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

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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).

least 30 seconds.[4] The cause of some of these discrepancies may be the failure to recognize aerobiosis or anaerobiosis as a factor affecting the nature and extent of the spectroscopic effects caused by illumination, even though this had already been shown to be of importance in the studies of *Rhodospirillum rubrum* (10). The possible importance of anaerobiosis was suggested by Duysens' recommendation that the cells be allowed to stand in the dark for "half a day" in order to enhance the spectroscopic effect of illumination and by his later note regarding the enhancement of the light-induced increase of absorption at 515 mμ by anaerobiosis (5).

The cytochromes that might participate in respiratory or in photosynthetic electron transfer in green and etiolated plants have been studied intensively by Hill and his collaborators (1, 2, 11). Cytochromes c, b_3, f, and b_6 have been isolated and interest has centered about the possibility of cytochromes f and b_6 participating in the photosynthetic process. The relative values of their oxidation-reduction potentials led Hill to speculate that b_6 would be completely reduced even in the presence of oxygen and that cytochrome f is oxidized in the illuminated leaf. An absorption band has been observed in the leaves of the golden varieties of certain plants in the position appropriate to reduced cytochrome b_6, although this absorption band was not observed in the chloroplast preparation unless dithionite was added.

It is apparent that spectroscopic data on the respiratory chain of the green cell together with the aerobic and anaerobic effects of illumination are necessary in order to give a comprehensive picture of the interaction of the respiratory and photosynthetic processes. While the oxidized minus reduced spectrum of Chlorella shows chiefly the 515 mμ pigment and very little indication of cytochrome, the studies on a pale green mutant of Chlamydomonas having low concentrations of carotenoids and chlorophyll (26) show clearly difference spectra of cytochrome, flavoprotein, and pyridine nucleotide components that are involved to varying extents in respiratory and photosynthetic reactions. The response of these components to aerobiosis and to anaerobiosis suggests their participation in the respiratory chain and their response to illumination under aerobic and anaerobic conditions may identify those components involved in photosynthesis. The importance of oxidation reactions caused by illumination is emphasized and the relative speeds of response of the components to illumination gives preliminary indication of the sequence of their reactions.

[4] Lundegårdh's recorder (8, 22) is a Leeds and Northrop device that plots one point every 2.2 seconds, and would require at least 30 seconds to record, at intervals of about 2 mμ, a spectrum from 540 to 570 mμ. The scanning mechanism described (22) requires an interval of 4 seconds between readings or a total interval of a minute. A personal communication from Lundegårdh verifies that by "a few seconds" (8) he meant 10 to 20 seconds. In this time interval after cessation of illumination, our rapid recordings show that the oxidation of cytochrome f directly caused by illumination would have fallen to a small fraction of its initial value, to less than 10 %. One could propose that Lundegårdh recorded this last 10 % of the light reaction, but his figure 3 (8) shows that ascorbate reduction gives "completely reduced" bands of cytochromes c and f and that these absorbancy changes are very nearly identical in magnitude to those recorded upon illumination. Thus, the hypothesis that Lundegårdh observed the same phenomenon as Duysens and we, appears to be untenable and the hypothesis proposed here that he observed the persistence of oxygenation of a previously anaerobic Chlorella suspension fits nicely with his own data. This confusion was caused by lack of adequate controls on oxygen concentration during experimentation with living cells.

Methods

Measurements were carried out with a double-beam differential spectrophotometer (12) fitted with an especially designed moist chamber described in the preceding paper (6). Since a wide range of wavelengths was covered in these experiments, a Wratten 39 and a Corning 978½ filter was used, the latter serving to eliminate the infra-red transmission of the former. This filter combination permitted measurements down to 320 mμ with an acceptable signal-to-noise ratio, and an excellent signal-to-noise ratio from 340 to 480 mμ. The filter combination also minimized stray light effects in the grating monochromators. For observations in the ultra-violet region, the tungsten source was operated at a considerable over-voltage in order to give a better emissivity. For studies of the visible region of the spectrum, the most satisfactory filter combination was Corning 430 plus 978½. For red illumination, Corning 2030 filter provided adequate intensity for saturating effects which were readily controlled by the insertion of neutral filters.

As in the previous paper (6), the oxygen concentrations were altered by passing either nitrogen or oxygen mixtures over the algal suspension. Although 5 % CO_2 was present in the nitrogen and oxygen, it was apparently not necessary for the effects upon cytochromes b and f. In one test, pure nitrogen was passed over the cells for two hours with no evidence of CO_2 lack, but it is possible that a longer interval might have produced a demonstrable effect.

The moist chamber was filled as described in the previous paper (6). The mutant cells settled to the bottom of the chamber in a uniform film of several tenths of a millimeter thickness. The wild type formed an irregular film. The respiration of the suspension was measured by the two types of platinum electrodes described previously (6). The Chlamydomonas cells respired so rapidly that records of their oxygenation and disoxygenation resemble those obtained with yeast (cf fig 2 of (6)). Prolonged oxygenation of the suspension was required to oxygenate completely the respiratory system and to eliminate the "anaerobic light effects."

Carbon dioxide utilization by the cells was measured in terms of the decrease of acidity. A suitable apparatus for such changes was described previously for simultaneous measurements of respiration and fer-

mentation in suspensions of yeast cells (12) and Spruit and Kok have applied similar methods to O_2 and CO_2 exchange in Chlorella (27, see also 13). Suitable controls (fig 2) provided direct calibration of the sensitivity of the bicarbonate-buffer cell suspension system. The effects of heating the solution by the lamp as well as loss of CO_2 to the atmosphere were found to be negligible compared to the changes caused by photosynthesis of the algae. The effect of electrode polarization upon the potential of the glass electrode was controlled by periodically removing such polarization voltage and noting the effect upon the electrode potential. The same 100-watt tungsten projection lamp was used to illuminate the moist chamber as was used to illuminate the cuvette in these studies.

Materials

The pale green mutant (strain no. 95) was obtained in 1951 after ultra-violet irradiation of the green alga *Chlamydomonas reinhardii* and has been maintained subsequently on an acetate medium in the dark. This strain has never back-mutated to the normal green. For these experiments cultures were grown in the dark on the acetate medium (25) and harvested at the end of the growth cycle. The cells were centrifuged and resuspended in their own medium or in 0.01 M $NaHCO_3$. The moist chamber requires 1.5 ml of a cell suspension the density of which was adjusted to approximately 20 micromolar chlorophyll.

For comparison of photosynthetic activity, the normal green algae were grown in the presence of acetate in the light and in the dark, and the cells were prepared as described above and suspended in a bicarbonate buffer. The normal green strain used was the one from which the pale green mutant had been obtained.

Results

Extractable Pigments: The carotenoid and chlorophyll content of the mutant is indicated by the graph of figure 1, which is a recording of the absolute spectrum of an ether solution of pigments extracted by treatment of the cells with 80 % acetone. It is seen that there are major absorption peaks near 660 and 430 mμ. At the sensitivity used in recording these peaks, no absorption bands can be detected that might be attributed to carotenoid, but if the scale is multiplied ten times, a small shoulder is seen on the 430 mμ peak at about 480 mμ which is probably due to carotenoid. In a study of pigment content of the pale green mutant (26), it was found to contain only 1/500 the total carotenoid of normal green dark-grown cells, and about 1/10 the chlorophyll. The only carotenoids detected in extracts of a one gram (dry wt) sample of pale green mutant cells were alpha and beta carotene. In the normal green cells, these two pigments account for about 65 % of the total carotenoids. The mutant has a chlorophyll to carotenoid ratio of 180 : 1 (mole basis) while the normal cells have a ratio of 15 : 1 when grown in the light and only 4 : 1 when grown in the dark. On the basis of beta carotene only, these ratios are respectively 240 : 1, 24 : 1, and 7 : 1. Thus the mutant has less than 1/30 the β-carotene relative to chlorophyll of the normal green dark-grown cells.

An approximate value of the cytochrome content relative to that of chlorophyll is provided by a measurement of the ratio of the absorbancy change at 430 and 405 mμ caused by the transition from aerobiosis to anaerobiosis to the chlorophyll absorption at 430 mμ in 80 % acetone. The ratio is 1:12 and has roughly the same value when converted to a mole basis. This value indicates the relatively favorable

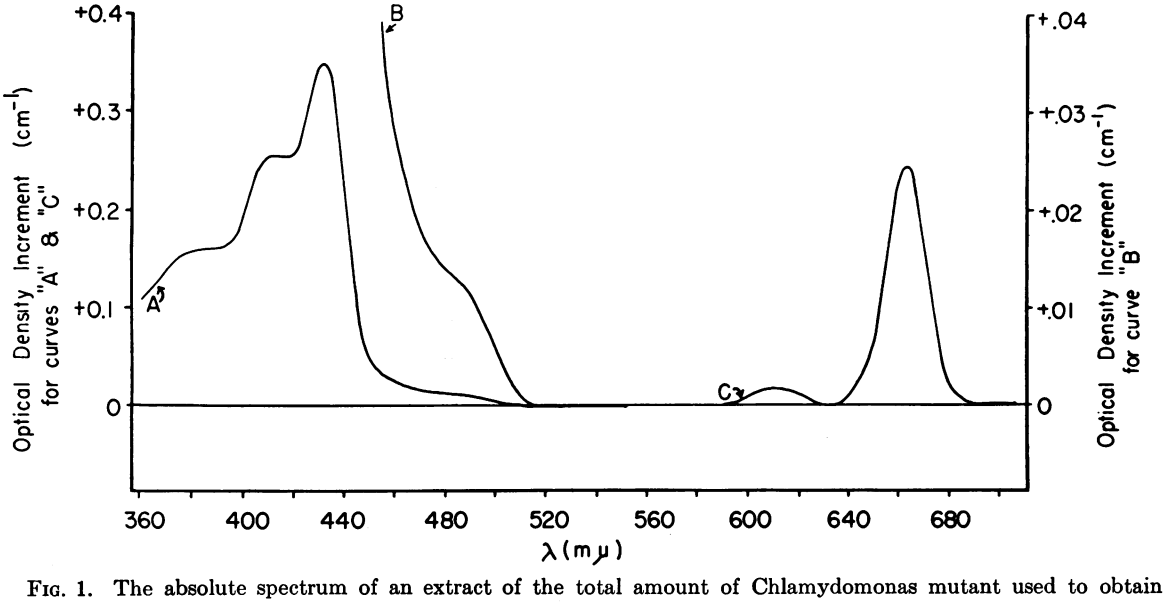

Fig. 1. The absolute spectrum of an extract of the total amount of Chlamydomonas mutant used to obtain the spectrum of figure 10. Curves A and C are recorded at the same sensitivity, and B is amplified 10-fold. (674 d).

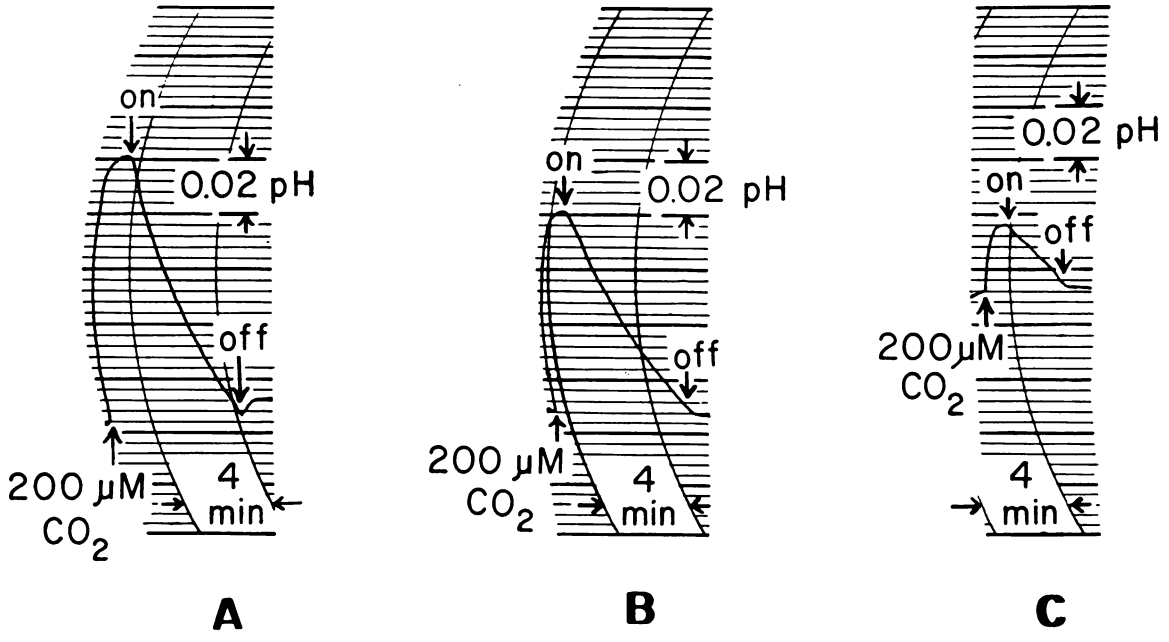

FIG. 2. A quantitative determination of the rate of CO_2 uptake by 3 different types of Chlamydomonas. The CO_2 uptake is measured by the pH change in a 0.01 M $NaHCO_3$ solution in a 2.8-ml cuvette. The electrode sensitivities and time scales are included in the diagram as well as the initial values of pH. The upward deflection of each of the three traces marks a calibration by the addition of 200 micromoles CO_2. The interval of illumination by 110 ft-c tungsten light is also indicated. A. Normal cells are grown in the light in acetate medium. B. Normal cells are grown in the dark in acetate medium. C. The pale green mutant is grown in the dark in acetate medium. (710 f).

conditions provided by the pale green mutant for observation of the cytochromes.

PHOTOSYNTHETIC ACTIVITY, *Carbon Dioxide Fixation:* The carbon dioxide fixation by three types of Chlamydomonas is illustrated by figure 2. The carbon dioxide uptake of the illuminated cells is measured in terms of the change of pH of 0.01 M $NaHCO_3$ solution by the glass electrode inserted into an open cuvette. As contrasted with the recent method of Rosenberg (13) whereby the conversion from pH change of carbon dioxide concentration was calculated for a solution free of interfering substances, we calibrate directly by the addition of a known volume of a saturated solution of carbon dioxide as do Spruit and Kok (27). Thus, the three records start with the addition of the calibrating solution to the dark cell suspension. This causes a decrease of pH (an upward deflection of the traces) and the amplitude of the deflection is indicative of the buffering capacity of the various suspensions. In the case of both the dark-grown green cells (B) and the pale green mutant (C), some acetate was present from the growth medium. The differences of these calibrations illustrate the importance of using this method (cf Rosenberg 13, table I).

As soon as the pH change due to addition of the calibrating solution is completed, the light is turned on and CO_2 uptake begins, prior illuminations having reduced the induction effects to a negligible value. The illumination is maintained until an amount of CO_2 equal to the calibration solution has been taken up. Then the light is turned off, and as shown by the traces, the CO_2 uptake comes to a halt. The rate of CO_2 uptake is simply the quotient of the concentration of CO_2 added in the calibrating solution (200 micromolar) and the number of seconds required to utilize that amount of CO_2. After the activities have been determined, the samples are treated with 80 % acetone, centrifuged, and the chlorophyll estimated in the Beckman spectrophotometer at 660 mμ. These values were divided by the molecular extinction coefficient for chlorophyll a (82 $cm^{-1} \times mM^{-1}$ (14)). In the normal cells, chlorophyll b is also present and the concentration found for chlorophyll a is multiplied by 1.7 to give the total concentration. In the mutant cells, the correction factor is only 1.1. The photosynthetic activity is given in terms of chlorophyll turnover—μM CO_2 sec^{-1} $chlorophyll^{-1}$. The data are summarized in table I, and it is seen that the turnover number of the chlorophyll increases from the normal, to the dark-grown green cells, to the pale green mutant cells. These turnover numbers for chlorophyll are the reciprocal of the "assimilation time" and an inspection of Rabinowitch's table 28 V (15) shows that the values of table I agree very closely with those obtained for a wide range of leaves and algae; the best value that he gives for Chlorella is 0.03 and agrees very well with the values obtained for the light- and dark-grown Chlamydomonas.

Since the ratios of chlorophyll to β carotene con-

TABLE I

COMPARISON OF CO_2 FIXING ACTIVITIES OF THREE FORMS OF CHLAMYDOMONAS

DESCRIPTION	CONDITIONS OF MEASUREMENT	INITIAL pH	$\mu M\ CO_2$/SEC IN LIGHT	D_{663} (CM^{-1})	ϵ_{663} ($CM^{-1}MM^{-1}$)	$CO_2 \times$ CHLOROPHYLL$^{-1} \times SEC^{-1}$	RATIO OF CHLOROPHYLL TO CAROTENE B	$CO_2 \times$ CAROTENE B$^{-1} \times SEC^{-1}$
Light grown normal	Aerobic	7.7	-0.5	1.9	82	-0.02	24	-0.48
Dark grown normal	Aerobic	7.6	-0.5	1.4	82	-0.03	7	-0.21
Dark grown pale green mutant	"Anaerobic"	7.4	-1.0	1.1	82	-0.10	240	-24.00

Cells resuspended in 0.01 M $NaHCO_3$. White light, ~ 1000 ft-c. (710 f).

centration in the three types of cells are available, it is possible to calculate the turnover number of β carotene, assuming that it is participating in photosynthesis. It is seen that the value for the pale green mutant would have to be 100 times greater than that for the dark-grown normal cells. The significance of this result is discussed below.

Oxygen Evolution: Due to the relatively high respiratory activity of the pale green mutant under the conditions of figure 2, these cells were anaerobic at the time of illumination and the increase of oxygen concentration in the medium was too small to be measured by the platinum electrode. By passing a mixture of 5 % oxygen and 95 % nitrogen over the surface of the washed cells in the moist chamber, the oxygen concentration in the medium was raised to the point where the spiral platinum electrode could be used to demonstrate evolution of oxygen upon illumination. A typical record of both platinum electrode and spectrophotometer traces is shown in figure 3. The record begins at the left-hand edge with the cell suspension in the dark and both traces horizontal. Upon illumination, there is a disturbance of the spectrophotometric trace and then an abrupt upward deflection which corresponds to a decrease of light absorption at 430 mμ measured with respect to 450 mμ. This corresponds to an oxidation of cytochrome of

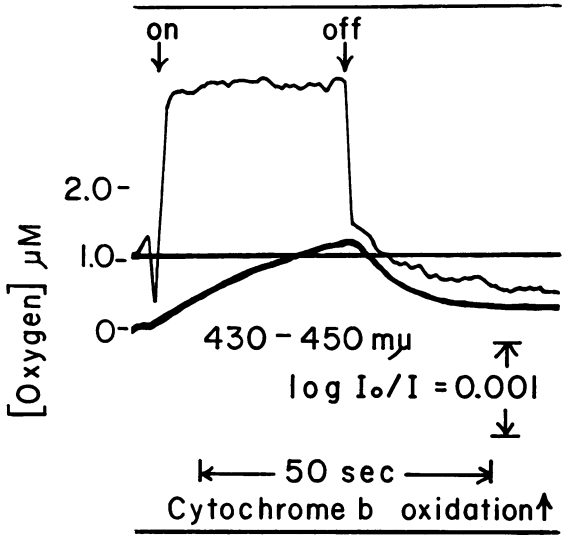

FIG. 3. Evidence for oxygen evolution caused by illumination of the pale green mutant. The cells are contained in the moist chamber described in the text and the oxygen concentration (lower trace) is measured by a spiral platinum electrode located in the lucite bottom of the chamber and polarized at -0.6 volts. The calibration for the oxygen concentration is given in the diagram and the upper trace represents a spectroscopic recording of the simultaneous absorbancy change by the double beam spectrophotometer. (702 b).

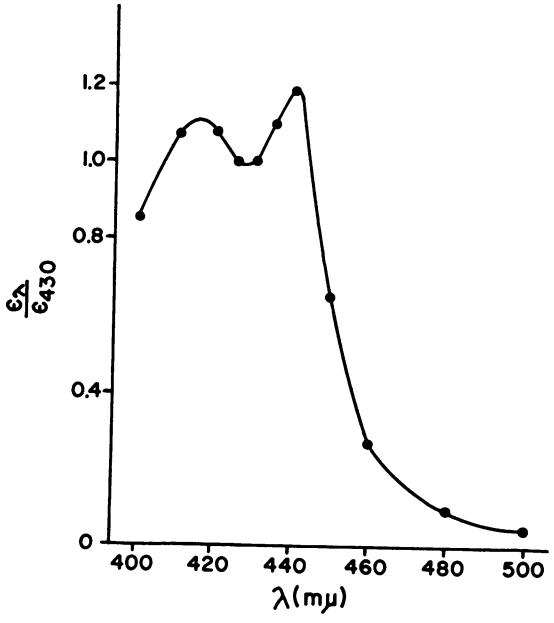

FIG. 4. The relative effectiveness of wavelengths of light appropriate to chlorophyll and carotenoid in photosynthetic oxygen evolution in the pale green mutant. The method used is that of Castor and Chance (17). (Experiments carried out in collaboration with Mr. Selwyn Ramsay.) (754).

type b. Shortly after illumination, the platinum electrode trace rises indicating an increased oxygen concentration due to photosynthesis in mutant cells. Upon turning off the light, an abrupt reduction of cytochrome b occurs and is followed by a fall of oxygen concentration due to dark respiration. The increase of oxygen concentration in the light and its utilization in the dark, together with the correlation of this change with the oxidation and reduction of intracellular cytochrome b, give further support to the idea that the mutant cells have an intact photosynthetic system.

Relative Effectiveness of Chlorophyll and Carotenoid Pigments in Photosynthesis: In order to determine the extent to which carotene pigments of the pale green mutant contribute to the photosynthetic activity, we have used the apparatus described by Castor and Chance (17) for measurement of the action spectrum for photosynthesis in the region of absorption maxima for chlorophyll and carotenoid pigments (fig 4). The wavelength of the peak of this spectrum indicates the participation of chlorophyll in oxygen evolution by the mutant cells. The sharp decrease of the effectiveness of longer wavelength of light indicates that carotenoid is not important in photosynthesis in the mutant cells.

Spectroscopic Effects of Aerobiosis and Anaerobiosis: The spectrum representing the absorbancy changes that occur in the transition from the steady state aerobic condition to the steady state anaerobic condition of the algal suspension is given in figure 5. It should be noted that the scale is broken at 500 mμ and the absorption bands in the visible region are plotted at twice the scale of those in the Soret and ultra-violet regions.

A number of components similar to those of the mammalian respiratory chain are immediately recognizable from the data.[5] Reduced pyridine nucleotide shows a symmetrical absorption band centered at about 345 mμ. Flavoprotein shows a distinctive trough at 470 mμ; the trough is caused by the disappearance of the oxidized form of flavoprotein. Cytochrome b shows a distinctive α band at 563 mμ and its corresponding Soret band at 430 to 431 mμ. An α-band attributable to cytochrome of type c shows clearly at 551 to 552 mμ. The Soret band of cytochrome c does not show clearly because it is relatively less distinctive. The cells show very unusual

[5] In order to clarify what may seem to be arbitrary relationships between the positions of the absorption bands of components of the electron transfer systems and the nature of the enzyme systems involved, we have made the following list:

a. Isolated diphospho- and triphospho-pyridine nucleotide have the absorption peaks of their reduced forms at 340 mμ and our spectra present the sum of the reduced forms of such pigments.
b. 430 and 563 mμ—these absorption peaks are attributed to cytochrome of type b without an attempt at this point to distinguish among cytochrome b of the respiratory chain, cytochrome b_3 and cytochrome b_6.
c. 424 and 555 mμ—absorption bands having their peaks at these wavelengths will arbitrarily be attributed to cytochrome f, although this may not be an unique or a final designation.
d. 420 and 550 mμ—pigments having their absorption bands at these wavelengths are identified with cytochrome c of the respiratory chain.
e. 450 to 470 mμ is the region where the oxidized form of flavoprotein absorbs and the pigment studied here is assumed to be that involved not only in the respiratory chain but also in the photosynthetic processes.

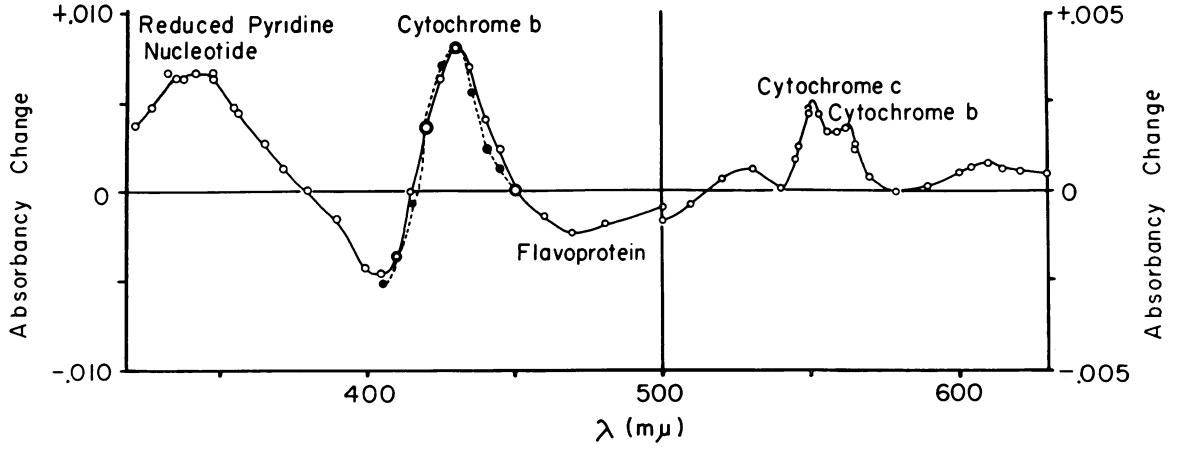

Fig. 5. The reduced minus oxidized difference spectrum for the respiratory components for the Chlamydomonas mutant. The open circles represent data taken by alternately flushing oxygen and nitrogen over the cell suspension with consequent oxidation and reduction of the pigments (for an example of a recording at a particular pair of wavelengths, see fig 6). A preliminary identification of the cytochrome components is included in the figure (cf footnote 3). Note that the magnification of the scale has been doubled in the visible region of the spectrum. The effects of alternately flushing CO and O_2 are illustrated by the closed circles in the region of the Soret band. (672).

TABLE II

RELATIVE ABSORBANCIES AND CONCENTRATIONS OF RESPIRATORY ENZYMES IN PALE GREEN MUTANT CHLAMYDOMONAS (672)

WAVE-LENGTHS USED (mμ)	PROBABLE IDENTITY	ABSORBANCY CHANGES RELATIVE TO c	ΔE(cm^{-1} × mM^{-1})	CONCENTRATIONS RELATIVE TO c
430–445	a_3CO (?)	< 0.7	90	0.15 (?)
562–575	b	0.8	20	0.8
551–540	c	1	20	1
470–510	Flavo-protein	1	11	1.8
370–374	Pyridine nucleo-tide	2.8	6	9

absorption characteristics with respect to cytochrome a, for in the region of the α-band of this pigment, about 605 mμ, there is a scarcely distinguishable peak. Furthermore, no absorption band attributable to cytochrome a_3 appears in the Soret region at 445 mμ. Since there is no evidence in favor of a terminal oxidase of the $a + a_3$ type, other possibilities for the terminal oxidase of these algae were considered; for example, the carbon monoxide binding pigment found in so many micro-organisms (16). If the carbon monoxide binding pigment were the terminal oxidase, a distinctive absorption band would be expected to appear at 415 mμ upon the addition of CO. The dashed portion of the trace in figure 5 shows that this is not the case; no distinctive band appears at 415 mμ, in fact, the absorbancy changes for CO addition are very nearly identical to those caused by nitrogen. There is, however, a small diminution of the intensity of the absorption band in the region of 445 mμ and a slight intensification in the region of 425 mμ. These effects suggest the presence of cytochrome a_3-CO (16). Thus, the small effects in the region of 615 mμ in the oxidized minus reduced spectrum, and at 445 mμ and 425 mμ in the oxidized minus carbon-monoxide-treated spectrum suggest the presence of a terminal oxidase system more nearly similar to cytochrome $a + a_3$ than to the CO-binding pigment. An unequivocal test of this conclusion would require a study of the photochemical action spectrum for relief of CO-inhibited respiration in these micro-organisms according to the method of Castor and Chance (17), but it is unlikely that this can be carried out satisfactorily because of the rapid oxygen evolution caused by illumination of the mutant.

It has been customary in studies of various cell types to represent the components of the respiratory chains as a sequence of optical density changes relative to a particular member of the chain, and also as a sequence of concentrations. This has been done for the pale green cells and the results are given in table II. The relative concentrations of cytochromes b and c are about equal as has been observed in many other types of intact cells (18), but pyridine nucleotide and flavoprotein are observed in somewhat lower concentrations relative to cytochrome than in other cells; for example, ratios of 20 and 3, respectively, are observed in isolated mitochondria. A partial explanation for the low concentration of pyridine nucleotide relative to cytochrome is that the spectroscopic measurements are based on the transition from the aerobic steady state to the anaerobic steady state, and it is probable that only a portion of the pyridine nucleotide is affected by this transition; substrate-free cells are desirable for measuring the total concentration of pyridine nucleotide.

The relative concentration of cytochrome a_3, as assayed from the very small spectroscopic effects caused by the addition of carbon monoxide, is so low that it is questionable as to whether this component would function together with the other cytochromes as a part of the respiratory chain. In other cells the relative concentration of cytochrome a_3 to c has never shown such a disparity as has been observed here. We conclude, therefore, that it is unlikely that we have yet identified the terminal oxidase of these cells.

SPECTROSCOPIC EFFECTS OF ILLUMINATION: A typical record illustrating the effect of light compared with that of oxygen upon cytochromes of type b is given in figure 6. The record begins after anaerobiosis of the cell suspension has been established. The cells are then illuminated and an absorbancy decrease

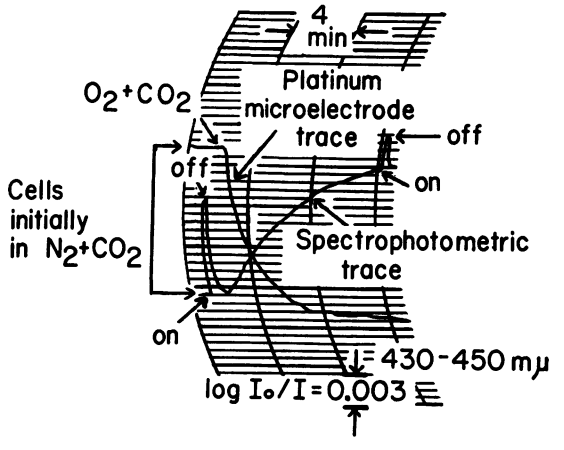

FIG. 6. The effects of light and oxygen concentration upon the absorbancy of the Chlamydomonas mutant suspension. A cell suspension, the difference spectrum of which is given in figure 7, is illuminated with red light at the moment marked "on" and the illumination is terminated at the moment marked "off." Such illumination causes an upward deflection of the trace which corresponds to a decrease of absorbancy at 430 mμ with respect to 450 mμ. The increase of oxygen concentration on changing the gas stream from $N_2 + CO_2$ to $O_2 + CO_2$ is indicated by the platinum micro-electrode trace. The corresponding oxidation of the cytochromes of the cells is indicated by the upward deflection of the spectrophotometric trace which corresponds to a decrease of absorbancy at 430 mμ with respect to 450 mμ. Illumination of the aerobic algae gives a small, further decrease of absorbancy at 430 mμ. (669 c).

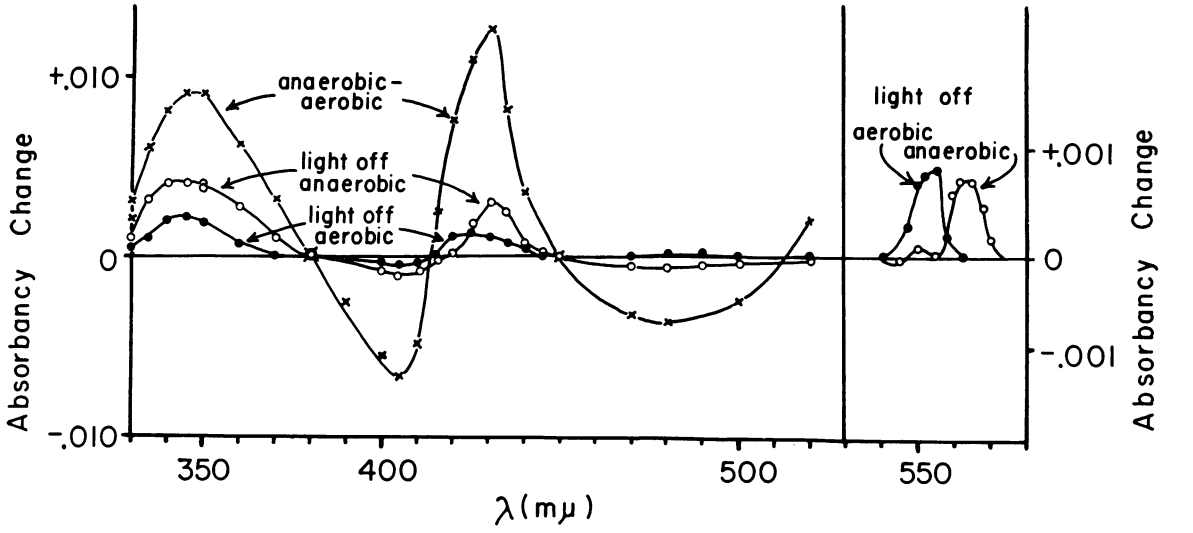

FIG. 7. Effect of aerobic and anaerobic illumination of the Chlamydomonas mutant compared with the effects of oxygenation. Repetitions of experiments similar to those of figure 6 give the difference spectra for aerobic and anaerobic light effects, as well as the aerobic minus anaerobic difference spectrum. The absorption peaks shown clearly are those due to pyridine nucleotide in the ultra-violet region and cytochromes in the region of the Soret band. In the visible region, only the aerobic and anaerobic light effects are shown because the amplification of the scale is such that the oxidized minus reduced spectrum would be far off scale. The color filter combinations appropriate to the wavelength regions are discussed in the text. (673).

occurs as is indicated by the upward deflection of the spectrophotometric traces. This decrease corresponds to an oxidation of cytochrome b. On cessation of illumination, the absorbancy increases towards the anaerobic value. The cells are then oxygenated with a mixture of O_2 and CO_2 as is indicated by a downward deflection of the patinum electrode trace and the absorption of 430 mμ decreases as the oxygen concentration increases. After the cells have been oxygenated, illumination causes a smaller absorbancy decrease. Thus an exhaustive study of the light effect requires recordings of the spectra in both the aerobic and the anaerobic states. Figure 7 illustrates such an experiment and, in addition, gives for comparison the oxidized minus reduced spectrum for the region of 330 mμ to 520 mμ. The absorbancy changes are plotted with the aerobic state as the base-line for the oxidized minus reduced spectrum and the dark state as the base-line for the light-on-light-off spectrum. Thus, the congruence of the traces shows that oxygenation and illumination or nitrogenation and darkening cause similar oxidation-reduction changes.

In the case of pyridine nucleotide, illumination of the anaerobic cells causes about half the oxidation that occurs with oxygenation. Nevertheless, illumination of the aerobic cells causes a further small increase of oxidation of pyridine nucleotide.

Studies have also been made on the effect of red illumination upon absorbancy changes in a Chlorella suspension placed in the moist chamber in the manner described above, other details of the experimental technique being approximately the same as those described above. In Chlorella the absorption of the material in this region of the spectrum is so large that the amount of measuring light falling on the phototube is small compared to the leakage of the red light through the filter combination. As shown in figure 8, illumination causes a transient downward deflection and cessation of illumination causes a transient upward deflection. These deflections represent the response of the resistance-capacitance coupling circuit of the amplifier to the square pulse of photocurrent caused by turning on and by turning off the light and do not represent spectroscopic changes. Following this transient deflection, true spectroscopic effects can be recorded and an absorbancy decrease at 340 mμ is observed upon illumination. Upon turning off the light, the absorption increases towards the initial level and the phenomenon may be repeated upon illuminating for a second time. Thus, in Chlorella we find

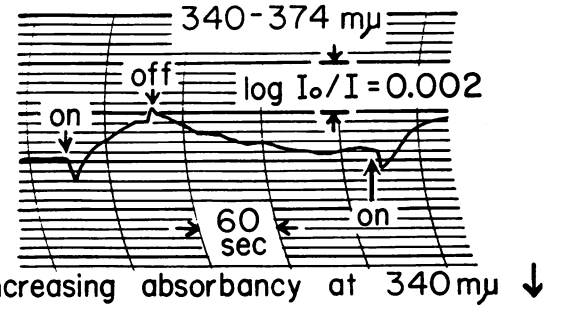

FIG. 8. The absorption spectra changes caused by red light illumination of the Chlorella suspension. For details see (6). (623 e).

confirmation of the phenomenon demonstrated clearly in the Chlamydomonas mutant.

While illumination causes qualitatively similar effects in both aerobiosis and anaerobiosis in the ultraviolet region, the effects are distinctly different in the region of the Soret band. Illumination under anaerobic conditions primarily affects cytochrome b as seen by the peak at 430 mμ, while illumination under aerobic conditions primarily affects cytochromes of type c or f as shown by the peak in the region of 425 mμ. The difference between the anaerobic and aerobic light effects is more clearly distinguished in the visible region of the spectrum where peaks attributable to cytochrome b at 563 mμ and to cytochrome f at 555 mμ are found. Flavoprotein, interestingly enough, although it shows a relatively large change of absorption in the oxidized-reduced spectrum, shows little or no effect upon illumination. This remarkable discrepancy is made clear by reference to figure 9, especially under anaerobic conditions where such a large oxidation of cytochrome b is observed. Effects upon cytochrome a_3, if they occur, are too small to measure.

It is found that the effect of light compared with that of oxygen varies with the characteristics of the cell suspension (possibly age and endogenous substrate content) and figure 9 represents a suspension which showed a very large anaerobic light effect. In fact, the oxidation of cytochrome b caused by illumination very nearly equals that caused by oxygen. This spectrum also clearly shows the distinction between aerobic and anaerobic light effects both in magnitude and in position of the peak; the Soret peak of the anaerobic light effect lies 4 mμ below that of the aerobic effect.

The data of both figures 7 and 9 indicate that no measurable absorbancy change has occurred at 515 mμ, a wavelength at which distinctive effects are observed in algae containing their full complement of pigments. Since these organisms carry out the photosynthetic process, the lack of the 515 mμ absorption band is of some significance.

The rather distinctive effects of oxygen upon the response to illumination at 554 mμ and 563 mμ is illustrated by figures 10 A and 10 B, respectively.

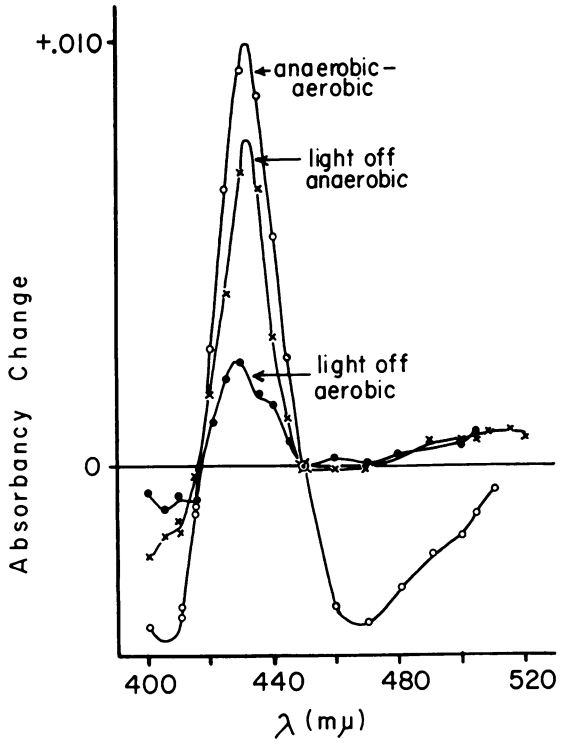

FIG. 9. Light and oxygen effects in an algal suspension in which the anaerobic light effect nearly equals the effect of oxygen. The difference spectra are obtained by repetition of experiments with the cell suspension of figure 6. Noteworthy is the very small effect of light upon the oxidation-reduction state of flavoprotein measured in the region of 470 mμ. (669 c).

These are the direct photographic recordings of the spectrophotometer output when the monochromators are set at wavelengths 554 and 540 mμ in figure 10 A and 563 and 540 mμ in figure 10 B. The trace begins with the cells in the aerobic state. Upon illumination the upward deflection indicates the decrease of absorbancy at 554 mμ. This absorbancy decreases rapidly and returns to the initial level upon cessation of

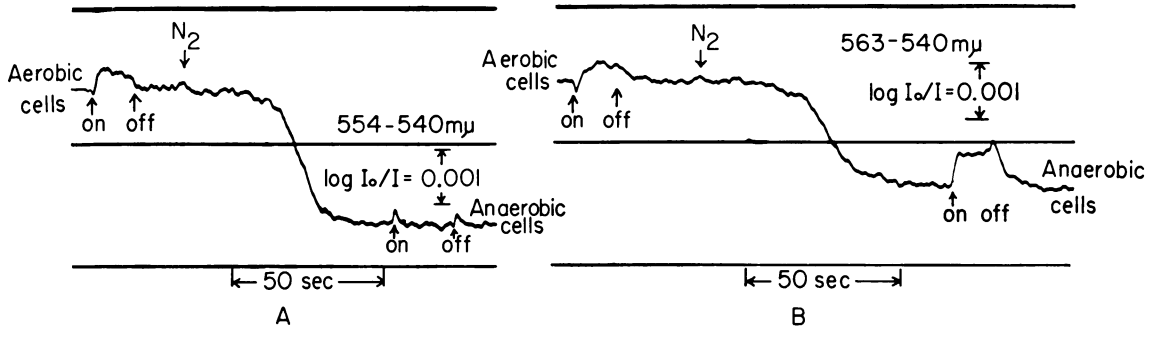

FIG. 10. The kinetics of the effects of illumination and disoxygenation measured at wavelength appropriate to cytochrome f (fig 10 A) and cytochrome of type b (fig 10 B). Other details of the experiment are included on the figure. (669 c).

the illumination. The gas passing through the moist chamber is then changed to nitrogen and the trace shows the absorbancy increase caused by the reduction of the cytochrome. Some seconds after anaerobiosis has been established, illumination is repeated and no significant change other than a brief transient is recorded. The record at 563 mμ differs in that there is a slight response to illumination in the aerobic state and a larger increase in anaerobiosis. These records also give some indication of the clarity of recording of these small absorbancy changes in the visible region of the spectrum. The noise fluctuation of optical density is less than 10^{-4} and yet the speed of response is considerably less than 1 second.

A detailed spectrum of the effects in the visible region is shown in figure 11. The anaerobic light effect shows the distinctive and sharp peak at 563 mμ. The subsidiary peak lies very close to 554 mμ (this peak shows somewhat less clearly in figure 7 and its magnitude relative to that of 563 mμ depends upon the interval of illumination as illustrated by figure 12 below). The aerobic light effect has a peak around 556 mμ which is the appropriate wavelength for cytochrome f.

KINETICS OF THE ANAEROBIC LIGHT EFFECTS: Figure 10 A showed that no appreciable absorbancy change occurs at 554 mμ for an illumination lasting a few seconds. Nevertheless, a definite peak shows in the spectra of figures 7 and 11. This is due to the longer interval of illumination used in those experiments and the two apparently divergent results may readily be correlated by reference to figure 12. This

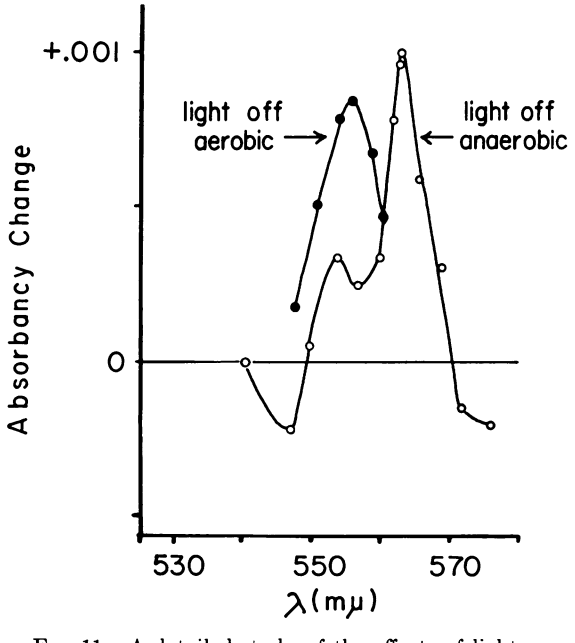

FIG. 11. A detailed study of the effects of light upon the α-bands of the cytochromes under aerobic and anaerobic conditions. The sensitivity used in this experiment is adequate to detect the small shoulder on the peak of cytochrome b in the anaerobic light effect. (674).

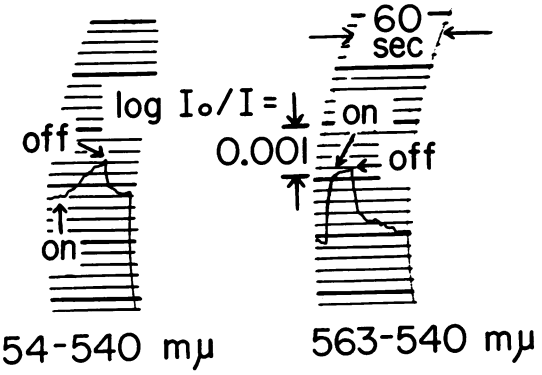

FIG. 12. A comparison of the kinetics of the light effect at wavelengths appropriate to cytochrome f (*left*) and cytochrome of type b (*right*). The same cell suspension was used in this experiment as in the experiment of figure 10. (674).

figure shows absorbancy changes in response to long period illumination of the anaerobic suspension. The typical rapid response measured at 563 mμ is shown in figure 12 and this agrees with that of figure 10 B which shows this response to occur in less than a second. The response measured at 554 mμ is much slower; immediately upon illumination there is no response at all (cf fig 10 A). After 30 seconds illumination, a considerable absorbancy decrease has occurred. Upon cessation of illumination, this change diminishes abruptly. Thus, the behavior of the two cytochromes upon illumination differs, that of type b reaches its steady state and returns therefrom symmetrically, while cytochrome f shows a lag in response to illumination of the anaerobic cells and a rapid response to cessation of illumination.

DISCUSSION

As stated in the introduction, there is little consistency of the various experimental studies on the spectroscopic changes caused by illumination of the green cell. Nevertheless, various conclusions have been drawn, and we propose to examine such conclusions in the light of the data obtained in these experiments.

1. THE OXIDATION OF CYTOCHROME f UPON ILLUMINATION: In Duysens' first paper he attributed a decrease of absorption based on a single point at 420 mμ to the oxidation of cytochrome f upon illumination of Chlorella (3). More points were obtained which confirmed the existence of such a peak at 420 mμ in later work on *Porphyridium cruentum* (4). However, 424 mμ is the correct location (see footnote 3) for the peak of the Soret band of cytochrome f (19), and Duysens' data can therefore be used only to support the supposition that cytochrome c is oxidized upon illumination of both Chlorella and Porphyridium. In Porphyridium, however, Duysens' data show the disappearance of a 555 mμ peak on illumination which he attributes to cytochrome f oxidation, and this is the correct wavelength for the band of cytochrome f (19).

The data of Strehler and Lynch (7), taken from the "negative overshoot" phase of their spectroscopic record, suggest that cytochrome f is *reduced* upon illumination. An alternative interpretation of their results, which is in no way inconsistent with the data they present, is that the "negative overshoot" phenomenon applies only to the 518-mμ band and does not apply to the 555-mμ band. We suggest that they were observing an oxidation of cytochrome f that persisted about 1 second after cessation of illumination, a supposition that is not ruled out by our data on cytochrome f (cf fig 10).

Lundegårdh (8) states that he measured an oxidation of cytochrome f "at illumination," but due to his slow recording method this result must (a) depend upon an extremely long persistence (30 seconds) of the oxidation of cytochrome f upon the illumination of Chlorella or (b) be related to indirect effects of illumination (see footnote 4). That the spectroscopic effect persists at any reasonable magnitude for approximately 30 seconds is a result contrary to our records of cytochrome f kinetics (see fig 10). Thus, it may only be a fortuitous circumstance that Lundegårdh's data agree with other experimental results.

In summary, Duysens showed that both cytochrome c and cytochrome f are oxidized upon illumination under his experimental conditions, a re-interpretation of Strehler and Lynch's result is also in favor of oxidation of cytochrome f, and the confirmation afforded by Lundegårdh's results remains of dubious value in view of the large interval between illumination and measurement. The results of this paper show that cytochrome f is oxidized upon illumination under aerobic conditions.

The lack of a measurable oxidation of cytochrome f under anaerobic conditions can be attributed to two factors: 1) that relatively more reductant is present anaerobically than aerobically and 2) that cytochrome of type b has a higher affinity for the oxidant than f. Both these factors are consistent with the considerable delay between the oxidation of cytochrome of type b and cytochrome f that is illustrated by figure 12. It is possible that Duysens' oxidation of cytochrome c occurred under anaerobic conditions, especially in his studies of Porphyridium where he allowed the cell suspension to stand "about half a day" (4) in order to enhance the spectroscopic effects.

2. The Oxidation of Cytochrome of Type b: It is not possible for us to determine which one of the three possible cytochromes of type b is actually under observation because of the similarity of their absorption bands (cytochromes b_6, b_3, and b of the respiratory chain), and our observations suffer from the same limitations as those of Hill on the leaves of golden varieties of certain plants. In such leaves, Hill observes cytochrome b_6 to be completely reduced even in the aerobic illuminated leaf. Our results on Chlamydomonas mutant show that the cytochrome of type b can show only a small further oxidation under aerobic conditions, suggesting that it is already largely oxidized, especially under illumination. Thus, these observations differ from those of Hill and do raise questions about the magnitude of the oxidation-reduction span between cytochrome of type b and cytochrome f in the illuminated cell. Our results suggest that both cytochromes of type b and cytochrome f are more oxidized in the aerobic illuminated green cell.

3. The Oxidation of Reduced Pyridine Nucleotide under Aerobic and Anaerobic Conditions: Duysens' experimental data, from which he has drawn the conclusion that pyridine nucleotide is reduced upon illumination of Chlorella and Porphyridium (4), are indistinct and are contrary to the results obtained here. His recordings cover the range from 370 to 320 mμ and show a general increase of absorbancy in this region upon illumination. When his curves are studied in detail, however, it is difficult to see how this general increase of absorption could be attributed specifically to the reduction of pyridine nucleotide. For example, his curve for Chlorella clearly shows the absorption at 350 mμ to exceed that at 340 mμ; the reverse is true for authentic DPNH. In the case of Porphyridium, the data show a small peak at 340 mμ but there is an equally large peak at 360 mμ. A part of this change could be attributed to the δ-band of cytochrome f which gives an absorbancy change upon illumination that is larger at 360 than at 320 mμ. Thus, the spectroscopic evidence does not support Duysens' interpretation in favor of pyridine nucleotide reduction. On the other hand, Duysens' data surely did not show a change of absorbancy that could be attributed to an oxidation of reduced pyridine nucleotide, and there is at the present time no explanation of the discrepancies between his results and ours. In our experiments on Chlorella at 340 mμ, the net effect of illumination was a decrease of absorbancy at 340 mμ measured with respect to 374 mμ (fig 8), indicative of an oxidation reaction. In the Chlamydomonas mutant, clear-cut results in the region from 330 to 370 mμ were obtained upon illumination under aerobic and anaerobic conditions. This result is adequately controlled by the response of the anaerobic cells to aerobiosis, i.e., the effects of illumination show the same sort of absorption maximum and are of magnitude that is consistent with the effect of oxygen. Thus Duysens' theory (4) that oxidized pyridine nucleotide reacts with excited chlorophyll to give reduced pyridine nucleotide lacks support.

One explanation of the oxidation of reduced pyridine nucleotide under aerobic conditions that is to be considered is that light-induced oxygenation of the interior of the cell causes further oxidation of pyridine nucleotide. If this were so, other respiratory components should also show further oxidations. Actually, cytochrome f is the only one that shows distinctive changes under aerobic conditions and cytochrome f is not considered to be a component of the respiratory chain. Thus, the pyridine nucleotide oxidized upon illumination of the aerobic cells is probably not that associated with the respiratory chain but with the photosynthetic mechanism of the chloroplasts. This result now appears to be opposed to the chemical

studies of the effect of illumination upon isolated chloroplasts; for example, Ochoa and Vishniac (20) used enzymatic methods for demonstrating pyridine nucleotide reduction upon illumination of chloroplast fragments, and more recently, San Pietro and Lang have obtained spectrophotometrically detectable amounts of reduced pyridine nucleotide at very high DPN and chloroplast concentrations (21). One suggestion that is compatible with both types of results is that both oxidation and reduction of pyridine nucleotide occur simultaneously in the whole cell and that the reduction reaction is completely overbalanced by the oxidative one. One must postulate that the chloroplast fragments are deficient in the oxidation reaction. In any case, the fact that the net effect of illumination of the aerobic photosynthesizing cell is an oxidation and not a reduction needs to be carefully considered in mechanisms of photosynthesis.

Both Strehler (28) and Duysens and Sweep (29) have attempted to use fluorimetric methods to demonstrate DPN reduction in Chlorella, but no measurable effects are obtained on red illumination of the cells under conditions suitable for photosynthesis. However, Duysens suggests reduction can be demonstrated in *Rhodospirillum rubrum,* although a discrepancy between this result and that of Chance and Smith (10) is apparent.

4. Effect of Illumination upon the Steady State Oxidation-Reduction Level of Flavoprotein: Whereas oxygenation of the anaerobic cells causes a large oxidation of flavoprotein as evidenced by the trough at 460 mμ in the oxidized minus reduced spectrum, there is a striking lack of effect of illumination on flavoprotein under both aerobic and anaerobic conditions. While this result might have been expected under aerobic conditions, as a portion of the flavoprotein might be auto-oxidizable, it certainly is not to be expected under anaerobic conditions where the flavoprotein associated with the respiratory chain is shown to be considerably reduced in other cells, and would be expected to be oxidized upon illumination together with the cytochromes. A working hypothesis is therefore proposed, that flavoprotein is a receptor of reducing equivalents and hence the lack of measurable changes in the steady state oxidation-reduction level of this component is due to the combined action of reducing power derived from the photolysis reaction and oxidizing power derived from photolysis and the oxidation of other respiratory components. If this hypothesis is applied to both pyridine nucleotide and to flavoprotein, the effect of reducing equivalents upon the latter is much greater.

A related effect of reducing equivalents may be found in the lag in the oxidation of cytochrome f upon illumination of the anaerobic cells, such as illustrated in detail by figure 12. It should be noted that the oxidation of cytochrome f proceeds slowly and reaches its steady state value after an illumination interval of about half a minute. On cessation of illumination, the reduction of cytochrome f proceeds very rapidly. This would suggest that cytochrome f is being oxidized against a preponderance of reducing substances which have accumulated during the dark interval. We have no explanation for the fact that similar kinetics have not yet been found in the response of flavoprotein and pyridine nucleotide to illumination under aerobic conditions.

5. The Role of Carotenoids in Photosynthesis in the Mutant Cells: Three results of this paper bear upon the participation of carotenoid in photosynthesis in the mutant cell. They are:

1) that the turnover number of chlorophyll in the mutant cell is higher than that of the normal cell in spite of a β carotene content relative to chlorophyll that is 1/30th that of the dark-grown normal cell;

2) that β carotene would have to turn over about 100 times faster in the mutant cell than in the normal to keep pace with the photosynthetic activity;

3) that the action spectrum for photosynthetic oxygen evolution shows a peak due to chlorophyll, but no detectable shoulder due to carotenoid, as would be expected if it participated in the activation of chlorophyll by light;

4) that the 515-mμ absorption band does not appear upon illumination of the mutant cells. It has been proposed elsewhere that this band is due to a compound of carotenoid and, on this basis, the lack of the 518-mμ band is consistent with a lack of carotenoid participation. Since about equal amounts of chlorophyll were used in experiments with normal and mutant cells, the 30-fold deficiency of β carotene relative to chlorophyll in the mutant cells would not be sufficient to prevent the detection of a 515-mμ absorption band upon illumination.

These data indicate that the mutant cells actively photosynthesize without any physical evidence for the participation of the small amount of carotenoid that they contain. A possible function of carotenoid is discussed below.

These studies with the mutant also shed considerable light on the nature of the chemical change that could give rise to the 518-mμ absorption band in the normal cells. From an observation of several components of the respiratory chain, it is shown here that the predominant effect of illumination of the aerobic or anaerobic cell is an oxidation reaction; reduction reactions can be identified only by a lag period in an oxidation of cytochrome f or the absence of an oxidation of flavoprotein: no direct reductions have been observed of any components of the whole cell. The presence of an oxidant and the lack of a reductant supports the hypothesis presented in the paper of Chance and Strehler (6) that the 518-mμ absorption band (phase 3 reaction) (6) results from an oxidation reaction rather than a reduction reaction.

Further evidence for the oxidation reaction may be inferred from the growth conditions for both this mutant and that of *Rhodopseudomonas spheroides;* they are sensitive to prolonged illumination to such an extent that the cells are killed. It is possible that the appearance of the 518 mμ absorption band in the normal type Chlamydomonas or in Chlorella is an in-

dication of a protective reaction in which excess oxidizing equivalents produced by the photosynthetic process react with a pigment, presumably carotenoid, in order to prevent damage to the cell, and such a hypothesis has recently been presented on the basis of work with *R. spheroides* (23). The possible nature of the reaction was discussed by Calvin (24) and it is probable that the oxidant involved is not molecular oxygen but rather an intermediate in the photolysis of water, since neither the Chlamydomonas nor the *R. spheroides* mutant is sensitive to oxygen itself.

6. Position of Cytochromes b and f in the Sequence of Illumination Reactions: The much more rapid oxidation of cytochrome b than f under anaerobic conditions in response to the illumination of the anaerobic cells suggests the manner in which these two cytochromes may react with the oxidizing and reducing equivalents produced by the photolysis of water. The reaction kinetics suggest that the oxidizing equivalents are received first by cytochrome b and then, with considerable delay, by cytochrome f. But such a sequential action of cytochromes b and f is unlikely in view of the difference in their oxidation-reduction potentials and it is possible that they are oxidized by different systems; b by the terminal, or oxygen, oxidase, and f by photolysis intermediates. A plausible hypothesis is that the oxidizing systems affect cytochromes b, and, under anerobic conditions, f, but f can respond only slightly because it is already reacting with reducing equivalents. Under aerobic conditions, cytochrome b responds only slightly to illumination, since it is already supplied with adequate oxidizing power from the terminal oxidase under these conditions. Under these conditions, the effect of light upon cytochrome f is rapid, presumably because less reducing power is present, for example, less reduced pyridine nucleotide is available (see fig 7) cytochrome b is already oxidized. Such a reaction sequence is in no way in accord with the oxidation-reduction potentials of the isolated pigments from which the opposite results would have been expected upon their reaction with oxidizing equivalents. Whether the cytochromes in the intact cell have different oxidation-reduction potentials from those obtained upon isolation, or whether some unknown factor is affecting the reaction kinetics is a point which cannot be decided at the present time, and further studies are needed.

7. Pathways for Respiratory and Photosynthetic Electron Transport and Phosphorylation: Since the components of the respiratory chain observed in the oxidized minus reduced spectrum for the Chlamydomonas mutant resemble, with the exception of the terminal oxidase, those of mitochondria capable of oxidative phosphorylation, we can presume that the respiratory chain of the mitochondria of the green cell consists of a similar series of components:

$O_2 \rightarrow$ oxidase $\rightarrow$ cytochrome c $\rightarrow$ cytochrome b $\rightarrow$ flavoprotein $\rightarrow$ pyridine nucleotide $\rightarrow$ substrate

While Hill has proposed that cytochrome f is more likely to react with the photo-produced oxidant than is cytochrome b, the delay in the oxidation of cytochrome f observed upon illumination of the anaerobic cells together with the rapid response of cytochrome b must be taken into consideration in any mechanism for photosynthetic electron transport and phosphorylation. Our data on flavoprotein suggest that this component is nearest to the source of the reducing equivalents. At the present time our inability to discriminate between components of the respiratory and photosynthetic chains is limited by the fact that our observations are based upon the whole cell. Further experiments on particles isolated from these cells will be necessary to distinguish between the enzymes that are involved in the pathways of photosynthesis and respiration.

Summary

Utilizing senstive spectrophotometric techniques and a pale green mutant of Chlamydomonas which carries out an active photosynthesis with a low chlorophyll content, the components of the respiratory chain have been investigated. It is found that pyridine nucleotide, flavoprotein and cytochromes b and c are present in amounts typical of other micro-organisms. The terminal oxidase is present in such a low concentration relative to the other cytochromes that it has not been surely identified.

Pyridine nucleotide is affected by light aerobically and anaerobically; flavoprotein is scarcely changed under either condition. Cytochrome b responds most sensitively to illumination anaerobically but responds very little to illumination aerobically. Cytochrome f responds rapidly to illumination under aerobic conditions and sluggishly under anaerobic conditions. No evidence of increased absorption at 518 mμ upon illumination is recorded. Since the mutant has a high photosynthetic activity per unit chlorophyll content, it is probable that the compound absorbing at 518 mμ and observed upon illumination of the normal cells is not an essential intermediate in photosynthesis.

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MALONYLTRYPTOPHAN IN HIGHER PLANTS[1,2]

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During the course of our study of indoleacetic acid metabolism (1, 6), we investigated the effect of exogenous tryptophan on the synthesis of indoleacetylaspartic acid in excised pea epicotyls. Tissues bathed for 24 hours in solutions containing 50 to 200 mg/l tryptophan accumulated considerable amounts of a substance which closely resembled indoleacetylaspartic acid in chromatographic mobility in several solvents, in acid strength, and in color reactions with the Ehrlich (*p*-dimethylaminobenzaldehyde) and Salkowski (acid-ferric chloride) reagents (fig 1). Consequently, in discussing metabolic precursors of indoleacetic acid at the 1956 Annual Meeting of the American Society of Plant Physiologists at Storrs, Connecticut, we reported erroneously the conversion of tryptophan into indoleacetylaspartic acid. Differences soon became apparent. The unknown substance, which occurred in several families of higher plants, had a slightly higher R_f in most solvents than had indoleacetylaspartic acid. Furthermore, although the color produced on paper with the Salkowski reagent was the purple of indoleacetylaspartic at very low concentrations, the color at higher concentrations was brown; in this respect the substance resembled acetyltryptophan. Basic hydrolysis of the unknown yielded tryptophan, not indoleacetic acid.

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ISOLATION

In order to assign a structure to the tryptophan derivative its isolation in a relatively pure state was necessary. This isolation was a somewhat laborious procedure since the solubilities of the derivative were similar to those of the bulk of the plant acids. However, it was possible to take advantage of the fact that it was a very strong acid and a considerable purification was achieved by partitioning the plant