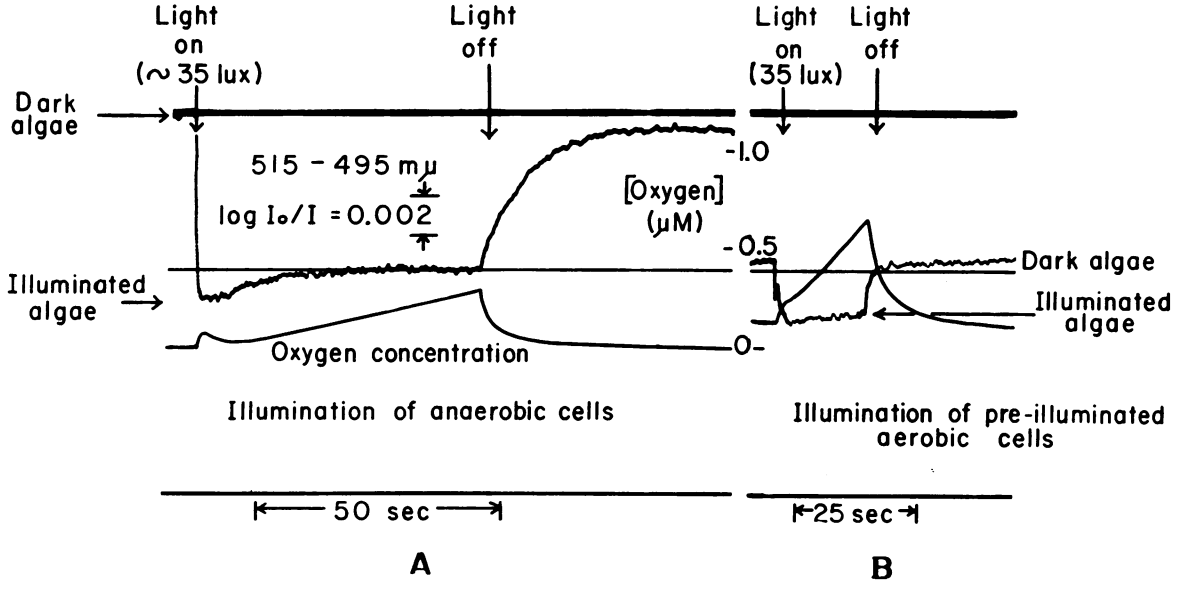

Fig. 3. Combined spectrophotometric and platinum electrode recordings of the effects of illumination upon Chlorella. This figure emphasizes the difference between illumination under anaerobic and aerobic conditions. At low oxygen concentrations in A, there is only the phase 3 transition upon cessation of illumination. Under highly aerobic conditions in B, on'y the rapid phase 2 transition is observed as described in text. (690).

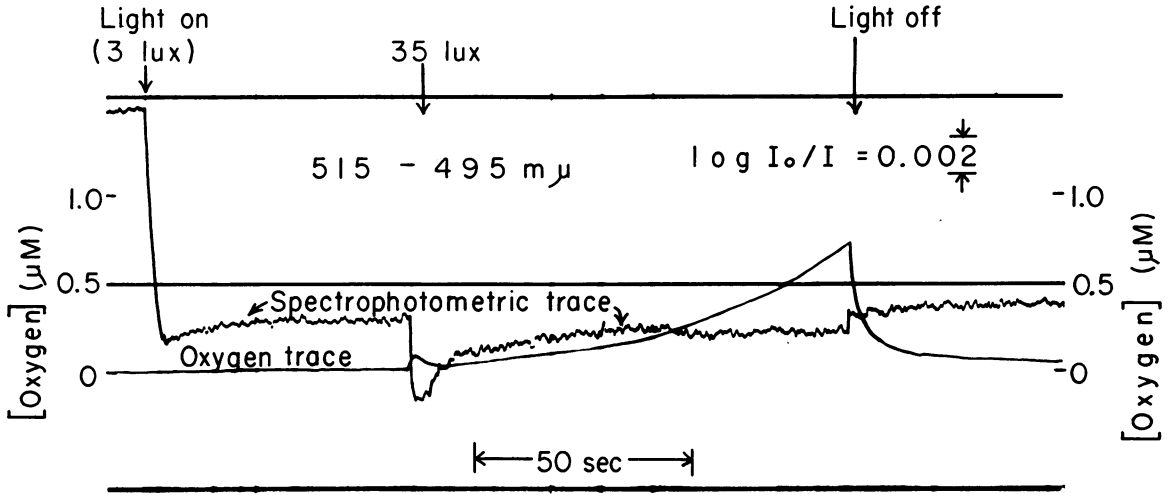

FIG. 4. Simultaneous platinum electrode and spectrophotometric measurement of the effects of illumination of a Chlorella suspension. The spiral platinum electrode is used to record the oxygen concentrations. This figure serves to distinguish between aerobic and anaerobic illumination of the algae. The phase 1 transition is represented by the slow decrease of the absorbancy at 515 mμ following illumination, and the phase 2 transition is the very abrupt decrease of absorbancy caused by cessation of illumination. The phase 3 transition is more clearly illustrated by figures 3 and 12. (690).

large rise indicating a decreased absorption at 515 mμ and at the end of about a minute has returned closely to the dark value. These spectroscopic effects are related to intracellular oxygenation of the Chlorella, due to photosynthetic activity. The decrease of absorption upon cessation of illumination is defined as a phase 3 transition.

The algae can be illuminated with such a low light intensity that intracellular spectroscopic effects are observed even though the extracellular oxygen concentrations cannot be recorded with the sensitivity available from the spiral electrode, and this is illustrated in figure 4. The algae are equilibrated with $N_2 - CO_2$ in the dark, and a light intensity of only 3 lux turned

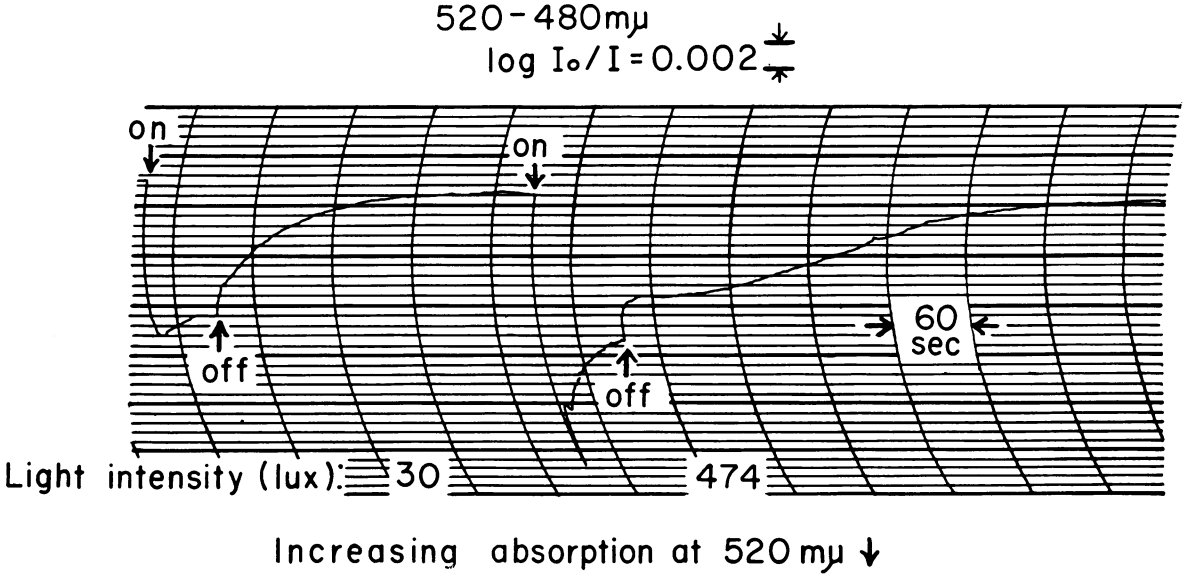

FIG. 5. Direct photographs of experimental records on the effects of red illumination upon Chlorella. On the left-hand side the light intensity is low and the absorbancy at 520 mμ relative to 480 mμ rapidly increases as indicated by the downward deflection of the trace. Following the steady state, the light is turned off and the trace returns to the base line. In the right-hand portion of the figure, an intensity of light is used that gives approximate saturation of the rate of oxygen production and absorbancy increases at 520 mμ are slightly larger than those at the lower light intensity. The reaction kinetics in this case involved the phase 1, 2 and 3 transitions. (623).

on. The characteristic increase of absorption at 515 mμ is observed although the oxygen trace remains horizontal. A ten-fold increase of light intensity causes an abrupt downward deflection of the trace, and in this case the deflection is definitely not constant, but falls back towards the level observed at the lower light intensity (this is a phase 1 transition). The oxygen trace, after a brief transient, shows a rise to a concentration of approximately 0.7 micromolar, at which point the light is turned off. The oxygen concentration under these conditions does not fall to zero but remains at a finite value (around 0.1 micromolar) until the end of the trace. The spectrophotometer shows only the "aerobic light effect" (phase 2 transition), and there is no phase 3 transition because enough extracellular oxygen remains to the end of the record to maintain the spectroscopic change.

Figures 3 and 4 should now afford a basis for understanding the complex cycle of spectroscopic changes that are recorded in figure 5. In this case the data are recorded on a slower time scale and at higher light intensities in order that the full cycle of events might be recorded. As in the previous figure, the cells are equilibrated with $N_2 - CO_2$. The left-hand portion of the record represents only a test of their response at a light intensity comparable to that used in figure 3, and the trace is in good agreement with the trace of figure 3 A. In the right-hand portion of figure 5 a much higher light intensity is used and the complex sequence of spectroscopic events that ensues is recorded. Upon illumination of the anaerobic cells the typical abrupt increase of absorption, measured in this case at 520 mμ with reference to 480 mμ, is observed, but the deflection is not stable and decreases to a steady value within a minute. This we define as the phase 1 transition and data below indicate that it may be related to the increase of the light requirement of the spectroscopic effect upon oxygenation of the cell suspension by photosynthesis (cf fig. 10). Upon cessation of illumination the small and rapid decrease of absorption at 520 mμ characteristic of the "aerobic light effect" (phase 2) is recorded. Since the cells were fully oxygenated by photosynthesis the trace is steady for about a minute while the excess oxygen is removed by dark respiration; then the absorption at 520 mμ slowly decreases as the extracellular oxygen falls to zero (phase 3 transition).

The absorbancy change that occurs upon illumination is not given a specific designation because it may consist of various combinations of the reverse of the phase 1, 2, and 3 transitions. For example, illumination of the anaerobic cells at low intensities (3 lux) as in figure 4 causes chiefly the phase 3 transition, while in figure 3 B only the phase 2 transition occurs.

With this introduction into the nature of the phenomena to be studied and the designation of the various spectroscopic changes, we shall now proceed to outline some control experiments and to indicate the spectra corresponding to one of the transitions caused by changes in illumination. We shall than return to the question of the relationship of the absorption changes and the oxygenation of the cells.

Effect of the Measuring Light: Since the response of the 515 mμ pigment to illumination under anaerobic conditions is extremely sensitive, there is a possibility that a portion of the spectroscopic change might already have occurred due to illumination with the measuring light and we have therefore repeated experiments similar to those in figure 3 at various values of the intensity of the measuring light and at a constant value of the exciting light. These data are plotted in figure 6 and it is seen that only at the highest value of the measuring light is there a measurable decrease of the spectroscopic effect. The conversion of the photocurrent at the photomultiplier anode to the light intensity in lux, as given in the figure legend, shows that 40 μa corresponds to only about 0.2 lux at 515 mμ.

The Effect of Light Intensity upon the Kinetics of the Absorbancy Change at 518 mμ. While Witt has made detailed studies of the kinetics of the absorbancy change caused by flash illumination of what are presumably the aerobic cells, no reports have yet appeared on the speed with which the absorbancy change occurs upon illuminating the anaerobic algae. A typical record of an experiment on the kinetics of this change is given by figure 7 which indicates the kinetics of the "on" and the "off" reactions for a given

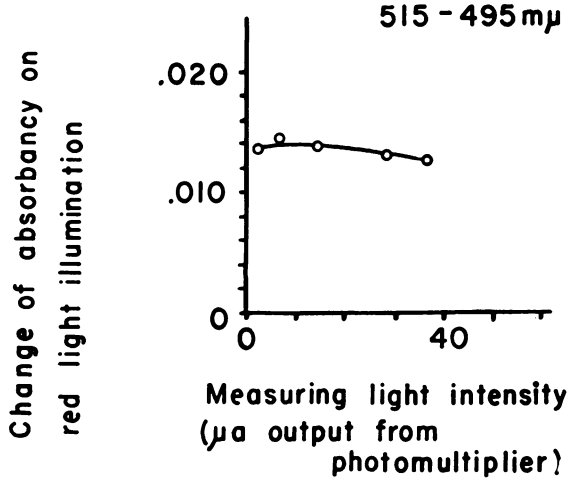

Fig. 6. The effect of the intensity of the measuring beam upon the change of absorbancy at 515–495 mμ at a constant intensity of red light illumination. The intensity of the measuring beam is given in terms of the photocell output current obtained through a Chlorella suspension. A developmental type K 1234 Dumont photomultiplier tube was used at 80 volts per stage, giving an approximate gain of 30,000. Thus the point 40 on the abscissa would correspond approximately to 1×10^{-9} amperes of primary photocurrent. At a sensitivity of roughly 2 amperes per lumen, the point 40 on the abscissa corresponds to 20×10^{-6} lumens. Averaged over the 1 cm^2 area of the moist chamber, 20×10^{-6} lumens corresponds to 0.2 lux. (62).

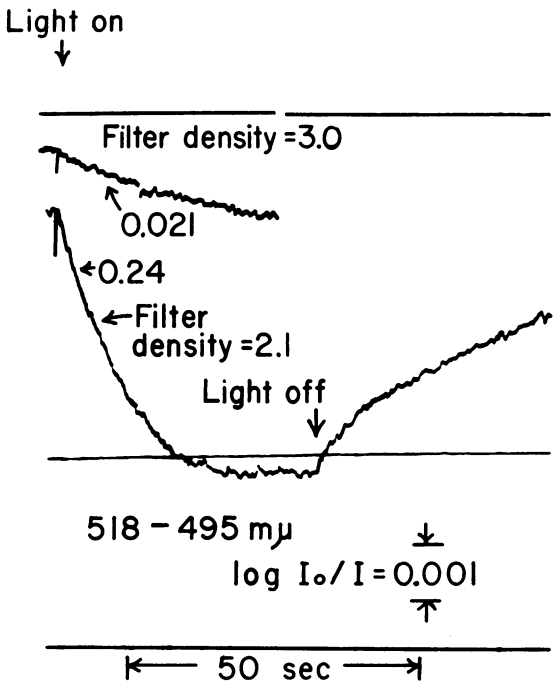

FIG. 7. Effect of a 10-fold variation of light intensity on the kinetics of the absorbancy increase at 515 mμ caused by illumination. The light intensity is varied by a neutral filter and the algae are initially in the anaerobic state. The slopes of the traces are in units of $10^3 \log \frac{I_o}{I} / \text{sec.}$ (625).

intensity of light and, in the upper portion, the kinetics of the "on" reaction for a ten-fold reduction of the exciting light intensity by means of a neutral filter. The ratio of the initial slopes of the kinetics of the two traces is 11:1, indicating reasonable proportionality between reaction kinetics and light intensity.

DIFFERENCE SPECTRA FOR THE EFFECT OF THE EXCITING LIGHT: In order to verify in detail the hypotheses based only on absorbancy changes measured at 515 mμ and 495 mμ, in the next two sections of this paper we compare the spectra for the illumination of the cells under anaerobic conditions with those obtained by oxygenation. Difference spectra corresponding to illumination of the anaerobic cells are necessary since it is uncertain as to whether cells were aerobic or anaerobic in previous works (3 to 8, 10), or in cases where the degree of aerobiosis was noted (7, 9), complete spectra have not been published. By taking measurements similar to those of figure 3 (left) (phase 3 transition) at a variety of wavelengths and by plotting the steady state deflection as a function of wavelength, we obtain difference spectra for the effect of low light intensities. This has been done for two types of algae.

Chlorella: In figure 8 A, we have plotted the absorbancy increases that occur upon illumination of Chlorella with red light. There is an absorption peak at 515 mμ, a plateau at 493 mμ, and a trough at 475 mμ. There is no distinctive effect in the region 550 to 555 mμ. The relationship of this spectrum to other work (3, 4, 6, 9, 10) is discussed below.

Chlamydomonas: The similar record for Chlamydomonas grown photosynthetically on a mineral medium shows a broader peak at approximately 518 mμ

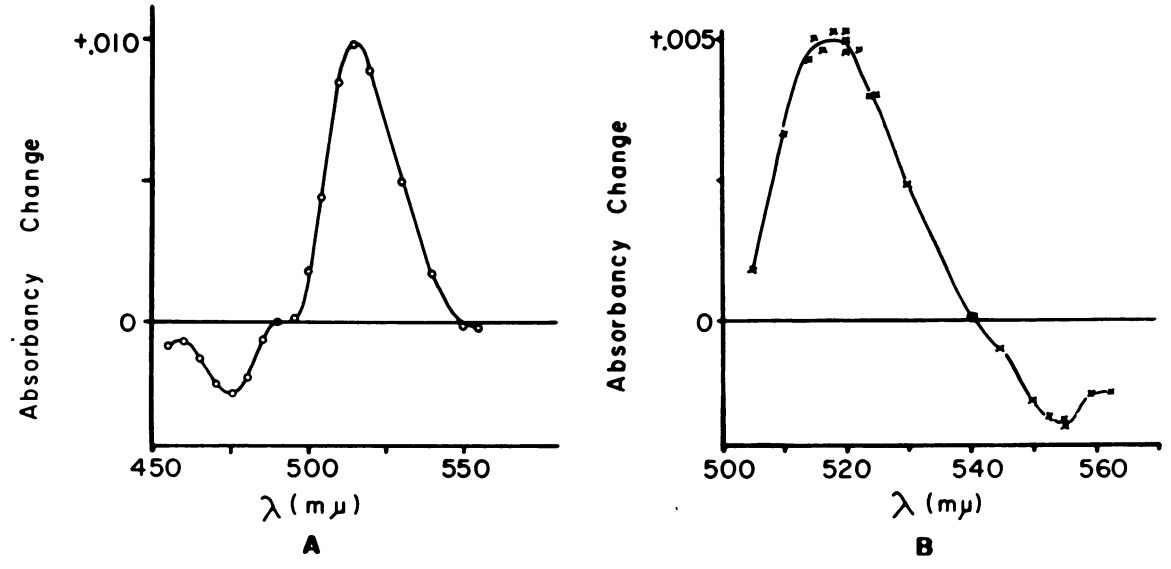

FIG. 8. Spectroscopic effects of illumination upon two types of green cell under anaerobic conditions (N_2 + 5 % CO_2). A. Chlorella. B. Chlamydomonas. The illumination used in these experiments was in general of lower intensity so that the kinetics resembled those of the left-hand portion of figure 5. The positive values of absorbancy change are plotted in an upward deflection, i.e., these are the absorption bands that appear upon illumination. Temperature in all experiments was 26° C. (668 and 666).

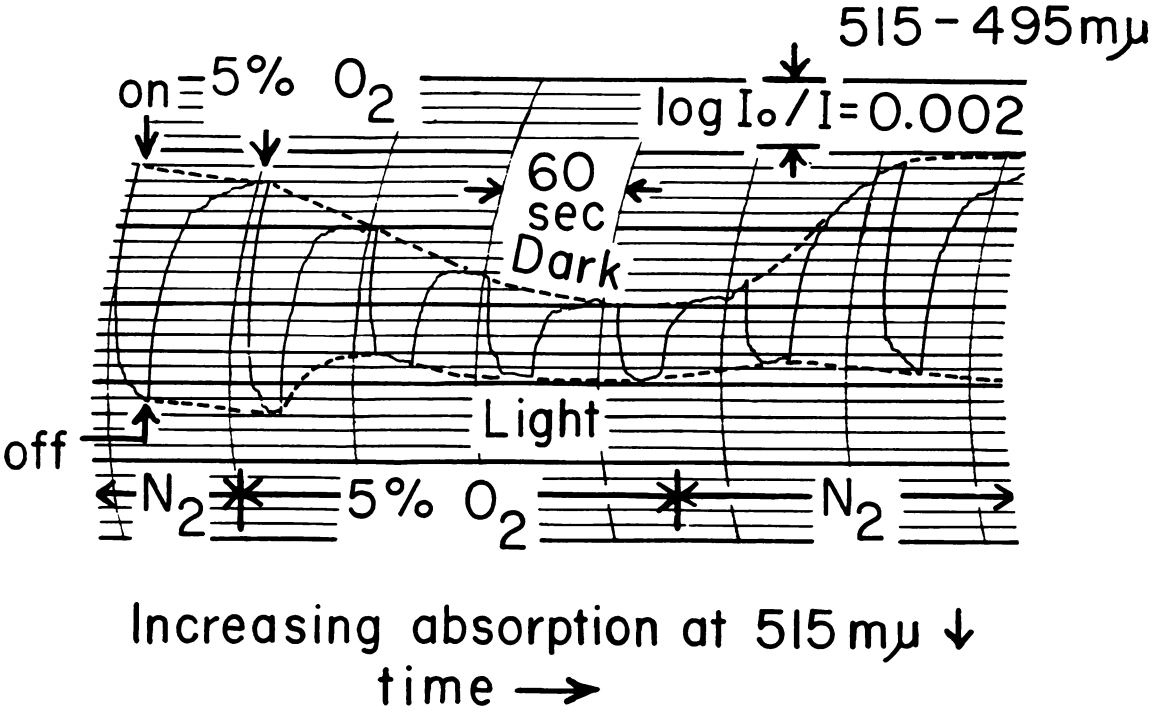

FIG. 9. The effect of oxygenation upon the absorbancy changes caused by low intensity illumination. The Chlorella suspension, initially in anaerobiosis, is repetitively illuminated throughout the experiment. After the first light-dark cycle, the gas mixture is changed to 5% oxygen and the attendant changes in responses to illumination are recorded. To serve as a control, 5% oxygen is replaced by nitrogen and the initial conditions are re-established. The conditions of the experiment are identical to those of the left-hand portion of figure 5. (626).

(fig 8 B), and in this case there is some absorption change in the region of 550 and 560 mμ. The trough between 550 and 555 mμ is so broad that it cannot be attributed solely to cytochrome f (see below).

EFFECT OF ADDED OXYGEN: We now return to the question of the relationship between oxygen concentration and spectroscopic effects. In figure 9 are recorded a number of cycles of spectroscopic changes caused by alternation of dark and light. The light intensity used is such that a phase 3 transition predominates (cf fig 3 A). After a trial period of light and darkness in the presence of nitrogen, the gas is changed to 5% oxygen, and after two minutes the amplitude of the light effect has diminished to about one-third and represents the smaller "aerobic light effect" (phase 2 transition). Upon readmission of nitrogen the larger phase 3 transition is established. The dashed curve is drawn on the figure to connect the extremes of the traces and to emphasize the change produced by oxygenation. The dashed line labeled "dark" indicates the increased absorbancy at 515 mμ, measured with respect to 495 mμ, caused by oxygen alone. The dashed trace labeled "light" indicates a change that may be related to the phase 1 transition —an increase of light requirement for the oxygenated cells, and hence a net decrease in the absorption of the illuminated cells in oxygen.

The phase 1 effect is explained by the graph of figure 10 in which the relationship between the intensity of illumination and the magnitude of the absorbancy change measured immediately after illumination under aerobic (open circles) and anaerobic (crosses) conditions. The latter is an unusual saturation curve, but is converted to a simple curve by subtracting the aerobic effect from the anaerobic one.

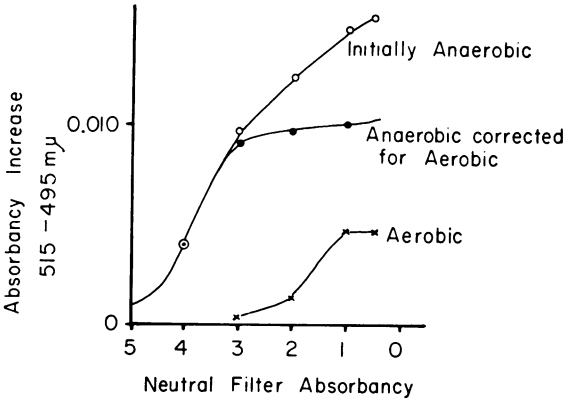

FIG. 10. Effect of illumination intensity upon the extent of the absorbancy changes at 515 mμ under aerobic and anaerobic conditions. The light intensities are varied by neutral filters. In order to compute the true anaerobic light effect the aerobic curve is subtracted from the anaerobic one. (701).

Thus we obtain the solid circles representing the basic anaerobic effect. It is now clear that approximately 100 times the light is required for half-maximal aerobic effects as for half-maximal anaerobic effects. Thus a cell suspension, initially anaerobic and illuminated with a strong light intensity, will show a slow decrease of absorption at 518 mμ as the photosynthesis proceeds. In figure 9, the lower dashed line indicates that oxygenation diminishes the response to light. The simplest hypothesis for the phase 1 effect is that the photosynthetically produced oxygen is the cause of the diminished response. However, illumination of the aerobic cells (fig. 4) gives an effect similar to phase 1. Thus the simple hypothesis must be extended to include the possibility that photosynthesis products other than oxygen can cause the phase 1 effect, or that more complex hypotheses need to be considered (6, 16).

So far as we have not shown that the phase 3 transition caused by illumination has a difference spectrum identical to that of the phase 3 transition caused by oxygenation by an external gas. This is taken up in the next two sections.

Oxidized Minus Reduced Difference Spectrum for Chlorella and Chlamydomonas: By repeating at various wavelengths the experiment of figure 9 without red illumination, we can record point by point the deflections obtained upon oxygenating the anaerobic algae in the dark. In figure 11 A, the oxidized minus reduced spectrum for a Chlorella suspension shows a peak very near to 515 mμ. Chlamydomonas grown in a medium containing acetate respire rapidly and become anaerobic soon enough after oxygenation to permit the use of a suspension of organisms in a 1-cm path cuvette. Since no red cross-illumination was used, no filters were needed to shield the photomultiplier. Thus the spectroscopic data of figure 11 B cover the range 490 to 648 mμ. This difference spectrum shows a sharp peak at 515 mμ, the typical shoulder at 530 mμ with a trough at 550 mμ, and no other very large changes out to 648 mμ. The 515-mμ peak and the 530-mμ shoulder are clearly identified with the characteristic light responses of the plants to oxygen. The 550 mμ trough is too broad to be attributable to cytochrome f alone, although it is possible that two or more cytochrome bands could fuse to give this broad trough.

A number of controls have been made to ensure that no artefact occurs during oxygenation in the dark. A more important one is that the absorbancy increase occurs just as rapidly and to the same extent if the measuring light is turned off during oxygenation.

Aerobic-Anaerobic Difference Spectrum Obtained by Illumination: Instead of oxygenating the Chlorella by a stream of gas as has been done in order to obtain the data of figure 11, it is possible to use the oxygen produced in photosynthesis for this oxygenation. In the first portion of figure 12, the measuring wavelength is at 507 mμ and in the second, 522 mμ, and 495 mμ is used as the reference wavelength in both cases. Illumination of the anaerobic algae for about 2 minutes at an intensity comparable to that of figure 5, right hand portion, causes a sharp increase of absorbancy at the measuring wavelengths, followed by the phase 1 decline of absorbancy attributed to the decrease of light sensitivity. On turning off the light, the abrupt phase 2 transition from il-

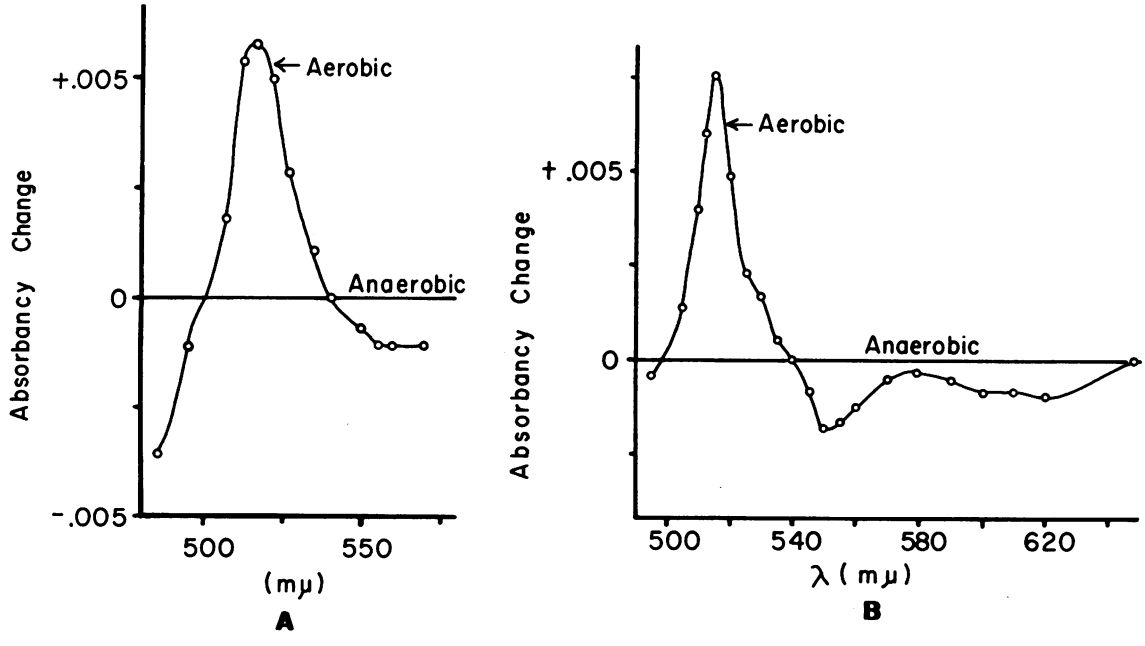

Fig. 11. Aerobic minus anaerobic difference spectra for Chlorella (A) and for acetate grown Chlamydomonas (B). The conditions of oxygenation were the same as those represented by figure 8 A. (666 and 668).

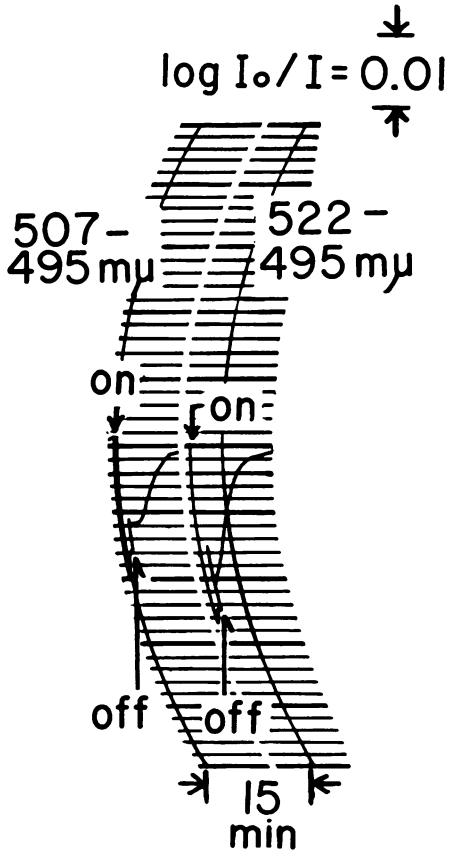

FIG. 12. Illustrating the use of the phase 3 transition to measure the oxidized minus reduced spectrum for Chlorella. Following the abrupt transition which occurs upon cessation of illumination (phase 2) the slow disappearance of the absorption band (phase 3 transition) can be followed at 507 and 522 mμ (cf fig 3). The measuring wavelength is shifted from 507 to 522 mμ at the dividing line in the figure. (707).

luminated to dark aerobic cells occurs. The cells are now in an aerobic dark state and the absorbancy changes in the next ten minutes represent phase 3 of figure 5 and are attributed to the disoxygenation of the cells. These two records show the effects of 522 mμ to exceed those at 507 mμ, and a repetition of these experiments at wavelengths in the region of 500 to 530 mμ gives a difference spectrum for the phase 3 transition that agrees with that of figures 8 A and 11 A to within the experimental error. Thus three methods for observation of the phase 3 effect give similar results: 1) oxygenation of the dark, anaerobic cells; 2) dim illumination of the anaerobic cells, and 3) the aerobic-anaerobic transition of cells following cessation of bright illumination.

DISCUSSION

The general effects of illumination of the green plant cell and purple bacteria present an interesting parallel that deserves discussion. Even though the bacteria do not form oxygen upon illumination, there is very clear evidence that an oxidant is produced, and, based upon recent information, this oxidant is powerful enough to oxidize not only the cytochromes of the respiratory chain but, in addition, to affect carotenoid pigments (Dr. Lucile Smith, (20)). It has also been found that the response to the cell depends upon the presence or absence of oxygen, since the spectroscopic effects observed in the anatrobic cells are greatly diminished and changed in nature by aerobiosis. In a companion paper (15), studies of a Chlamydomonas mutant are reported and there the illumination of the anaerobic cells oxidizes pyridine nucleotide and cytochrome b in a manner similar to, but less extensive than, that caused by oxygenation. These oxidations caused by illumination under anaerobic conditions are also diminished and changed in their nature under aerobic conditions. In studies of the normal green cell, we find a similarity of the basic phenomena. First, the magnitude of the spectroscopic effects produced by illumination is sensitive to added oxygen, as in the case of the two other systems: in Chlorella over 50 % of the absorbancy change at 515 mμ produced by illumination is caused by oxygenation in the dark. Second, oxygenation in the dark causes a spectroscopic effect in the region 475 to 540 mμ similar to (but smaller than) that caused by illumination with red light.

In the Chlamydomonas mutant, oxygenation in the dark causes spectroscopic changes similar to those recorded in other microorganisms and there is no difficulty in recognizing most of the pigments as members of the respiratory chain of the photosynthetic cells. Since illumination causes similar spectroscopic changes, identification of most of the pigments active in respiration and photosynthesis is not difficult.

In the mutant, the nature of the photochemical reaction can be elucidated: for example, the formation of an oxidant upon illumination is easily proved because the pigments involved are oxidation-reduction indicators (pyridine nucleotide, cytochrome b, etc.). But in the normal green cell, the pigment responding most clearly to red illumination is not recognizable as a member of the respiratory chain of any non-photosynthetic cell, nor are its properties as an oxidation-reduction indicator known. Thus, various criteria must be considered in order to establish the relative oxidation state of the substance responsible for the 518-mμ band:

1) Physical evidence for the formation of an intracellular oxidant upon illumination is obtained from observations of respiratory carriers in purple bacteria, a Chlamydomonas mutant, Porphyridium, and with less certainty, Chlorella (3).

2) No unequivocal evidence for the formation of the photochemical reductant has yet been obtained from spectroscopic studies of respiratory carriers of the uninhibited cells, purple bacteria, a Chlamydomonas mutant, or Chlorella. Although Duysens obtains suggestive evidence in recent fluorimetric studies of *Rhodospirillum rubrum* (18).

3) The 518-mμ absorption band persists after cessation of illumination when extracellular oxygen can be demonstrated (fig 4) and disappears approximately as the extracellular oxygen concentration falls to zero (fig 3 A).

4) An absorption band very similar to that obtained by low intensity illumination (phase 3 effect) is obtained by oxygenation of Chlorella and Chlamydomonas in the dark.

5) Studies of the respiratory carriers of various cells and mitochondria isolated therefrom have not shown any reductant to be produced upon oxygenation of the anaerobic cells.

On the basis of these data it is probable that the 518-mμ band identified in the phase 3 transition corresponds to a higher oxidation state. Since the disappearance of a band at 475 mμ is simultaneous with the appearance of the 518-mμ band, the 475-mμ is tentatively attributed to the reduced form of the pigment. The oxidant may be molecular oxygen or intermediates produced in water splitting (OH, etc.).

The phase 3 transition may differ from the "aerobic light effect" or phase 2 transition. First, about a 100 times more light is required for phase 2 than for phase 3. Second, extensive oxygenation of Chlorella does not suppress the phase 2 effect, as would be expected if the phase 2 effect were just a further oxygenation of the cells. Third, the difference spectra for high intensity illumination of the aerobic cells show bands that are clearly absent in the aerobic-anaerobic difference spectra (compare Kok's figure 1 (10) with our figure 11 and note the absence of the multiple bands in the region 560 to 640 mμ in our figure 11 B). It is very likely that additional components are involved in the phase 2 effect.

Witt (7) finds the phase 2 effect to disappear more rapidly on cessation of illumination in the presence of 2,6-dichlorophenol indophenol. While he interprets this result as evidence for a more reduced state in the phase 2 518 mμ-compound, the lack of an effect of ferricyanide or quinone requires an explanation. In fact the action of 2,6-dichlorophenol indophenol on energy-linked reactions is rather poorly understood (21, 22), and may not afford a definitive test of the oxidation state of the 518-mμ compound involved in the phase 2 effect. Thus theories which identify this substance with the reduced intermediate XH may be premature.

The dark form of the pigment from which the 518-mμ compound is derived in the phase 3 transition is unknown. It is very significant, however, that this absorption band does not appear upon illumination of a carotenoid-deficient Chlamydomonas mutant, while it shows clearly in the normal cells. A very similar relationship between a carotenoid-free mutant of purple bacteria and the normal cells has been demonstrated by Dr. Lucile Smith (personal communication). On this basis we can conclude that carotenoid is necessary for the 518-mμ band. It should be pointed out that the presence of carotenoid may not be a sufficient condition for the appearance of the 518-mμ absorption band because Duysens failed to demonstrate this band upon illumination of *Porphyridium cruentum* (4).

The function of the 518-mμ compound in photosynthesis has not been demonstrated, but there have been proposals that it may represent spectroscopic evidence of the primary process in photosynthesis (7, 10), and a proposal for its interaction with chlorophyll has recently been made (16). In addition, recent data show chlorophyll-carotenoid interaction in vitro (23). The phase 3 transition may not be involved in a primary process since it can be caused by a dark reaction (oxygenation). Neither the phase 2 or 3 transition is observed in the Chlamydomonas mutant in which active photosynthesis occurs, and hence the 518-mμ compound may not be required for photosynthesis. A possible function for this compound in the protection of the cell from excess oxidizing equivalents is discussed elsewhere (15).

There now exist a number of difference spectra for the illumination of Chlorella and significant differences are beginning to be established. The two spectra which differ markedly from those obtained by Duysens (4), Spruit (9) and this work are those obtained by Strehler and Lynch (6) and by Kok (10). The last two studies have an important feature in common: they were both based on measurements of the amplitude of the dark reaction at arbitrary times after bright illumination. Thus a wide range of wavelengths was covered. A disadvantage is that the complete reaction kinetics in light and dark could not be recorded. Strehler and Lynch used a flow system and Kok used a rotating shutter; both precluded obtaining data during illumination. Thus the relative sizes of the peaks in their spectra depend upon the assumption that the nature of the reaction kinetics does not change with wavelength. For example, Strehler and Lynch recorded a portion of the dark reaction kinetics that they termed "negative overshoot" which causes their results to be inverted (cf reference 5). In addition, Strehler and Lynch find that the "negative overshoot" has different kinetics at 525 and 648 mμ (6). In Kok's method, a decrease in the rate of the dark reaction at different wavelengths would cause an increase in the reading given by his instrument and thereby over-emphasize the relative magnitude of the slower dark reactions. Technically this is because Kok's first electronic switch cannot open "immediately after the flash"; a certain delay must surely be present, during which a variable decay of the spectroscopic effect may occur. Thus, although the methods cover a wide range of wavelengths at high sensitivity, the relative sizes of the peaks should be controlled by an apparatus of the type used by Duysens or by the double-beam instrument of the type described here.

The "negative overshoot" phenomenon (6) does not appear in these experiments in Chlorella, a result that is apparently in agreement with those obtained by others (7, 9, 10, 17). Thus further studies of the

conditions under which Strehler and Lynch obtained their results are desirable.

Spruit (9) finds that anaerobiosis causes a marked decrease of the 518-mμ band, a result contrary to Duysens' (8) and to the more detailed results of this paper. Since Spruit has not yet published a spectrum for the effect he has observed, it is difficult to compare his result with ours. On a technical basis it should be pointed out that the compensating beam method he used is very sensitive to non-specific light absorption effects that might have been altered by the establishment of anaerobiosis in Spruit's studies. This is because the "compensating beam" does not pass through the sample.

Lastly our data show no change in either the aerobic-anaerobic or the phase 3 difference spectra that can surely be attributed to an oxidation of cytochrome f upon illumination of the anaerobic cells. The absorption changes in this region of the spectrum are too broad to be attributed with any assurance to cytochrome peaks. Nevertheless the evidence from other workers for the participation of a cytochrome in the phase 2 effect is convincing (4). Whether this cytochrome is of type f or c is not clear (for a discussion, see (15)).

In summary, the spectroscopic changes that are caused by illumination of Chlorella are complex and depend upon the metabolic state of the cell which is very sensitive to illumination. On the technical side, these effects are large enough so that they can be measured by a number of spectrophotometric methods, each capable of giving somewhat different results. It is a matter of great importance to control both the biological and the physical aspects of studies of this interesting phenomenon so that meaningful and consistent data can be obtained, and that concordant interpretations of the nature of these effects can be achieved.

Summary

The spectrum corresponding to the differences of absorption between aerobic and anaerobic suspensions of Chlorella and Chlamydomonas have been recorded with a double beam bichromatic instrument. Instead of a cytochrome spectrum we find that oxygenation causes a major absorption band to appear at 518 mμ and a small band to disappear in the region of 550 mμ; the latter band is considered to be too broad to be attributed with certainty to cytochrome oxidation. These absorption bands are very similar to those which appear upon dim illumination of anaerobic cells (phase 3 transition). It is concluded that the absorption changes caused by dim illumination are associated with oxygenation of the cells. The spectroscopic effects caused by oxygenation and by low intensity illumination are found to differ in significant details from those caused by high illumination of the aerobic cells (phase 2 transition) where considerably higher light intensities are required for saturation and in which additional absorption bands are observed by other workers. A comparison of the properties of normal cells showing light- and oxygen-induced spectroscopic changes at 518 mμ with those observed in a carotenoid-deficient mutant which does not show such a change suggest that carotenoid is required for the 518-mμ absorption band (cf 6). Similar considerations suggest that the 518-mμ absorption band observed on oxygenation or low intensity illumination may not be required for photosynthesis. The identification of the phase 2 transition with the primary process of photosynthesis, as postulated by other workers, requires further study.

These studies emphasize the need for proper choice of the physical method and adequate control of the metabolic state of the photosynthetic and respiratory systems of the cell in order that concordant interpretations of the spectroscopic data can be made.

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OXYGEN AND LIGHT INDUCED OXIDATIONS OF CYTOCHROME, FLAVOPROTEIN, AND PYRIDINE NUCLEOTIDE IN A CHLAMYDOMONAS MUTANT[1,2]

BRITTON CHANCE AND RUTH SAGER
THE JOHNSON FOUNDATION FOR MEDICAL PHYSICS, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA, PENNSYLVANIA AND DEPARTMENT OF ZOOLOGY, COLUMBIA UNIVERSITY, NEW YORK, NEW YORK

In green cells direct spectroscopic studies of the respiratory pigments involved in oxidation-reduction reactions are difficult because their absorption bands are obscured by those of the photosynthetic pigments. Visual spectroscopy has been used by Hill and by Davenport (1, 2) on etiolated leaves and on cytochromes extracted from them and from green algae, but no studies comparable to the classic ones of Keilin on non-photosynthetic systems have yet been carried out. Spectra representing absorbancy changes caused by illumination have been reported by Duysens (3, 4, 5) and by Chance and Strehler (6) for Chlorella. Spectra representing changes immediately following cessation of illumination have been reported by Strehler and Lynch (7), and long persistent changes following a previous illumination are reported by Lundegårdh (8). Witt has studied in detail the kinetics of spectroscopic changes at 520 and 480 mμ (9). Inconsistencies in the results so far obtained indicate the necessity for a comprehensive study of spectroscopic effects in the intact cell. Duysens, who first found increased absorbancy at 515 mμ upon illumination of Chlorella, later found the effect to be lacking in Porphyridium. He further reported that cytochrome f was oxidized upon illumination of Chlorella on the basis of an absorbancy decrease at 420 mμ, an observation which was later verified in Porphyridium, but, in that case, he found, corresponding to the Soret band at 420 mμ, an α band at 555 mμ. This α band agrees with that of purified cytochrome f, but the Soret band differs by 4 mμ, a discrepancy beyond the experimental error.[3] This inconsistency in the identification of the cytochrome involved also applies to the question of whether pyridine nucleotide has been observed to be affected by illumination (4). Broad and non-specific increases of absorption in the ultra-violet region were observed for Porphyridium (4) and were attributed to increased reduction of pyridine nucleotide even though no 340 mμ peak was observed. Lundegårdh, using slower methods than any of the other authors, finds that oxidation of cytochrome f following illumination must have persisted (according to our estimates) for at

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[3] A close reading of Davenport and Hill's graph (19) gives 424 mμ as the correct wavelength and this value is used by Duysens (4).